Por el pasado, a mi abuela Agustina

Por el futuro, a mi hermana Alba

T1G3 bladder tumors: at the crossroads of molecular pathways

by Elena López Knowles

Barcelona, 2006

phD program in Health and Life Sciences
Pompeu Fabra University
(2001-2003)

Dep. Ciències Experimentals i de la Salut
Doctorat en Ciències de la Salut i de la Vida

Molecular and Cellular Biology unit
IMIM

C S B Consorci Sanitari de Barcelona
IMAS
Institut Municipal d'Assistència Sanitària

EPICUR red

EPI CUR

T1G3 bladder tumors: at the crossroads of molecular pathways

Thesis presented by Elena López Knowles
to receive a phD
by the Department of Health and Experimental Sciences
Pompeu Fabra University

Thesis under the direction of Dr. FX. Real
Molecular and Cellular Biology unit
Institut Municipal d'Investigació Mèdica

Dr. FX. Real
Thesis director

Elena López Knowles
phD student

AMPK	AMP activated protein kinase
APAF-1	apoptotic peptidase activating factor
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
BAC	Bacterial artificial chromosome
BAX	BCL-2 associated X protein
BCG	Bacillus Calmette Guerin
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like
CGH	Comparative genomic hybridization
CHK-1	Checkpoint Kinase 1
CIS	Carcinoma In situ
DBCCR1	Deleted in bladder cancer chromosome region candidate 1
DHFR	Dihydrofolate Reductase
DP1	Dimerization partner 1
ERCC1	Excision repair cross-complementing rodent repair deficiency, complementation group 1
ERK	Extracellular regulated MAP kinase
FDA	Food and Drug Administration
FGFR	Fibroblast growth factor receptor
FRS2	Fibroblast growth factor receptor substrate 2
G	Grade
GADD45	growth arrest and DNA damage-inducible protein 45
GAP	GTPase activating protein
GLUT4	Glucose Transporter 4
GST	Glutathione S-transferases
GTP	Guanosine triphosphate
Gy	Grays
HDAC	Histone deacetylase
hTERT	human Telomerase Reverse Transcriptase
IARC	International Agency for Research on Cancer
ID4	Inhibitor of DNA binding 4
ISUP	International Society of Urological Pathology
LOH	Loss of heterozygosity

LZTS1	Leucine zipper, putative tumor suppressor 1
NAT	N-acetyltransferases
NMP22	Nuclear Matrix Protein 22
PCNA	Proliferating cell nuclear antigen
PDK1	Pirucate dehydrogenase Kinase 1
PLC gamma	Phospholipase C gamma
PTCH	Patched homologue
PUNLMP	Papillary urothelial neoplasm of low malignant potential
Rac	Related to A and C kinase
RSK1	Ribosomal S6 kinase 1
SFRP	Secreted frizzled-related protein
SHP1	Suppressor of high copy PP1
SNP	Single Nucleotide Polymorphism
T	Stage
TCC	Transitional Cell Carcinoma
TNM	Tumor Node Metastasis
TRAP assay	Telomerase Repeat Amplification protocol assay
TRP channels	Transient receptor potential channel
TURBT	Transurethral resection of bladder tumor
UC	Urothelial carcinoma
UCC	Urothelial cell carcinoma
VEGF	Vascular Endothelial Growth factor
WHO	World Health Organization

INTRODUCTION

Bladder cancer is a major health burden in the Western world. In Europe, in 2002, with a male:female ratio of 3.7:1, 109,871 new bladder cancer cases were diagnosed in men (38,272 deaths) and 29,102 in women (12,500 deaths) (Ferlay et al., 2004). In Spain, it is the 5th leading cause of cancer-related death in men and the 13th in women. It is estimated that approximately 14,400 new cases are diagnosed per year (Coleman et al., 2003). During 2002, with a male:female ratio was of 7:1, there were 3,492 deaths in men and 703 deaths in women due to bladder cancer, accounting for 6% and 2% of cancer deaths in men and women, respectively. In the US, the estimated number of newly diagnosed urinary bladder cancer cases is 61,420 with a male:female ratio of 2,6:1 (Jemal et al., 2006).

Because patients have to be monitored regularly and have a long-term survival, bladder cancer is one of the most expensive cancers from diagnosis to death when calculated on a per patient basis (Botteman et al., 2003). Overall, bladder cancer is the fifth most expensive cancer in terms of total medical care expenditures. Bladder cancer patient management includes diagnosis, treatment, follow-up and maintenance treatment, and, for those for whom there is no specific therapy, palliative care. The average per patient cost for the 3 periods of treatment is $16,500 for initial treatment, $13,200 for maintenance and $36,500 for the terminal period (Nicolaas Bouwes (EPA WAM), 2006).

1. Anatomy and physiology of the bladder

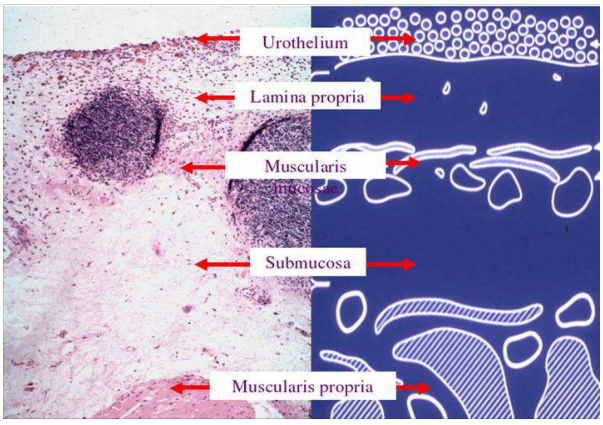


Figure 1. Low magnification photomicroscopy and diagramatic representation of normal bladder tissue.

The bladder is a hollow, muscular and distensible organ that sits on the pelvic floor, which collects urine from the kidneys and stores it until it is full enough to empty through the urethra. Organs closest to the bladder include the rectum, which is the most posterior organ in the pelvis, the prostate and seminal vesicles (in males), and the uterus, ovaries and fallopian tubes (in females).

The bladder is made up of four layers (Figure 1). These layers are important landmarks in determining how deeply the tumor has invaded.

1) Epithelium. The epithelium, which lines the bladder and is in contact with the urine, is referred to as transitional epithelium or urothelium. Most bladder cancers originate from the cells of this transitional epithelium. The urethra, ureters and the pelvis of the kidney are also lined by this transitional epithelium; therefore, the same types of cancers seen in the bladder can also occur in these sites. The urothelium is formed by approximately 3-5 layers of cells and it contains basal cells, intermediate cells and umbrella cells (Apodaca, 2004). Cell replacement occurs by cell fusion of the basal cell layer to form intermediate cells and cell fusion of intermediate cells to form umbrella cells (Lewis, 2000). The surface of umbrella cells is covered by hinges and plaques. The hinge areas are plasma membrane domains that separate plaques, and contain urohinging (Yu et al., 1992) (Figure 4). Plaques are composed of 1000-3000 asymetric unit membrane (AUM) particles. An AUM is composed of uroplakins (Ups). Four highly conserved UP proteins have been characterized. On the basis of their urothelial specificity and differentiation-restricted expression, the uroplakins have been described as urothelial differentiation-related membrane proteins and proposed as markers of advanced urothelial cytodifferentiation (Wu et al., 1990;Xu et al., 2001;Romih et al., 2005).

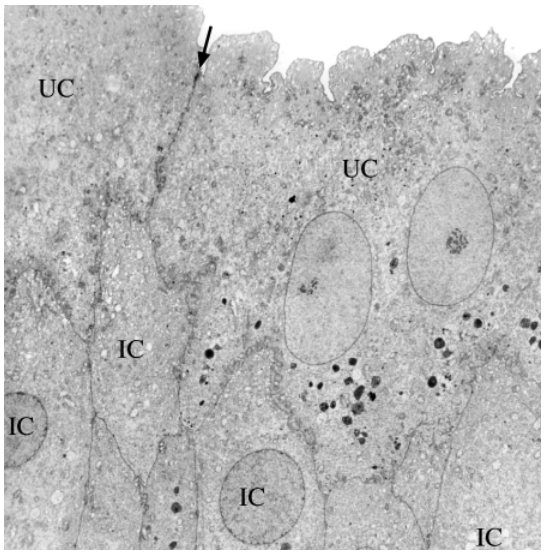


Figure 3. Transmission electron micrograph showing a binucleate umbrella cell (UC) and underlying intermediate cells (IC). The location of the tight junction between adjacent umbrella cells is marked with an arrow.

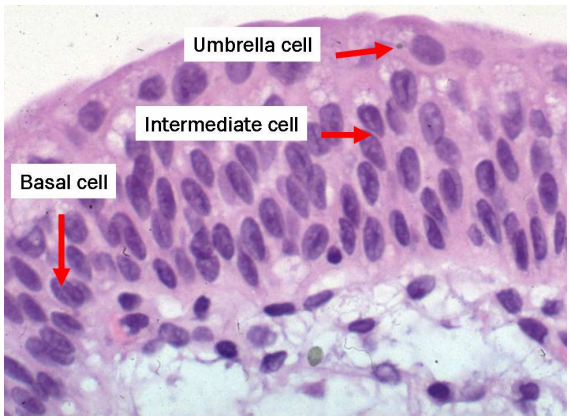


Figure 2. Optical microscopy image of normal bladder epithelium.

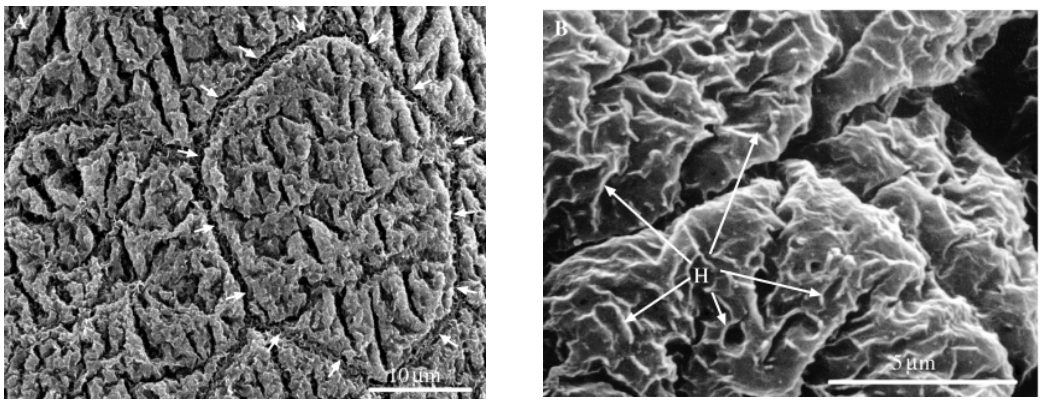


Figure 4. Umbrella cells A) Scanning electron micrograph of umbrella cell layer. The tight junction is demarcated by arrows. B) High magnification view of apical surface of umbrella cell. Examples of Hinges (H) are marked by arrows (Apodaca, 2004).

The urothelium has been traditionally viewed as a passive membrane. However, recent findings support that it is a responsive structure capable of detecting mechanical, chemical and thermal stimuli in addition to releasing a number of putative signaling molecules as tissue plasminogen activator and urokinase, and contain receptor/ion channels: bradykinin, TRP channels, purines (Birder, 2006). The umbrella cells function as a barrier against most substances found in urine, protecting the underlying tissues (Apodaca, 2004) owing to their unique lipid and protein composition. The function of plaques is to help maintain the permeability barrier associated with the apical membrane of umbrella cells and modulate the apical surface area by regulation, insertion and recovery of plaque membrane. When injury or inflammation derives in the passage of toxic substances into the underlying tissue it results in urgency, frequency or pain during voiding.

2) Lamina propria. Under the epithelium is the lamina propria, a layer of connective tissue and blood vessels. Within the lamina propria, there is a thin and seemingly discontinuous layer of smooth muscle called the muscularis mucosae. This superficial layer of smooth muscle is not to be confused with the true muscular layer of the bladder called the muscularis propria or detrusor muscle.

3) Muscularis propria or detrusor muscle. This deep muscle layer consists of thick smooth muscle bundles that form the wall of the bladder. For purposes of staging bladder cancer, the muscularis propria has been divided into a superficial (inner) half and a deep (outer) half.

4) Perivesical soft tissue. This outermost layer consists of fat, fibrous tissue and blood vessels.

2. Classification of bladder tumors

Approximately 90% to 95% of malignant bladder tumors are urothelial cell carcinomas (UCC). These tumors arise in the urothelium. Traditionally, the urothelium has been called a transitional epithelium to reflect its intermediary morphology between stratified and pseudostratified epithelium. The remaining 5% to 10% of tumors comprise epithelial tumors of other types (squamous cell carcinoma, adenocarcinoma, small cell/neuroendocrine carcinoma, carcinoma with mixed epithelial features, sarcomatoid carcinoma, lymphoepithelioma-like carcinoma, nested, micropapillary, microcystic or plasmacytoid) and mesenchymal neoplasms (1 book).

Based on their gross morphology, urothelial carcinomas can be classified as papillary, solid or Carcinoma In Situ (CIS).

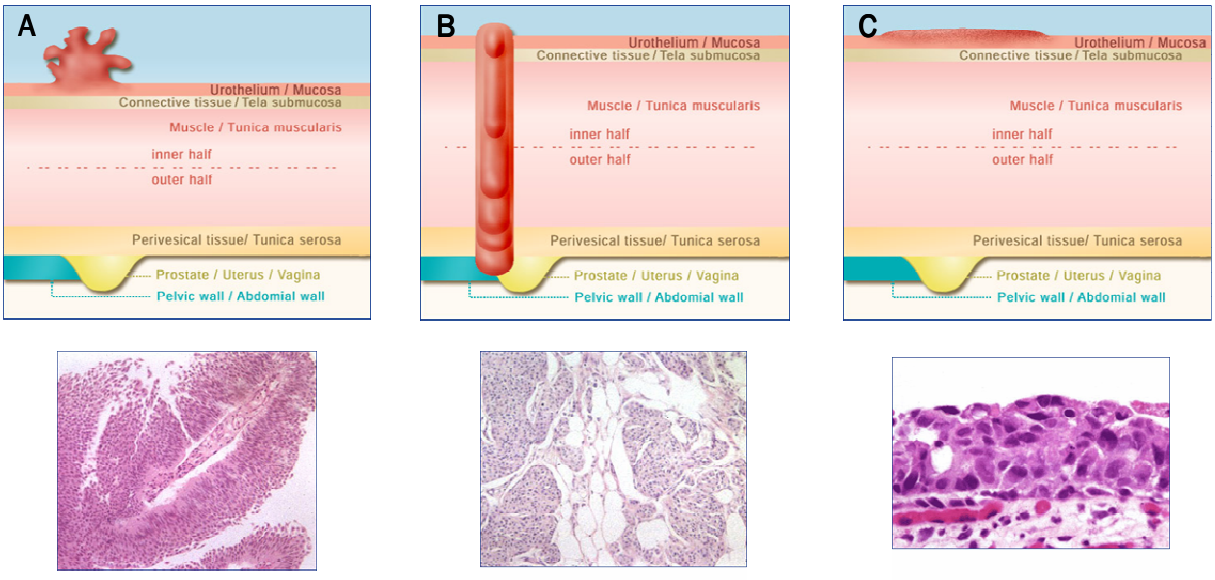


Figure 5. A) Papillary tumor, B) Solid tumor and C) Carcinoma in Situ.

Papillary tumors are the most common type and they tend to grow slowly. Papillary tumors usually grow towards the inside of the bladder. Solid tumors are less frequent but tend to be more aggressive. CIS is a high-grade non-invasive transitional cell carcinoma, a very aggressive cancer that involves only the inner

lining of the bladder. CIS does not look like a tumor, but more like a flat, red area on the bladder wall.

2.1 Staging. Tumors can be classified according to their depth of invasion into superficial (Ta, T1) and invasive (T2, T3, T4). The 2002 Tumor, lymph node, metastasis (TNM) staging system defines pTa as those tumors which have an exophytic growing part of papillary ("fingerlike") configuration and an endophytic growing part, restricted to the urothelium; pT1 tumors as those penetrating below the basement membrane and invading the lamina propria but not the muscularis propria; pT2 tumors as those invading the muscularis propria; pT3 tumors as those invading perivesical tissue; and pT4 tumors as those invading other organ structures (prostate, uterus, vagina, pelvic wall, or abdominal wall) (Sobin and Wittekind CH, 2002). A simplified staging into superficial and invasive disease has been used as a prognostic factor, although accurate staging is a more powerful prognostic factor and is critical for patient management.

pT1 tumors. The diagnosis of pT1 tumors is often difficult, with substantial interobserver variability (Lopez-Beltran and Cheng, 2003). The proposal by some urologists to treat early invasive tumours more aggressively, makes the accurate detection of pT1 lesions a relevant subject in clinical practice. In recent years, the morphologic features and patterns of invasion of tumour into the lamina propria have been more accurately characterised and standardised and, although some of the features are subtle, they can be of great utility in the accurate assessment of pT1 staging. Several studies have explored the utility of invasion into the muscularis mucosae for subclassification of the pT1 tumors (Holmang et al., 1997;Hermann et al., 1998). Muscularis mucosa consists of thin and wavy fascicles of smooth muscle, which are

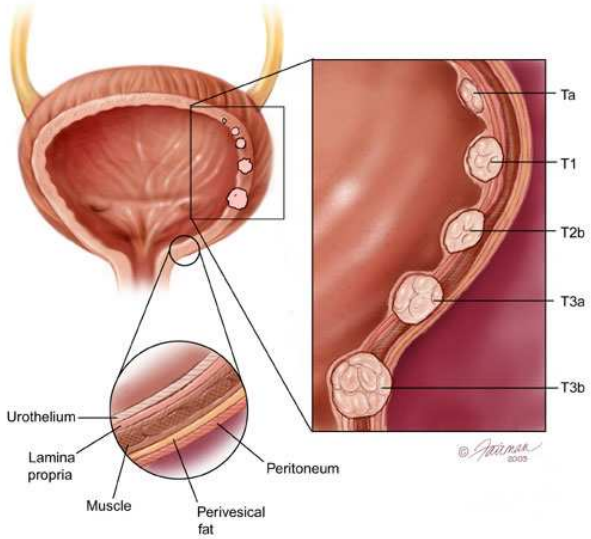


Figure 6. Bladder cancer staging-

frequently associated with large-caliber blood vessels. Muscularis mucosa can be identified in only 15-83% of biopsy specimens and 6% of radical specimens do not have muscularis mucosa (Cheng et al., 1999b). A more simple approach for subdividing papillary pT1 tumors may be to measure the maximum length of the invasive front, parallel to the overlying epithelium, to distinguish microinvasion from extensive infiltration. The extent of invasion is an important independent prognostic factor for progression-free survival (van der Aa et al., 2005).

pT2 tumors. The 2002 TNM system subclassifies pT2 tumors into 2 categories: cancer invading <50% of the depth of muscular propria (pT2a) and cancer invading >50% of the depth of muscularis propria (pT2b). A number of studies have compared clinical outcomes between patients with pT2a and pT2b tumors and have failed to find a difference in prognosis between patients in these 2 tumor categories (Cheng et al., 1999a).

pT3 tumors. This category is also divided into 2 subcategories: pT3a tumors display microscopic extravesical tumor extension and pT3b tumors display gross extravesical extension. Quek et al (Quek et al., 2004) examined 236 patients with pT3 tumors and found no difference in recurrence or survival between patients classified as pT3a or pT3b.

2.2 Grading. Grading is a method by which pathologists evaluate the cytologic and/or growth pattern characteristics of a tumor to predict its biologic potential. Multiple grading schemes have been developed throughout the years (Figure 7) (Reuter, 2006). Classifications differ in their recognition of a benign tumor and the categories into which TCCs (transitional cell carcinoma) are graded. Mostofi et al. (Mostofi et al., 1973) established a classification for the World Health Organization (WHO), distinguishing a class of papillomas from TCC grades 1, 2, and 3. Their definition of papillomas was restrictive, being applied to <1% of patients who had a papillary tumor, usually solitary, with no more than 8 cell layers of normal-appearing transitional epithelium on a fibrovascular core. Subsequent clinicopathologic studies suggested that many of the grade 1 papillary “carcinomas” as defined by this classification scheme were in fact biologically benign.

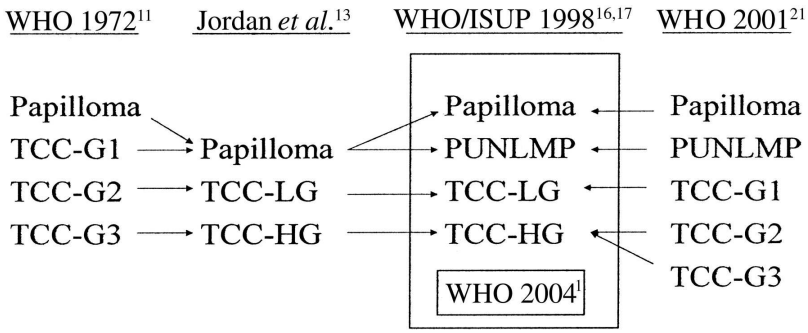


FIGURE 7. Grading of urothelial carcinoma: a historical perspective. G grade; HG high grade;LG low grade; PUNLMP papillary urothelial neoplasm of low malignant potential; TCC transitional cell carcinoma; ISUP International Society of Urological Pathology; WHO World Health Organization. (Adapted from Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs (1book), WHO 11 (2book), Cancer 13 (Jordan et al., 1987), Am J Surg Pathol 16 (Epstein et al., 1998), Hum Pathol 17 (Reuter et al., 1999), and Urology 21 (Samaratunga et al., 2002)).

Jordan et al. studied 400 patients with urothelial tumors classified by WHO criteria and monitored for 10 to 20 years. Of 91 with grade 1 TCC, only 7 (8%) subsequently developed more advanced tumors; the remaining 84 had no progression of disease and had normal life expectancy, although some had recurrent tumors of the same stage and grade. All patients whose cancer progressed had an increase in grade before or at the time of invasion. None of the patients with grade 1 TCC presented with invasion and 55 of these patients (60%) had no recurrences (Jordan *et al.*, 1987). The available data suggested that up to 20% of papillary bladder tumors were not malignant and should not be classified as carcinomas. In December 1998, members of the WHO and the ISUP published a consensus classification of urothelial neoplasms of the urinary bladder due to the need to develop a universally acceptable classification system for bladder neoplasia that could be used effectively by pathologists, urologists and oncologists. This classification not only covered neoplastic conditions but also the nomenclature of preneoplastic lesions (Epstein *et al.*, 1998;Reuter *et al.*, 1999). Subsequent studies validated the clinical significance of this classification and, in 2004, it was formally adopted as the WHO classification of bladder tumors (Eble J et al., 2004).

According to this classification, grading is as follows:

1) Flat and papillary hyperplasia. Flat hyperplasia consists of a markedly thickened mucosa without cytologic atypia. Papillary hyperplasia is characterized by urothelium of variable thickness exhibiting a slightly undulating growth.

2) Flat lesions with atypia which can be classified as reactive atypia, dysplasia and carcinoma in situ (CIS). Dysplasia is a lesion with cytologic and architectural abnormalities. CIS is a flat lesion of the urothelium characterized by normal urothelial thickness and cells which are highly anaplastic.
3) Papillary neoplasms which can be classified as papilloma, papillary neoplasm of low malignant potential (PUNLMP), low grade papillary carcinoma and high grade papillary carcinoma. Papilloma is a lesion where normal appearing urothelium lines papillary fronds. PUNLMP describes lesions that do not have cytologic features of malignancy yet have thickened urothelium. Low grade papillary carcinoma exhibits an overall orderly appearance but has minimal anomalies in architecture and/or cytologic features. High grade papillary carcinoma is characterized by a disorderly appearance due to marked architectural and cytologic abnormalities.

This classification separates carcinomas into two groups, which simplifies the grading process and improves interobserver reproducibility. However, high grade tumors may include cases that are less aggressive than others.

3. Epidemiology of bladder cancer

3.1 Age, sex and race. Overall, bladder cancer is observed in three times as many men as women although there is substantial geographic variation in this ratio. As with most cancers, it also occurs with much greater frequency among the elderly. The average age at diagnosis is 70 years old. The incidence and mortality of bladder cancer increases with age. Black patients are less likely than whites to develop bladder cancer. However, once they are diagnosed, they experience poorer survival (Prout, Jr. et al., 2004). This difference is due to the greater extent of disease at diagnosis and a higher percentage of aggressive histologies compared to white patients.

3.2 Lifestyle and environmental risk factors: tobacco, occupation, drinking water, hair dyes, diet, geographic variation. Bladder cancer development is affected by contact between the vesical epithelium and carcinogenic substances excreted in

urine. Such substances may be directly ingested or inhaled, or come from the metabolism of other products in the body. The two lifestyle/environmental risk factors acknowledged as being most important for this type of cancer are smoking and occupational exposure to aromatic amines.

Tobacco: cigarette smoking is the best established risk factor for bladder cancer with the risk increasing in proportion to the intensity of smoking habit (Lopez-Abente et al., 1991;Brennan et al., 2000). Tobacco accounts for 50% of cases in men and 35% in women (Zeegers et al., 2000). Bladder cancer risk declines sharply after cessation by approximately 30% after 1-4 years; however, even after 25 years the risk does not reach the level of non-smokers among users of black tobacco (Samanic et al., 2006). The precise mechanisms by which smoking causes bladder cancer have yet to be fully clarified. Tobacco smoke is known to contain many potential human carcinogens, including aromatic amines and N-nitrosamines (Johansson and Cohen, 1997;Phillips, 2002), compounds that have been linked to DNA adduct formation and bladder cancer development. Cigarette smoke also generates large quantities of free radicals (Pryor and Stone, 1993), which are highly reactive species that induce base modifications and strand breaks. Thus, smoking may induce bladder cancer through different types of DNA damage.

Occupations associated with increased risk of bladder cancer are those in the dye, textile, leather, chemical and rubber industries.

- Dye industry: benzidine and 2-naphthylamine are human carcinogens found in the dye industry. The first evidences associating bladder cancer to dye manufacturing workers date to the 1950s (SPITZ et al., 1950). Subsequent studies have confirmed this association (Piolatto et al., 1991;Naito et al., 1995). Rosenman et al (Rosenman and Reilly, 2004) have recently confirmed the high risk of bladder cancer even after exposure to benzidine had ceased.

- Textile industry: exposure to azo dyes have been associated with bladder cancer in numerous industries. Siemiatycki et al (Siemiatycki et al., 1994) found a weak association between bladder cancer and work with acrylic fibres and polyethylene. Long-term workers in this industry presented a 10-fold excess risk for bladder cancer. Similar findings have been reported by other authors (Band

et al., 2005), although opposite studies are also noted (Silverman et al., 1989). A recent study (Serra et al., 2000) describes no overall excess risk of bladder cancer in the textile industry. However, some elevated risks were observed among the workers with the highest exposures, therefore results remain controversial (Consol et al. unpublished).

- Rubber industry: antioxidants containing 2-naphthylamine have been used in rubber manufacturing industries. In 1954, it was observed that British rubber workers had twice the expected bladder cancer mortality rate (CASE and HOSKER, 1954). Since then many studies have found an association between bladder cancer and the rubber industry in the Lombardy region in Italy (Amendola et al., 2005), in British rubber workers (Veys, 2004), and in a cohort of 12 studies from 9 countries (Kogevinas et al., 1998).

Drinking water: an association between by-products of chlorination in drinking water and bladder cancer risk was first suggested in the 1980s (Crump and Guess, 1982). Water is disinfected by chlorination, ozonization, and upon contact with organic matter, by-products are generated which can increase bladder cancer risk. A recent pooled analysis of 6 case-control studies of bladder cancer showed total fluid intake was associated with an increased risk of bladder cancer in men, confirming previous results obtained by the same group (Villanueva et al., 2004). Increased bladder cancer risk was shown to be associated to intake of more than 5 cups of coffee a day (vs. less than 5) and to Trihalomethane exposure. Also, an association of bladder cancer risk with tap water consumption and exposure through inhalation/dermal absorption has been recently described (Villanueva et al., 2006). The association between drinking water and bladder cancer risk may also be due to contamination with arsenic, nitrites and other metals in water. Arsenic association to bladder cancer comes from studies of high level exposure in Taiwan, China, and Argentina (Hopenhayn-Rich et al., 1998;Wang et al., 2003;Chen et al., 2003), although other countries also have high levels of arsenic in their groundwater. Increased levels of arsenic exposure in bladder tumor patients have been linked to high levels of chromosome instability, suggesting that these tumors may behave more aggressively than tumors from unexposed patients (Moore et al., 2002).

Hair Dyes: the risk of bladder cancer associated with hair dyes is uncertain. Several studies starting from the 1980s have found no association between bladder cancer risk and hair dye daily use (Hartge et al., 1982;Takkouche et al., 2005). However, others differ. A case-control study in 2001 found a 2 fold risk for once-a-month dye users and a 10 fold risk for hairdressers (Gago-Dominguez et al., 2001). A meta-analysis in 2005 found that permanent hair dye users experienced a higher risk of bladder cancer (Huncharek and Kupelnick, 2005). Many articles analyzing the association with hair dyes are pooled analysis of previous studies. Recently Kogevinas et al have studied 150 patients and 160 controls and have not found an association between the use of hair dyes and bladder cancer risk (Kogevinas et al., 2006).

Diet: coffee drinking, artificial sweetners, alcohol consumption, fruit and vegetable intake and dietary supplements have been studied in relation to bladder cancer risk. An association with coffee was suggested in 1971 (Cole, 1971). Since then many studies have analyzed the relationship and the conclusion of the contradictory data implies that if coffee is a carcinogen it is a weak one. A meta-analysis of 6 dietary factors has shown an increased risk of bladder cancer associated with diets low in fruit intake or rich in fat intake and a slight increase in risk with low vegetable intake (Steinmaus et al., 2000). This suggests that a diet high in fruits and vegetables and low in fat intake may help prevent bladder cancer.

Geographical Variation: bladder cancer incidence rates vary worldwide (Ferlay *et al.*, 2004), Western Europe and America show the highest incident rates and mortality rates are higher in Western Europe and North Africa. In Spain, a recent article has analyzed the municipal distribution of bladder cancer mortality. It has pointed to a few areas where mortality by bladder cancer is particularly high. Cadiz, Seville and Huelva show highest risks possibly due to environmental pollution of the river Guadalquivir and mining industry; the Barcelona area also shows higher risk, possibly due to the textile and mining industries (Lopez-Abente et al., 2006).

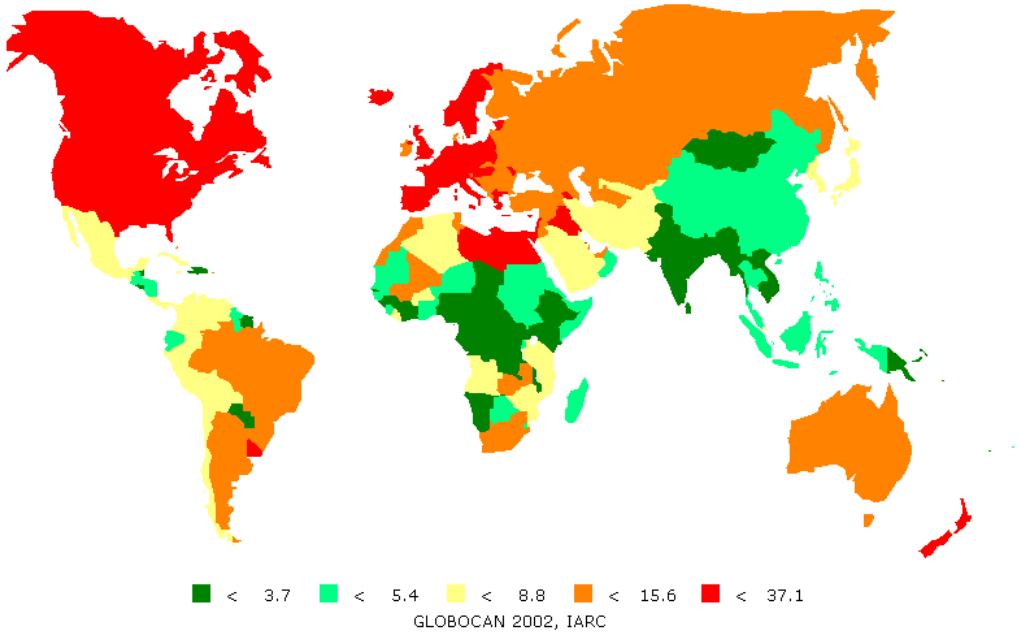


Figure 8. Age-standardized incidence rate of bladder cancer (males) per 100,000.

3.3 Urinary conditions. A positive association has been reported between urinary bladder infection and risk of bladder cancer. A type of urologic condition associated with bladder cancer is schistosmiasis of the urinary tract. The association of bladder cancer with schistosomiasis seems to be related to the endemicity of the parasite (World Health Organization, 1994;Pisani et al., 1997). Bilharzial bladder cancer is most common in Northern Africa. In Egypt, this neoplasm accounts for 31% of the total cancer incidence and is ranked first among all types of cancer recorded in Egyptian males and second only to breast cancer in females (Kahan et al., 1997). This type of bladder cancer has a clinicopathologic pattern that differs from that seen in North America and Europe. The peak age of individuals diagnosed is younger and most tumors present invasive lesions with deep muscle infiltration and are mainly of the squamous cell variety (El Sebaie et al., 2005).

3.4 Familial Bladder cancer.

a) Familiar aggregation in bladder cancer. Although bladder cancer is not known as a familial cancer, several case reports and population based studies have described familial clustering of the tumor (Kramer et al., 1991;Kunze et al., 1992;Kiemeney and Schoenberg, 1996). The largest bladder cancer familiar study was undertaken

in 1985 (Kantor et al., 1985). Nearly 3000 patients were compared against 5782 controls. A positive family history was found. A study by Aben et al (Aben et al., 2002) studied 1193 patients and 853 controls and found an increased risk of 1.8 in first degree relatives. Other groups have been unable to confirm these findings (Najem et al., 1982). A study based on the Icelandic population describes a slight increase among relatives of patients. However, the greater risk of more distant relatives raises questions about the association (Kiemeney et al., 1997). In a twin study from Scandinavia, the concordance rate of bladder cancer among monozygotic vs. dizygotic twins was 3 times higher, suggesting a genetic etiology (Lichtenstein et al., 2000) however, no single gene has been associated to bladder cancer.

b) High penetrance genes and familiar cancer syndrome. Patients with hereditary nonpolyposis colon cancer (HNPCC) have more than 20 times the risk of the normal population for bladder cancers (Lenz and Harpster, 2003). Another group of patients with a higher risk of bladder cancer are retinoblastoma patients, a highly penetrant gene which is known to increase the risk of bladder cancer is the Rb1 gene (Tarkkanen and Karjalainen, 1984). However, overall, the findings reported support the existence of a familial subtype of bladder cancer that appears to be site-specific and not part of any known hereditary cancer syndrome.

One argument accounting for the disparity seen between studies has been the lack of controlling of known risk factors such as smoking and susceptibility genes associated to carcinogens at which these patients are exposed. Work by Aben et al (Aben et al., 2001) shows that genetic susceptibility may partly explain the familial clustering (Murta-Nascimento et al submitted).

c) Low penetrance susceptibility. The first genes studied in this group were those related to carcinogen metabolism. Many carcinogens are chemically inert and require metabolic activation. Subsequently, these carcinogens may also undergo detoxification. Imbalance between activation and detoxification may result in an increased bladder cancer risk via accumulation of active metabolites, increased DNA adduct formation, and mutations. Genetic variation in genes that encode activation and detoxification enzymes has been of special interest because certain variants may be associated with a differential ability to metabolize carcinogens.

Cytochrome P450 enzymes are activator enzymes involved in the metabolism of aromatic amines and Polycyclic Aromatic Hydrocarbons (PAHs). N-acetyltransferases (NAT) are detoxifying enzymes involved in the metabolism of aromatic amines and hererocyclic amines. NAT1 and NAT2 genes are highly polymorphic, with over 40 human alleles identified (Hein et al., 2000). Several alleles have been shown to vary in enzymatic activity and are referred to as slow or rapid aceylators. Glutathione S-transferases (GSTs) are also detoxifying enzymes that catalyze the conjugation of reduced glutathione to electrophillic substrates (Mannervik, 1985). The most studied are GSTM1 null, GSTP1 and GSTT1 null polymorphisms. Polymorphisms in NATs (NAT1, NAT2) and GSTs (GSTM1, GSTM3, GSTP1, GSTT1) have been widely studied and results are controversial. Garcia-Closas et al (Garcia-Closas et al., 2005) have recently carried out a large bladder cancer case-control study of polymorphisms in these genes in a large group of patients. A significant association has been found between bladder cancer risk and GSTM1 null and NAT2 slow genotypes; the effects of the NAT2 slow acetylator genotype were restricted to cigarette smokers (Gu et al., 2005).
The overall evidence for the contribution of low penetrance susceptibility alleles to bladder cancer genes and the clear demonstration of a gene-environment interaction between NAT2 and tobacco smoking make bladder cancer one of the best model systems to study the contribution of genetic polymorphisms to a complex disease.

4. Diagnosis of Bladder cancer

4.1 Signs and symptoms. The most common symptom of bladder cancer patients is painless hematuria, which occurs in about 85% of patients. Bladder cancer may also cause bladder irritability and urinary frequency, urgency and dysuria. These signs and symptoms are not specific to bladder cancer, and are also caused by noncancerous conditions, including prostate infections and cystitis. Other, less common, symptoms include flank pain caused by ureteral obstruction, lower extremity edema and a palpable pelvic mass. Very rarely, patients present with symptoms of advanced disease such as weight loss and abdominal or bone pain from distant metastases.

4.2 Cystoscopy. It is the gold standard for the diagnosis of bladder cancer, but it is invasive and relatively expensive (Lotan and Roehrborn, 2002). Appearance of bladder tumors by cystoscopy is important for diagnosis and treatment. Information such as number, size, shape and location of the tumor(s) is easily obtained. Cystoscopically, bladder tumors can be classified according to their appearance, surface characteristics, and tumor base. Approximately 70% of urothelial tumors are papillary, 10% nodular and 20% are mixed. Because of the frequency of tumor recurrences, cystoscopic surveillance is essential after tumor resection, after intravesical prophylaxis or treatment, and during the maintenance prophylaxis period. The assumption is that frequent cystoscopies allow treatment of recurrences at an early stage, lowering recurrence and progression rates. Unfortunately, cystoscopy can fail to detect the most important indicator of dangerously unstable urothelium: CIS (Witjes, 2004). Areas of CIS can be visually indistinguishable from the surrounding normal bladder. To detect CIS, Hexyl aminolevulinate, a photosensitising agent, can be used. This agent induces the accumulation of a photoactive agent that emits red fluorescence when illuminated (Frampton and Plosker, 2006). Other compounds such as pirarubicin hydrochloride (Uchikoba et al., 2005) are also used.

4.3 Imaging of bladder cancer at diagnosis. For the evaluation of upper urothelial tract cancer IntraVenous Urography (IVU) is performed. IVU is a radiographic examination of the urinary tract with intravenous injection of contrast medium.
To stage bladder cancers, 11-C choline positron emission tomography (PET) (Picchio et al., 2006), computed tomography (CT) (Blanco et al., 2003) and magnetic resonance (MRI) are used to delineate the perivesical tissue but their accuracy is variable, ranging from 40 to 98% (Paik et al., 2000). The extent of disease evaluation usually includes chest radiography, liver function tests, and ultrasound (Lerner and Skinner, 2000). Abdominal and pelvic imaging (CT or MRI) is often reserved for patients with abnormal liver function tests or advanced local cancer based on TURBT and bimanual examination.

4.4 Bladder cancer screening. The goal of screening is to improve survival by detecting cancer at an earlier stage. The ideal test should be noninvasive, inexpensive and exhibit high sensitivity, specificity, and accuracy. The performance

of a screening test improves as the prevalence of the disease increases. In the general population, the low prevalence of bladder cancer limits the utility of screening. However, patients at high risk for bladder cancer have a high prevalence of the disease, leading to a more favorable performance by a screening test. Screening studies have been conducted in high-risk populations exposed to occupational carcinogens, including β-naphthylamine (Cassidy et al., 2003;Felknor et al., 2003), 4,4'-methylene-bis-2-chloroaniline (Ward et al., 1990), benzidine (Carreon et al., 2006), and coal-tar pitch volatiles (Tremblay et al., 1995) but it has not been proven that screening improves outcome in high-risk patients.

Tests that may be used for screening include assays to detect hematuria, cystoscopy, bladder imaging, urine cytology and tumor markers in biological fluids. Although no tests have been adequately evaluated for bladder cancer screening, voided urine cytology and chemical dipstick for hematuria are the only ones for which screening trials with a large sample size or with long-term follow-up have been conducted. Recently, the U.S. Food and Drug Administration has approved screening patients at high-risk for bladder cancer using the urine-based marker NMP22 (Lotan et al., 2006). Table 1 shows a list of tests used for bladder cancer detection. A review of bladder markers has been published recently (Konety, 2006).

Table 1. Tests for bladder cancer detection (modified from (Lokeshwar et al., 2005)).

Test /Marker	Marker detected	Sensitivity (%)	Specificity (%)
Cytology	Tumor cells	11-76	>90
Hematuria detection	A: Hemoglobin B: Red blood cells	50-90 100	Low 100
BTA-Stat	Complement factor H-related protein	36-89	50-70
BTA-TRAK	Complement factor H related protein (different assay type)	57-83	50 benign 90 healthy
NMP-22	Nuclear mitotic apparatus protein	47-100	55-80
BLCA-4	Nuclear matrix protein	96.4	81-100
Survivin	Apoptotis inhibitor	100	87-100
UBC	Cytokeratin 8 and 18	36-79	88-92
Cytokeratin 20	Cytoskeletal protein	82-87	55-70
CYFRA 21-1	Cytokeratin 19	75-97	67-71
HA-Haase	Hyaluronic acid and hyaluronidase	88-94	84
Microsatellite DNA test	Microsatellite markers on chromosomes	72-97	>95
TRAP assay	Telomerase enzyme activity	70-90	60-70
hTERT	hTERT	83-95	60-70
ImmunoCyt	Carcinoembryonic antigen, MUC2, bladder tumor cell-associated mucin	38-90	73-80
DD23	185 kDa tumor-associated antigen	73-100	33-67.5
Quanticyt	Mean nuclear shape and DNA content	-	-
Urovysion	Alterations in chromosomes 3, 7, 17, 9p21	68-87	>90

A recently developed method for screening of recurrences among patiens with bladder cancer is the Urovysion test. It is a multicolor, multitarget, interphase fluorescent in situ hybridization (FISH) probe mix containing centromere probes for chromosomes 3, 7, and 17, and a locus specific probe to the band 9p21 (p16). This has proven to be highly sensitive in voided urine samples and bladder washings (Bubendorf et al., 2001). FISH testing in urinary specimens of patients under surveillance could help to classify patients with different risks of recurrence or progression, allowing the reduction in the number of cystoscopies in low risk patients (Sarosdy et al., 2006;Zellweger et al., 2006).

5. Therapy

The treatment of bladder cancer is based on stage and grade of the tumor. Three major questions in patient management are: 1) which patients will not recur after initial treatment, 2) which patients with non-muscle invasive bladder cancer will have progression to invasive disease and 3) which patients with invasive tumors will develop metastatic disease after cystectomy. Stage, tumor grade, size, multifocality, presence of CIS and lymph node status are currently the most useful parameters for making therapeutic decisions and evaluating the prognosis.

5.1 Transurethral resection of bladder tumor (TURBT).

It is the standard of care of all superficial tumors. It provides diagnostic information and generally achieves therapeutic benefit. The goals are to determine the stage and grade of the tumor and to resect all grossly visible tumors when indicated. Bladder wash cytology may be obtained before TURBT by irrigating the bladder with saline through a catheter or cystoscope sheath. Bladder wash cytology is the gold standard for detection of CIS (Planz et al., 2005), even when the urothelium appears grossly normal. There are two basic techniques for performing TURBT: staged and en bloc.

1) Staged resection. It consists of several phases. The first one is resection of tumor that protrudes into the bladder lumen. The surgeon begins resecting superficially, starting at one side of the tumor and gradually progressing to the other side. Resection of the next layer of tumor is done in the same way. This process is continued until the base is reached. The second phase is resection of the base of the tumor and of a portion of the underlying bladder.

The third phase is resection of tissue surrounding the tumor base. Tissue removed during the third phase determines the status of the lateral margins of resection.

2) En bloc resection. The resection loop is aproximately 1 cm in diameter. Therefore, tumors <1 cm may be resected in a single specimen using the standard loop. Techniques for en bloc resection of tumors that are up to 3 cm in dimension have also been described (Saito, 2001). Proponents of en bloc resection believe that it may permit more accurate pathologic assessment by preventing tumor fragmentation, preserving the orientation of the tumor relative to the bladder wall, and decreasing cautery artifacts at the tumor base.

Initial TUR might not remove all disease, specially when there are multiple or invasive tumors, leading to an increased risk of early tumor recurrence and stage progression. Recent studies show that a second or re-staging TUR can efficiently detect residual disease, including invasive cancer in a substantial proportion of patients (Herr and Donat, 2006).

5.2 Intravesical therapy. Patients at high risk for recurrence or progression, identified according to the clinical-pathological parameters stated above, should receive additional intravesical therapy. Patients with high grade tumors are generally considered candidates to receive intravesical therapy. The two major modalities are intravesical chemotherapy and intravesical immunotherapy.

Intravesical chemotherapy: all drugs tested have been shown to reduce recurrence rates. Mitomycin C is widely accepted as an efficient therapy (Clarke et al., 2006) used for low risk tumors. Epirubicin has also been shown to reduce the recurrence of bladder tumors with few side effects (Liu et al., 2006). Various multicenter, randomized, prospective studies demonstrate that the risk of recurrence can be reduced by 50% at 2 years and by $\geq$ 15% at 5 years with a single dose of adjuvant intravesical chemotherapy (Oosterlinck et al., 1993;Tolley et al., 1996;Solsona et al., 1999;Bartoletti et al., 2005a). There are some side effects such as irritation, pain, bladder inflammation.

Intravesical immunotherapy with Bacillus Calmette-Guérin (BCG) is the most effective therapy in the treatment of high risk non-invasive bladder cancer (Sylvester et al., 2005;Han and Pan, 2006). Intravesical BCG instillation stimulates

a local cellular immune response and the release of a wide variety of cytokines such as interleukin 2, tumor necrosis factor and interferon gamma. The immune stimulation persists for many months and results in tumor destruction, probably through non-specific defence mechanisms. BCG attaches to tumor cells by means of specific receptors (fibronectin and integrin) and can penetrate deep into the bladder wall and be carried to regional lymph nodes and beyond. The onset of action is slow, 6 weeks are required for optimum stimulation and it sometimes takes 6 months for immune destruction to be complete. Although patients treated by initial intravesical therapy have a high likelihood of achieving a complete remission on the short-term, there is debate as to the long-term risk of progression. Cookson et al. reported that after 15 years of follow-up, 53% of patients with initial high-risk superficial bladder cancer progressed to muscle-invasive disease, 36% eventually underwent cystectomy and 34% died of bladder cancer. Only 27% were alive with an intact bladder (Cookson et al., 1997). Similar results have been described by Shahin et al (Shahin et al., 2003). There are significant side effects associated with intravesical therapy. Up to 90% of patients have irritative lower urinary tract symptoms, and a small proportion has serious complications such as sepsis and contracted bladders.

5.3 Cystectomy. The benefits of cystectomy must be balanced against the morbidity and mortality of the procedure. Radical cystectomy is now a relatively common procedure in most major medical centers and carries a perioperative mortality rate of approximately 2-3% and a complication rate of 28% (Konety et al., 2006). However, the results of therapy are largely surgeon-dependent. Disease-specific survival after cystectomy for non-muscle invasive urothelial carcinoma is higher than that for patients with muscle-invasive disease but it is not 100% due largely to understaging. A recent review on the largest series of patients treated with cystectomy confirmed long-term survival in patients with extravesical and/or lymph node disease of 25-47% at 5 years and 17-27% at 10 years (Izawa et al., 2006). There is significant evidence supporting the use of early cystectomy for T1 tumors, most of which are high grade, due to the failure of intravesical therapy and the reported good quality of life after cystectomy (Malavaud, 2004). However, the proponents of an aggressive initial approach acknowledge that a significant number of patients will be rendered disease-free with bladder-sparing strategies. Current

indications for surgery are proposed in the case of young patients with T1 tumors with >1 bad prognostic factor: multifocality, CIS, prostatic involvement, and a tumor of difficult position to resect. Bianco et al. performed a multivariate analysis to identify risk factors that influenced cancer-specific survival in patients undergoing cystectomy (Bianco, Jr. et al., 2004). They found that patients with concomitant CIS and with persistent disease after initial BCG therapy were at significant risk.

New advances in surgery for bladder cancer include orthotopic bladder substitution, which may improve patients' quality of life. An orthotopic bladder is a reconstruction surgery that allows normal urination through the urethra after bladder removal and replacement by using parts of the small intestine to make a new pouch. A recent published retrospective comparison of orthotopic versus ileal conduit urinary diversion describes that the type of diversion does not affect the oncologic outcome of radical cystectomy (Yossepowitch et al., 2003). Another advance in surgical technique has been the nerve sparing approach to maintain potency and continence postoperatively.

The critical issue for cystectomy is how to choose the right time to perform surgery: not too early to avoid its use in patients who have a low risk of progression after intravesical therapy and not too late so the risk of regional spread and metastases does not increase, since tumors of stage T2 or greater represent a life-threatening disease. There is yet no complete consensus on the indications for cystectomy. According to one study (Herr and Sogani, 2001), 15 year survival is 92% if cystectomy is performed within 2 years of initial therapy and 56% if it is performed after 2 years in patients in which BCG treatment failed.

5.4 Radiotherapy. It is rarely used alone for medically fit patients with invasive carcinoma of the bladder in the United States but it is still commonly used in Canada and Europe. There is no absolute consensus regarding field selection and field size, but there is a general agreement that the whole pelvis should receive approximately 50-55 Gy, with a boost to the tumor of an additional 20 Gy. Radiation therapy as a single modality achieves local control in 30-50% of patients with muscle invasive bladder cancer (Birkenhake et al., 1998).

5.5 Chemotherapy. Cytotoxic chemotherapy has, in general, well defined mechanisms of action such as alkylation (mitomycin, thiotepa) or intercalation and

topoisomerase II inhibition (doxorubicin). Cell destruction depends on direct contact with the cancer cell and is proportional to drug concentration and duration of exposure. However, toxicity is also proportional to these parameters. Chemotherapeutic drugs are non-specific and penetrate the bladder by concentration gradient diffusion. The deepest tumor cells therefore unfortunately receive the least effective drug treatment. The onset of action is quick, the drugs can be retained for 2 hours and little remains after voiding.

Systemic chemotherapy is used to treat locally advanced and metastatic disease and may be useful perioperatively as neoadjuvant (to shrink a tumor before surgery with the hope of downstaging the tumor) or adjuvant (to destroy leftovers after surgery) therapy for patients at significant risk for relapse. Since the eighties, MVAC (methotrexate, vinblastine, adriamycin and cisplatin) has been considered the best standard systemic chemotherapy for advanced disease (Connor et al., 1989;Sternberg et al., 1989). A multicenter, prospective, randomized trial has found neoadjuvant MVAC to be beneficial over cystectomy alone (Sternberg, 2002). Adjuvant chemotherapy after cystectomy has been reported to be beneficial in multiple studies (Borden, Jr. et al., 2003). Metastatic urothelial cancer has been treated with MVAC with a response rate of 40-70% and median survival of approximately 1 year (Calabro and Sternberg, 2002). Because of the toxicity of MVAC, attention has turned to other combinations. Gemcitabine and taxanes have demonstrated excellent activity alone and in combination with each other, and also as doublets with cisplatin. In fact, gemcitabine-cisplatin combination has been proposed as an alternative to MVAC (von der et al., 2000). Therefore, systemic chemotherapy is also an evolving area of clinical research.

The incapacity to predict the outcome from chemotherapy regimes, may be a function of the heterogeneity of response to the cytotoxic agents employed. A number of studies have focused on the molecular basis of tumor response to chemotherapy. Raghavan et al summarized some of these studies (Table 2 (Raghavan, 2003)).

Table 2. Molecular Parameters Predictive of Response to Chemotherapy in Bladder cancer patients.

Agent	Molecular index	Bladder cancer studies
Cisplatin	*ERCC1*	no
Cisplatin	*P53*	yes
Cisplatin	Glutathione	yes
Cisplatin	Metallothionein	yes
Methotrexate	DHFR	no
Doxorubicin	MDR	yes
Doxorubicin	*P53*	yes
Vinca alkaloids	MDR	yes
Taxanes	MDR	yes
Taxanes	*BAX, BCL 2*	yes
5-FU	TS	no
5-FU	DPD	yes
gemcitabine	Deoxycytidine kinase	no
gemcitabine	Deoxycytidine deaminase	no

5-FU: 5-fluorouracil; TS: thymidylate synthase; DPD: dihydropyrimidine dehydrogenase.

Intracellular glutathione, which decreases the cytotoxic activity of agents, such as cisplatin, on tumor cells has been studied in bladder cancer (Pendyala et al., 1997). Another predictor of chemosensitivity are the multidrug resistant genes (MDR). The expression of the mdr1 gene and multidrug resistance associated proteins (Mrap) may lead to a cell phenotype with high multidrug resistance. Overexpression of the mdr1 gene leads to drug resistance via up-regulation of a membrane-bound 190kDa phosphoglycoprotein that serves as an energy-dependent drug flux pump and eliminates toxic metabolites out of the cell. Petrylak et al (Petrylak et al., 1994) demonstrated enhancement of P-glycoprotein expression, P-glycoprotein is an mdr family member, in tumors associated to MVAC treatment. In contrast, Siu et al (Siu et al., 1998) did not find any prognostic significance in the expression of P-glycoprotein.

Currently, there is a great interest in targeted therapy i.e. therapy selected on the basis of the molecular genetic changes associated with a given tumor in a given patient. Among the targets of interest is for example the epidermal growth factor receptor (Dominguez-Escrig et al., 2004). Phase I trials for patients with a variety of solid tumors are ongoing (Janmaat and Giaccone, 2003). There are 2 main classes of inhibitors of EGFR. 1) Monoclonal antibodies directed against the extracellular domain of the receptor that block binding of the ligand and 2) small molecules that inhibit the tyrosine kinase activity. Gefitinib (Iressa ZD 1839) is an orally active, EGFR1-specific tyrosine kinase inhibitor (Sordella et al., 2004). Recent work in lung

cancer has shown that an increase in kinase activity resulting from increased copy number or mutation has very distinct pharmacological meaning: the latter makes the receptor hypersensitive to the effects of kinase inhibitors as gefitinib (Endo et al., 2006). Other examples include BCL-2 in which phase III clinical trials with antisense oligonucleotides are underway (Nicholson, 2000), Vascular endothelial growth factor (Xia et al., 2006), TRAIL (libro 10) and the AKT/PTEN pathway. Small-molecule drugs that inhibit AKT function or exogenous introduction of PTEN, via an adenoviral vector-mediated delivery have successfully inhibited tumor growth and reversed drug resistance to doxorubicin in bladder cancer cell lines (Tanaka et al., 2000). Table 3 gives a selection of antibodies and small-molecules, potential targets for bladder cancer, currently in phase II and phase III clinical trials (Cote and Datar, 2003).

Table 3. Potential bladder cancer drugs.

	Target	Drug	Reference
Antibody drug	EGFR TK	IMC-225 (cetuximab) OSI-774	(Baselga, 2001)
	Her2/neu TK	Herceptin (trastuzumab)	(Ciardiello and Tortora, 2002)
	VEGF	Bevacizumab (Avastin)	(Jain, 2002)
Small-molecule drug	EGFR TK	ZD1839 (Iressa)	(Natale and Zaretsky, 2002)
	BCR-ABL TK	STI571 (Gleevec)	(DeMatteo, 2002)
	EGFR, Her ½	AG1478	(Normanno et al., 2003)
	Topoisomerase	Irinotecan	(Ulukan and Swaan, 2002)
	VEGFR1 and 2	SU5416	
	VEGF,bFGFR and PDGFR	SU6668	(Shepherd, 2001)
	AP-2alpha degradation	PS-341	(Nyormoi et al., 2001)
	Protein:farnesyl transferase	SCH 66336	(Cohen et al., 2000)
	Bcl-2	GEnasense	(Jansen et al., 2000)

5.6 Management of superficial bladder tumors. Because bladder tumors have a long natural history (including medical intervention in the term “natural”), their management is complex and can change significantly over time. Treatment is generally selected on the basis of the risk of progression to invasive disease, which is a life-threatening condition. The following proposal has been taken from a recent review of bladder tumor management (Chopin and Gattegno, 2002). Patients are distributed into 3 categories: low risk tumors include TaG1 or TaG2/3 without a recurrence in the first 3 months after initial treatment; intermediate-risk tumors include multifocal TaG2, TaG3 and T1G2; high-risk tumors include T1G3 and multifocal T1 tumors.

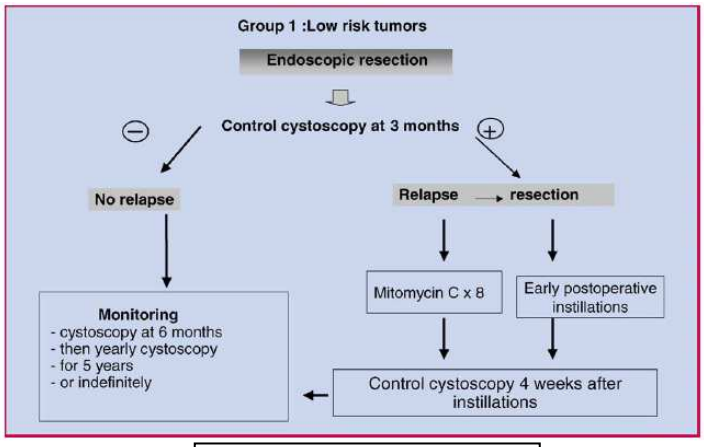


Figure 9A. Low risk tumors.

Low risk tumors: treatment is endoscopic resection. Control cystoscopy has to be carried out 3 months after primary treatment. If it is negative, the patient will be followed up at 6 months, then every year for 5 years. If positive, resection of the new tumor followed by intravesical instillations will be proposed. After 4 weeks of instillations, a new cystoscopy is performed. If no recurrence is observed, the patient will be monitored as in the low risk groups. However, if a recurrence occurs the patients enter the intermediate risk group.

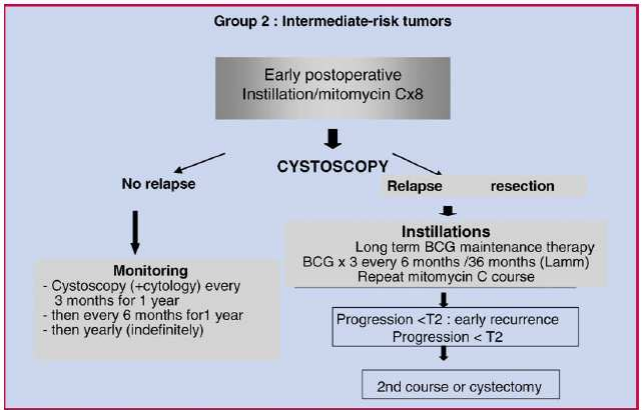


Figure 9B. Intermediate risk tumors.

Intermediate risk tumors: after complete resection, intravesical instillations should be performed. For pTa tumors, mitomycin C is preferentially used; for pT1 tumors, BCG induction treatment is used. If a control cystoscopy, performed after 4 weeks, shows no tumor, the patient is monitored by cystoscopy every 4 months during the first year and then every 6 months during the second year and once a year thereafter. In the case of a positive cystoscopy, the question arises whether conservative or radical treatment should be used.

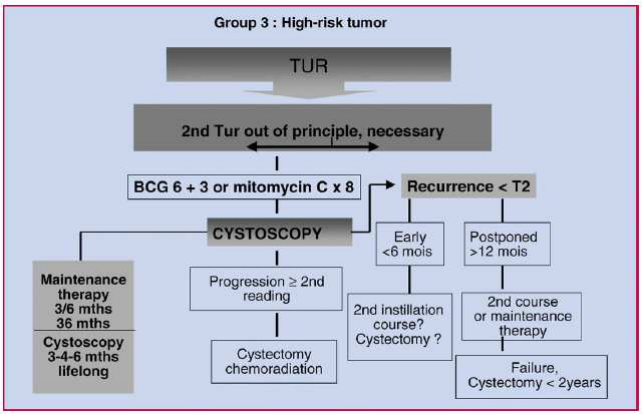


Figure 9C. High risk tumors.

High risk tumors: these tumors need a second resection to ensure resection is complete. Following BCG treatment, a control cystoscopy is essential. If it is negative, maintenance therapy is continued under life-long cystoscopic monitoring. If a tumor of lesser risk than pT2 appears within 6 months, cystectomy could be recommended due to the risk of progression. If recurrence is delayed, maintenance therapy or a second course is initiated. In case of failure, cystectomy is proposed within less than 2 years of initial therapy.

6. Bladder carcinogenesis

The requirements to make a cancer cell. Cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis. The vast catalog of cancer cell genotypes has been proposed to be a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth (Hanahan and Weinberg, 2000):

- Self-sufficiency in growth signals: alteration of cell surface receptors and components of the downstream cytoplasmic circuitries that receive and process the signals (Ras/Raf/MAPK and PI3K/PTEN/AKT pathway)
- Insensitivity to growth–inhibitory signals (pRb pathway)
- Evasion of programmed cell death (p53 and PI3K/PTEN/AKT pathways)
- Limitless replicative potential (Telomerase)
- Sustained angiogenesis
- Tissue invasion and metastasis

For many years, it has been clear that bladder cancer represents a complex disease (possibly more than one disease) and a paradigm of tumor progression. In more recent years, the advent of molecular techniques such as the polymerase chain reaction (PCR), comparative genetic hybridization (CGH), FISH, microarrays, laser-

capture microdissection, or tissue microarrays have led to an increased recognition of both the existence of more than one disease under the designation of “bladder cancer” and the progressive nature of the tumor. It is currently possible to characterize in depth the molecular alterations present in a given tumor or its recurrences. The challenge is to integrate this information in the clinical setting in an efficient and useful manner. To avoid reviewing the current status of knowledge on bladder cancer genetics as a list of molecular alterations, I have opted for presenting the information according to molecular/biochemical/functional pathways. Obviously, these pathways are not linear and many gene products participate in more than one of them and there is extensive cross-talk between them. Therefore, this approach represents an oversimplification applied here for the sake of clarity and brevity.

6.1 Gene level

A list of the most common oncogenes and tumor suppressor genes associated with bladder cancer has been published recently by Mhawech-Fauceglia et al and is shown below, with some modifications (Mhawech-Fauceglia et al., 2006b).

Table 4. Oncogenes Involved in Urothelial Bladder Carcinoma

Oncogenes	Locus	Gene alteration	% Freq. of amplification (a) / protein expression by IHC (%)	Association with tumor behavior	Function	Proposed prognostic value
TP73L, p63	3q27	Promoter methylation	63b/16-68		Cell maturation/differentiation	Poor prognosis
EGFR-1	7p12	Amplification/overexpression	4.60/23-100		Receptor protein tyrosine kinase	Poor prognosis /target for drug therapy?
FGFR3	4p16.3	Mutation	41-59/51	Low stage and grade	Tyrosine kinase receptor	Favorable clinical outcome, low rate of pathologic Ta recurrences
ERBB2	17q21	Amplification/overexpression	2-7/37-57	Invasive tumor	Receptor protein tyrosine kinase	Survival, anti- ER2/*Neu* therapy?
STK15	20q13	Amplification/overexpression	35/20	Invasive tumor	Centrosome duplication	Overall survival, metastasis-free interval
TRIO	5p15	Amplification/overexpression	1.5-17.0	Invasive tumor	Cell cycle, proliferation, cell cell interaction	None?
E2F-3	6p22	Amplification/overexpression	7-11.3/33-70	Invasive tumor	Cell cycle, DNA replication	Not known
MET	7q31	Amplification/overexpression	4.9-22.5c/5-100		Receptor protein tyrosine kinase	Poor long-term survival
MYC	8q24	Amplification/overexpression (d)	3.4-13.0/15-60	Invasive tumor	Cell proliferation	None?
FGFR1	8p13	Amplification/overespression (d)	6-25		Receptor protein tyrosine kinase	Not known
CCND1	11q13	Amplification/overexpression	13-33/13-25	Low grade and stage	Cell cycle: G1-S progression	None?
MDM2	12q13	Amplification/overexpression	1-5/9-29	Low grade and stage	Cell cycle, control p53	None?
TOP2A	17q21	Amplification/overexpression	1.5-3.4/73	Invasive tumor	DNA replication	Poor prognosis, response to chemotherapy
CDC91L1	20q11	Amplification/overexpression	33-36c		Urokinase receptor regulation, STAT3 activation?	Disease-related death?

a Amplification was measured by using fluorescent in situ hybridization; b measured by quantitative PCR analysis; c Amplification measured by Northern blot analysis and reverse transcriptase-PCR analysis; d There was no association between amplification and overexpression.

Table 5. Tumor suppressor genes Involved in Urothelial Bladder Carcinoma

TSG	Locus	Gene alteration	% Freq. of mutation and deletion (a) / loss of protein expression by IHC (%)	Association with tumor behavior	Function	Proposed prognostic value
FHIT	3p11	Deletion/promoter methylation	Deletion, 25; methylation, 16/50- 70		Apoptosis regulation	Poor patient survival
sFRP1	8p12	Promoter methylation	Methylation, 29b/66		Antagonist to Wnt pathway	Overall survival
TSC1	9q34	Mutation	6-12	Low grade and stage	Regulate cell proliferation	Recurrence in subset of superficial tumors
KISS1	1q32	Deletion	—/62 (ISH)	Late stage	Suppressor of metastasis	Prediction of poor outcome?
RAF1	3p25	Deletion	2-15		Signal transduction, proliferation	Progression of T1 tumors?
KAI/CD82	11p11	Deletion	—/38	Late stage	Suppressor of metastasis	Prediction of recurrences in Ta/T1 tumors
ARHGDIB	12p12	Deletion/mutation	—/14		Suppressor of metastasis	Death-related disease?
CD9	12p13	Deletion	—/62-49		Cell motility regulation	Prediction of progression in Ta/T1 tumors
P33ING1	13q34	Deletion			Cell proliferation	Overall patient survival?
RAD51	15q15	Deletion			DNA repair	Not known
E-cadherin	16q22.1	Hypermethylation	Methylation, 35/60-80	Late stage	Cell adhesion	Poor patient survival
Nm23-H1	17q21	Deletion/mutation	—/40-15	Late stage	Suppressor of metastasis	Conflicted results

a Amplification was measured by using fluorescent in situ hybridization; b Measured by quantitative polymerase chain reaction analysis

6.1.1 The Ras/Raf/MEK/ERK pathway. The Ras/Raf/MEK/ERK intracellular signalling cascade couples cell surface receptors to transcription factors, which regulate expression of genes involved in cell cycle progression. This pathway also has effects on the regulation of apoptosis by the post-translational phosphorylation of apoptotic regulatory molecules including BAD, caspase 9 and BCL-2. This pathway is often activated in tumors by mutations or overexpression of upstream molecules such as EGFR or downstream due to mutations at *Ras* and *B-Raf*. Among the major growth factor receptor tyrosine kinases activating the pathway are the Epidermal growth factor receptor (EGFR) and Fibroblast growth factor receptor (FGFR) family of proteins.

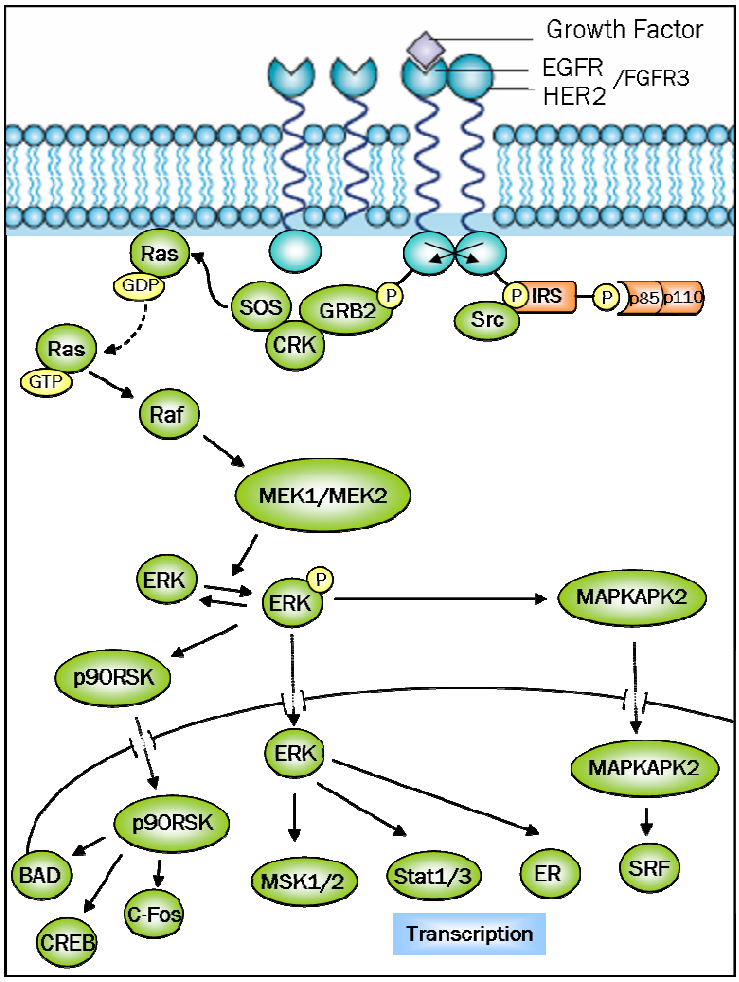


Figure 10. Ras/Raf/MEK/ERK pathway.

EGFR family. It consists of four highly similar proteins, encoded by the c-erbB oncogenes, that are apical signaling proteins in a phosphorylation cascade that regulates cellular proliferation. **EGFR1** (7p12) is the protein product of the c-erbB-1 proto-oncogene and consists of 3 distinct structural parts. The extracellular component region forms the ligand binding region that is activated by one of several ligands (epidermal growth factor [EGF] and transforming growth factor-alpha [TGF-α] among others) whilst the intracellular component region contains 2 domains: a catalytic domain with tyrosine-kinase activity and an autophosphorylation domain. Activation of EGFR leads to homo- and heterodimer formation which leads to the recruiment of a number of signal transducers to the phosphorylation form of EGFR1

such as adaptor proteins growth-factor-receptor bound-2 (GRB2) and Src-homology-2-containing (Shc) which are responsible for the recruitment of Ras and activation of MAPK cascade and PI3K (Yarden and Sliwkowski, 2001). Compartmentalization is a central mechanism that controls output from the EGFR network. Ligand binding to EGFR1 and their subsequent dimerization induces receptor internalization into endosomes, which is followed by recycling back to the cell surface in a kinase-independent manner (Citri and Yarden, 2006). Ligand-induced receptor endocytosis downregulates growth-factor signalling but internalized receptors might also activate signalling pathways distinct from those that are activated at the cell surface (Wiley, 2003). EGFR can also form ligand-independent dimerization but the formed dimer is inactive (Yu et al., 2002). An important defining feature of the ERBB network is that two members ErbB2 and ErbB3 are non-autonomous. **ErbB2** (17q11), also known as HER2/neu, encodes the ErbB2 receptor, located on the cell surface, where it associates with other similar receptors of the EGFR family. ErbB2 functions as the preferred heterodimeric partner of the other EGFR members. ErbB2 binds to a much larger subset of phosphotyrosine-binding proteins that the other ligand-binding receptors of the family (Jones et al., 2006). Growth factors bind to these similar receptors triggering the receptor complex to relay a signal inside the cell activating certain genes that promote cell growth.

FGFR family. It is composed of four members, each consisting of an extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic domain containing the kinase core as well as additional regulatory sequences (Schlessinger, 2000). FGFR1-4 differ in tissue expression, ligand specificity, signal pathway activation and biological effect (Powers et al., 2000). Binding of ligands to FGFRs induces receptor dimerization, resulting in transphosphorylation of the kinase domain and an interaction with effector proteins such as PLC gamma, Src family kinases, FRS2 and PI3K. FGFRs play an important role in regulating biological processes such as proliferation, differentiation, angiogenesis and embryonic development.

FGFRs interact with fibroblast growth factors (FGF). FGFs are a family of 22 distinct members whose pattern of expression ranges from nearly ubiquitous (FGF2) to highly restricted within particular cell subsets at specific developmental stages (FGF4). FGFs play a major role in the development of the skeletal and nervous

systems in mammals. An important hallmark of the FGFR family is that a variety of isoforms are generated by alternative splicing of FGFR transcripts (Eswarakumar et al., 2005). FGF specificity is conferred by the receptor family member and also by the alternative splicing of FGFRs (Ornitz et al., 1996). The large number of FGFs and the fact that many of them can stimulate the same receptor, implies that the FGF system has a lot of redundancy (Dailey et al., 2005).

Recent work has focused a special interest in one member of the FGFR family, **FGFR3**. There are two major isoforms of FGFR3 transcripts that are generated from a mutually exclusive splicing event in which the second half of the third Ig-like domain is encoded by either the 151 nucleotides of exon 8 (FGFR3b) or the 145 nucleotides of exon 9 (FGFR3c). FGFR3b is the main form found in epithelial cells, whereas FGFR3c is the predominant form found in mesenchymal cells.

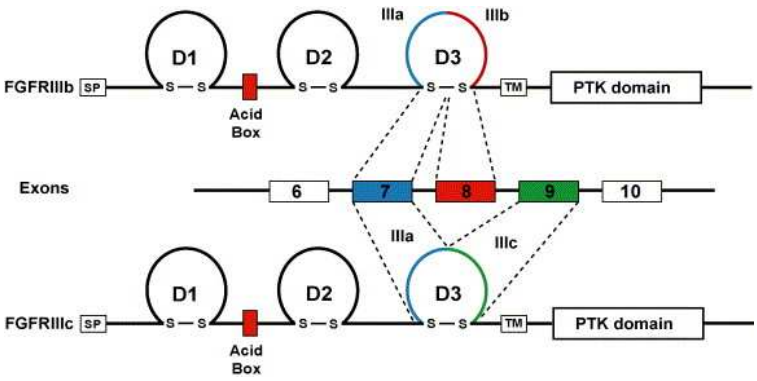


Figure 11. FGFR3 alternative splicing.

After the signal has been transmitted into the cell the main members of the signalling pathway are the Ras oncogenes, the Raf family of proteins and the MAP kinases.

Ras oncogenes. The ras genes encode a group of closely related proteins with GTPase activity. Three Ras proteins genes have been identified, H-Ras (11p15), K-Ras (12p12) and N-Ras (1p13) which oscillate between an active – GTP-bound – state and an inactive – GDP-bound - state. GTP binding induces Ras activation by causing a marked conformational change which leads to the activation of downstream signaling elements. Ras proteins show varying abilities to activate the Raf/MEK/ERK and PI3K/AKT cascades. For example, K-Ras is more efficient in recruiting and activating Raf 1, whilst H-Ras is a more potent activator of PI3K (Yan et al., 1998). Once Ras is activated, it recruits Raf-1 to the cell membrane. Ras

mutations cause a reduced GTPase activity due to resistance to the activity of GAPs, leading to continuous cell proliferation.

Raf family. The mammalian Raf gene family consists of A-Raf (Xp11), B-Raf (7q34) and C-Raf (Raf-1, 3p25). Raf is a serine/threonine kinase that is normally activated by recruitment to the plasma membrane by interaction with Ras, dimerization of Raf proteins, phosphorylation or dephosphorylation on different domains, or dissociation with the Raf kinase inhibitory protein and association with scaffolding complexes (McCubrey et al., 2006). Once active, Raf-1 phosphorylates and activates MEK (MAPK/Erk kinase), a dual specificity tyrosine/threonine kinase, that in turn phosphorylates and activates Erk1 and Erk2. Raf can phosphorylate proteins which control apoptosis. Raf is believed to directly antagonize the death-promoting activity of apoptosis signal-regulating kinase 1 through a physical interaction between the two proteins (Chen et al., 2001).

MAP kinases. The mitogen activated protein kinases are a family of proteins that regulate the activity of downstream kinases or transcription factors. Its members are ERK1/2 responsible for mitogenic response, ERK5/BMK1, and p38 and JNK associated with inflammatory or stress response. ERK1/2 is cytoplasmic in quiescent cells and translocates to the nucleus upon activation (Kondoh et al., 2005). EGFR activation of ERK is terminated by ERK-dependent feedback inhibition of SOS (Waters et al., 1995). ERKs phosphorylate consensus sequences in a large number of substrates leading to diverse cellular outcomes. To date, over 70 ERK substrates have been identified. ERK induces the expression of immediate early genes (IEG), such as c-fos, c-Myc, Jun and this expression is implicated in regulating subsequent induction of the delayed early genes, including cyclin D1. For example, ERK phosphorylates and stabilizes C-Myc, a transcription factor that induces the expression of Cyclin D1 and suppresses that of CDK inhibitors leading to cell cycle progression (Sears and Nevins, 2002). Recent studies have identified a number of Ras/ERK signaling-related proteins, such as scaffold proteins and inhibitor proteins of this pathway. These proteins provide variations in ERK signaling by modulating the duration, magnitude and subcellular compartmentalization of ERK activity (Ebisuya et al., 2005). The duration of ERK activity is a key factor for ensuring G1 phase progression. Transient and sustained ERK activation induce expression of

IEG, but only sustained activation induces sustained phosphorylation leading to stabilization and activation of the genes. Environmental stresses induce transient ERK activation. In some cases ERK activation leads to cell cycle arrest. High level activation of ERK upregulates CDK inhibitor p21, however this ERK mediated cell cycle arrest can be bypassed by the PI3K signaling pathway (Torii et al., 2006).

The Ras/Raf/MEK/ERK in bladder cancer. The major genes involved in the Ras/Raf/MEK/ERK pathway involved in bladder cancer are *FGFR3* and *H-Ras.*

FGFR3. Several human skeletal dysplasias have been linked to activating point mutations in *FGFR1*, 2 and 3 (Webster and Donoghue, 1997). Germline point mutations in *FGFR3* have been linked to the short limb syndromes of achondroplasia, hypochondroplasia and thanatophoric dysplasia. Mutations so far are mainly missense that result in amino-acid substitutions in the extracellular, transmembrane and/or cytoplasmic domain. The most common mutation involves the conversion of a non-cysteine residue to a cysteine in the extracellular loop, potentially creating a de novo intermolecular disulphide linkage. This linkage can lead to ligand-independent receptor dimerization and autophosphorylation of the intracellular kinase domain. Alternatively, the mutated receptor exhibits increased stability and decreased translocation to the lysosomal degradative pathway. Both mechanisms lead to increased and prolonged activation of the receptor (Cappellen et al., 1999;Cho et al., 2004) which is associated with increasing severity of the disease (Webster et al., 1996;Naski et al., 1996). Mutations in *FGFR3* have also recently been

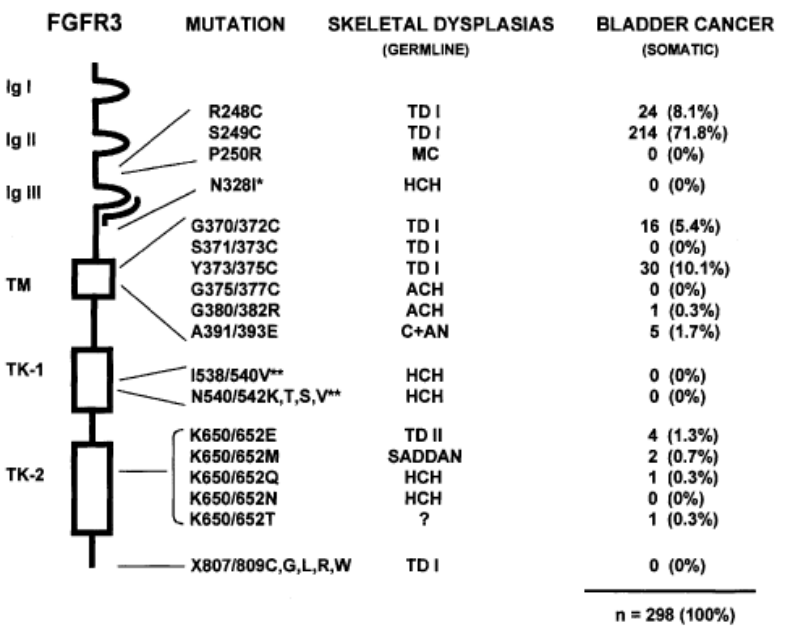


Figure 12. FGFR3 mutations in skeletal dysplasias and bladder cancer.

associated to epidermal nevi, and seborrheic keratoses (Logie et al., 2005;Hafner et al., 2006a;Hafner et al., 2006b). The gene encoding *FGFR3* has been implicated in urothelial carcinoma, being activating mutations - rather than amplification - responsible for its oncogenic role (Cappellen et al., 1999;Munro and Knowles, 2003). The same mutations described in bladder are also found in the non-lethal skeletal disorders (van Rhijn et al., 2002). Mutations have been described to be more frequent in low grade or papillary superficial tumors (Kimura et al., 2001;Billerey et al., 2001) and to decrease with stage and grade. This is thought to be due to the emergence of tumors following a different molecular pathway with no FGFR3 mutations in more invasive tumors. *FGFR3* mutations have also been observed in patients with favorable disease characteristics (van Rhijn et al., 2001;van Rhijn et al., 2003), however, *FGFR3*-mutated tumors have malignant potential.

Mutations have shown to fail to predict the risk of recurrence and progression (Zieger et al., 2005) in a small group of superficial and invasive tumors, however in a larger study *FGFR3* mutations have been associated to recurrence in TaG1 bladder tumors (Hernandez et al., 2006). *FGFR3* mutations apparently are not directly involved in cancer progression. It is speculated that mutation alters cellular proliferation and growth, but does not affect apoptosis and instability. FGFR3 overexpression has been shown to be more frequent in low grade and stage, and not correlated to recurrence and progression (Mhawech-Fauceglia et al., 2006a). A fast and easy- to- use method has been developed to detect 9 mutations in bladder cancer DNA and voided urine (van Oers et al., 2005). It is a multiplex PCR which analyzes the 9 most common mutations with a sensitivity of 62% in voided urine which can be explained because most mutations occur in low stage/grade tumors which shed less cells into the urine.

EGFR. Amplification of *EGFR1* and *EGFR2* is reported in 4.6% and 3.4% of bladder tumors and protein overexpression has been described in 48% of tumors (Neal et al., 1990) and 41% respectively (Ohta et al., 2001;Leonardo et al., 2005). This amplification/overexpression of EGFR is associated with tumor proliferation, aggressive behaviour and poor prognosis (Chow et al., 2001). The mechanism by which expression is associated with poor prognosis is not entirely clear, although there is some evidence linking EGFR stimulated activation of activator protein-1

transcription factor with induction of matrix metalloproteinase activity (Nutt et al., 1998). Unlike in lung cancer, mutations within the kinase domain of *EGFR1* have not been detected in bladder cancer cell lines and tumors (Blehm et al., 2006). HER2/neu overexpression or amplification is seen frequently in advanced-stage bladder carcinoma and appears to be an independent predictive factor of disease related-survival (Kruger et al., 2002). Its association with the presence of metastasis is unclear (Wulfing et al., 2005;Rotterud et al., 2005) but it has been associated to stage and grade (Toncheva and Zaharieva, 2003;Eissa et al., 2005).

Ras oncogenes. Amplification of Ras proto-oncogenes and activating mutations that lead to the expression of constitutively active Ras proteins are present in approximately 30% of human cancers (Eisenmann et al., 2003;Lee et al., 2004;Vicent et al., 2004). Point mutations have been identified in codons 12, 13 and 61 in *H-Ras* (Haliassos et al., 1992) and in the *N-Ras* gene at an overall frequency of 10-20% (Przybojewska et al., 2000;Prat et al., 2001). The commonly occurring mutations that render Ras oncogenic are those that make the GTPase insensitive to the action of GAPs and thereby lock it in the GTP-bound, active state. These mutations leave the GTP-binding properties intact but inhibit GTP hydrolysis. In this way, a constant activation of the protein and continuous mitogenic signaling is induced (Barbacid, 1987). FGFR3 and Ras activation have been analyzed in a subgroup of urothelial cell lines and tumor samples, as they both would activate the MAPK pathway and could have redundant functions. Mutations have been shown to be mutually exclusive, although not segregated with tumor stage and grade (Jebar et al., 2005).

Raf. *B-Raf* mutations have been described in 7% of tumors (Davies et al., 2002) with a frequency ranging from 27%-70% in melanoma, 36-53% in thyroid cancer, 5-22% of colorectal cancer, 30% in ovarian cancer and 1-3% in various other cancers (Garnett and Marais, 2004). The majority of the mutations are activating B-Raf. C-Raf overexpression in human malignancies has been described in cases of acute myeloid leukemia (Schmidt et al., 1994) and ovarian cancer (McPhillips et al., 2001). *B-Raf* mutations, although common in other cancers, have not been found in bladder cancer (Stoehr et al., 2004). Raf1 amplification has been associated to grade, stage and poor survival (Simon et al., 2001) and overexpression has been

associated to grade, not stage, in a further study (Mhawech-Fauceglia et al., 2006c).

As the MAPK signaling pathway promotes proliferation, mediates cell survival and is upregulated in cancer cells, it seems to be a good therapeutic target. Several inhibitors of MAPK signalling have been developed (Sebolt-Leopold and Herrera, 2004).

6.1.2 The PI3K/PTEN/AKT pathway. Receptor tyrosine kinases can also activate PI3K (phosphatidylinositol-3-kinase) by two mechanisms. First, a phosphorylated Y residue of EGFR serves as a docking site for the p85 regulatory subunit of PI3K (Chang et al., 2003). This recruits the catalytic subunit p110 to this complex and activates PI3K. Alternatively, upon activation of the cytokine receptor, the Shc protein binds the receptor to enable the Grb-2 and SOS proteins to form a complex which activates Ras. Ras is then able to induce the membrane translocation and activation of p110 of PI3K. Activated PI3K in turn activates many downstream molecules by binding to their pleckstrin-homology domain. AKT is the principal target of PI3K, which is recruited to the membrane and leads to phosphorylation by the mTOR (mammalian target of rapamycin)-rictor kinase complex. This leads to full activation of AKT, which in turn phosphorylates many target proteins.
The PI3K signalling pathway is crucial to many aspects of cell growth and survival. AKT promotes G1-S cell-cycle transition by blocking FOXO (forkhead)-mediated transcription of cell-cycle inhibitors, such as p27. Through AKT signalling, it inactivates several proapoptotic factors such as FasL and Bim. Through AKT phosphorylation of the tuberous sclerosis complex 2 (TSC2) protein tuberin it leads to protein synthesis, and it has an effect on glucose metabolism by interacting with GSK3β and GLUT4 (Hennessy et al., 2005).

Abnormalities in the PI3K pathway are common in cancer (Hanahan *et al.*, 2000). This pathway can be activated by mutation or amplification of PI3Ks, and AKT, and inactivated by PTEN and TSC2. In the following section there is a brief description of some of the members of this pathway. The main component is the PI3K family of proteins.

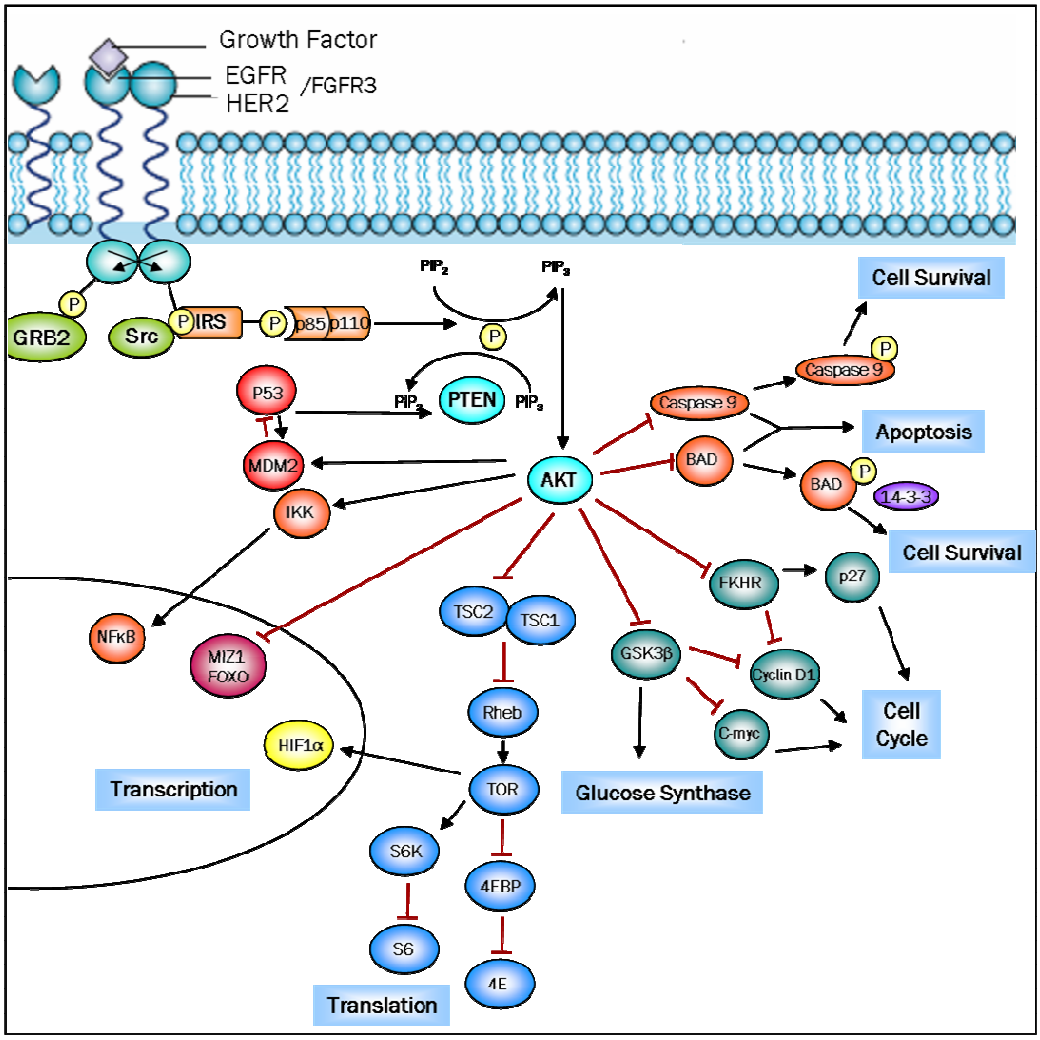


Figure 13. The PI3K/PTEN/AKT pathway.

PI3K. Phosphatidylinositol 3-kinases (PI3Ks) phosphorylate phosphatidylinositol and its phosphorylated derivatives at the 3' position of the inositol ring, generating second messengers that control proliferation, survival, motility and cell shape (Bader et al., 2005). PI3Ks are a family of lipid and serine/threonine kinases distributed in three classes. The most important class involved in proliferation and tumorigenesis is class IA. Class IA enzymes are constituted by a heterodimer formed by the catalytic subunit p110 and an adaptor subunit (p85, p50 o p55). P85 binds and integrates signals from various cellular proteins including transmembrane tyrosine kinase-linked receptors and intracellular proteins such as protein kinase C, SHP1, Rac, Rho, hormonal receptors, Ras and Src, providing an integration point for activation of p110 and downstream molecules (Hennessy et al., 2005). P110α phosphorylates phosphatidylinositol 4,5-biphosphate to phosphatidylinositol 3,4,5-triphosphate which then recruits proteins containing a pleckstrin homology domain

as AKT and PDK1 to cellular membranes (Corvera and Czech, 1998) where they are activated by phosphorylation.

The tumor suppressor genes Serine-Threonine kinase 11, Phosphatase and Tensin Homolog (PTEN), Neurofibromatosis 1 (NF1) and tuberous sclerosis complex (TSC) exert interacting inhibitory effects on signalling through the PI3K/AKT pathway.

PTEN. It is a lipid phosphatase located at 10q23. It is a negative regulator of PI3K pathway by dephosphorylating PIP_3 to generate PIP_2 and it physically interacts with p53 in the nucleus, which in turn stabilizes p53 and affects its levels and transcription activity (Freeman et al., 2003). PTEN has also been shown to form a complex with p300, playing a role in the maintenance of high p53 acetylation (Li et al., 2006a). PTEN has an important function regulating cell cycle arrest and anchorage-independent growth when forced to localize in the nucleus by downregulating mTOR and p70S6K in an AKT independent manner, losing however its capacity to inhibit cancer cell invasion (Liu et al., 2005).

TSCs. There are two TSC genes, *TSC1* (9q34) encoding hamartin and *TSC2* (16p13) encoding tuberin. TSC1 forms a complex with TSC2, and although independent functions have been proposed for each protein, it is not clear whether their coordinated action is absolutely required for function. TSCs receive inputs from at least three major signaling pathways, TSC2 is phosphorylated by AKT, ERK1/2, RSK1 and AMPK (Potter et al., 2003) and TSC1 is phosphorylated by CDK1 (Astrinidis et al., 2003). The TSC proteins have been implicated in the regulation of different cellular functions such as transcription, neuronal differentiation or cell adhesion. TSC1/TSC2 acts as GTPase activating protein for Rheb-GTP, which can bind directly to mTOR-raptor complex and enhances its kinase activity and lead to cell size increase and cell growth (Kwiatkowski and Manning, 2005). Overexpression of TSC1 or TSC2 negatively regulates cell cycle progression and tuberin regulates the activity of cdk2 and p27 (Soucek et al., 1998;Rosner et al., 2006).

The PI3K/PTEN/AKT pathway in bladder cancer. The PI3K pathway is activated in 30% of human cancers (Luo et al., 2003). The pharmacological inhibition of the

pathway drastically has been shown to reduce the invasive capacity of bladder cancer cell lines, suggesting that aberrant activation of this pathway may contribute to invasion of a subset of bladder tumors (Wu et al., 2004).

PI3K. Mutations and amplifications in *PIK3CA*, gene encoding p110α have been described in a number of cancers at high frequencies (Karakas et al., 2006). Mutations occur at various hotspots of the gene: E542K and E545K in exon 9 and H1047R in exon 20. Proteins with these mutations show a gain of enzymatic function in vitro and are oncogenic in cell culture and in vivo (Bader et al., 2006). P85α mutations and translocations, although rare, have also been described (Jucker et al., 2002). Primary human bladder tumors of all stages overexpress PI3K, analyzed by western and immunohistochemistry, and have a significantly higher activity than adjacent normal epithelium (Benistant et al., 2000). The chromosomal region containing the gene *PIK3CA* has been shown gained in urothelial carcinomas (Simon et al., 1998;Koo et al., 1999;Veltman et al., 2003a;Hurst et al., 2004;Blaveri et al., 2005).

PTEN. Loss of function mutations or reduced expression of the PTEN gene are found at high frequency in a wide variety of human tumors. *PTEN* is mutated or deleted in 14% of invasive bladder cancers with 40% LOH at 10q (Cappellen et al., 1997;Cairns et al., 1998;Aveyard et al., 1999;Wang et al., 2000). PTEN protein downregulation has been described in 13% of tumors, most of which were muscle-invasive urothelial carcinomas (Koksal et al., 2005).

TSC. Germline mutations in *TSC1* and *TSC2* cause TSC, an autosomal dominant disease characterized by the presence of hamartomas. Twelve percent of bladder tumors have been shown to contain mutations in *TSC1* (Knowles et al., 2003) and LOH at 9q34 is present in 32% of cases (Adachi et al., 2003). There is no data for TSC2 and bladder cancer.

A well described crosstalk has been identified between the Ras/Raf/MEK/ERK and PI3K/PTEN/AKT pathway. Many growth factors activate receptor tyrosine kinases (RTKs) located at the plasma membrane which – upon dimerization and transphosphorylation - activate two key signal-transduction components: the small

GTPase Ras and the lipid kinase PI3K. The PI3K pathway can also be activated by Ras and ERK has been described to inhibit the TSC1/2 complex. AKT, meanwhile, can inhibit B-Raf and Raf-1.

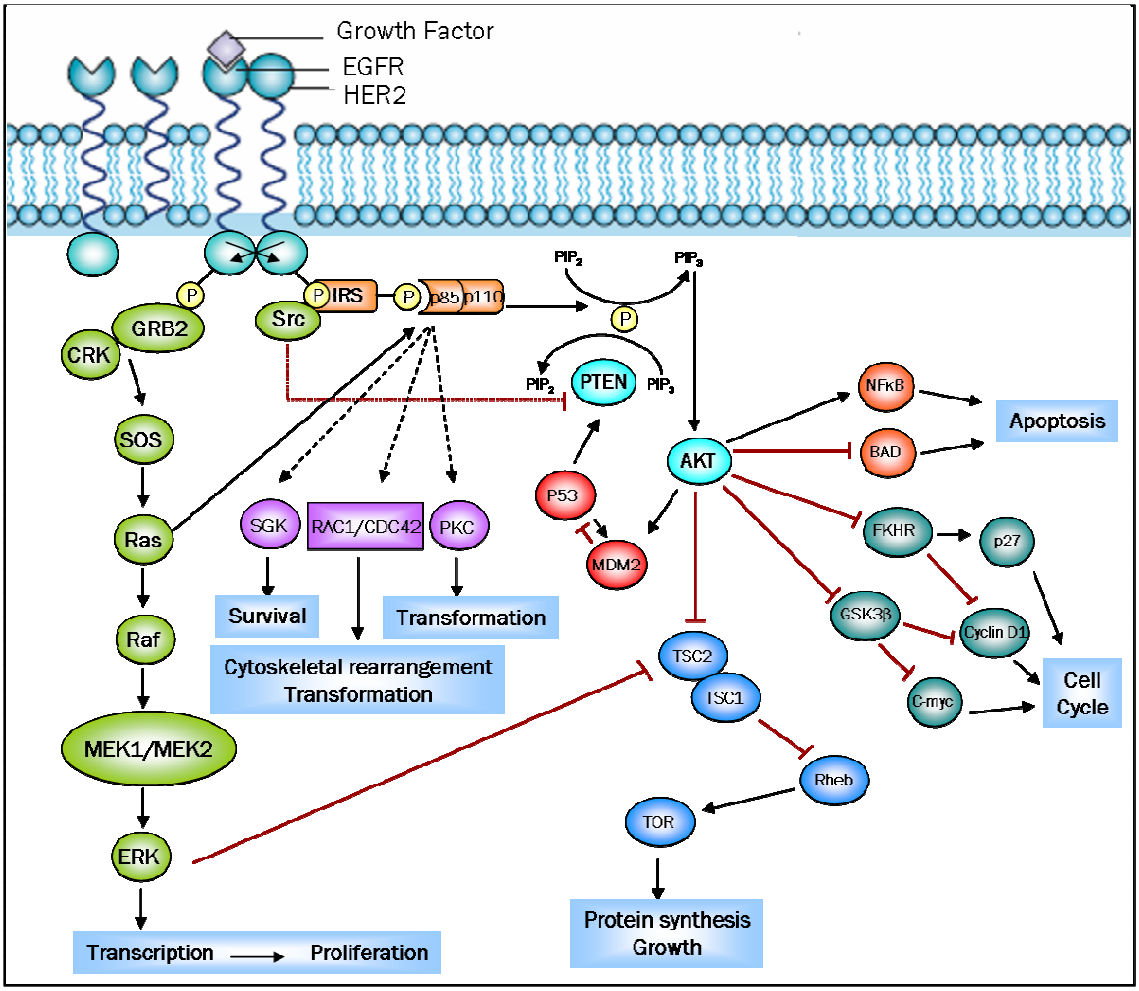


Figure 14. The Ras/PI3K crosstalk.

6.1.3 The Rb pathway and cell cycle control. The mammalian cell cycle can be divided into four phases: G_1, S, G_2 and M. In G1 (gap1) the cell is preparing for DNA synthesis, during S phase DNA replication occurs, in G2 the cell prepares for mitosis and in M, nuclear division occurs. The restriction point (R) is defined as a point of no return in G1, following which the cell is committed to enter the cell cycle. Additional controls or checkpoints exist in the cell cycle ensuring an orderly sequence of events. Checkpoints are located at G1-S or at G2-M and different stimuli can trigger checkpoint controls: DNA damage, contact inhibition and replicative senescence which induces the INK4 or Kip/Cip families of cell cycle kinase inhibitors, and

growth factor withdrawal which activates GSK3β, which phosphorylates cyclin D1 leading to its ubiquitination and degradation.

Cell cycle progression is controlled by a series of phosphorylation events implicating the major proteins of the cell cycle machinery: cyclin-dependent kinases and cyclins, the regulatory subunits of cdks. CDK protein levels remain stable during the cell cycle, in contrast to their activating proteins, the cyclins. Cyclin protein levels rise and fall during the cell cycle and in this way they periodically activate CDK. Cyclin/cdk complexes are formed during distinct phases of the cell cycle and are involved in the phosphorylation of specific target proteins (Vermeulen et al., 2003). **Cyclin D1** (11q13) functions as a positive regulator of CDK kinases. It forms a complex and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for G1/S transition. Cyclin D1 interacts with Rb and its expression is positively regulated by Rb. **Cyclin D3** is also involved in progression from G1 to S phase of the cell cycle.

CDK activity is abrogated by cell cycle inhibitor proteins called CDK inhibitors (CDKI). CDKI bind to CDKs or CDK-cyclin complexes and regulate CDK activity. There are two distinct families of CDKIs: the INK4A family and Cip/Kip family.

The INK4A family specifically inactivates G1 CDK (CDK4 and CDK6). The **INK4a/ARF** locus, located at 9p21, is unusual in that it encodes $p16^{INK4a}$ and $p14^{ARF}$, two cell cycle regulatory proteins. The two resulting cDNAs use distinct exons 1, are translated in alternative reading frames and encode proteins bearing no amino acid homology (Figure 15. (Sharpless, 2005)). P16 is a CDKI that blocks cyclin D-dependent kinase activity thereby inhibiting the phosphorylation of pRb; its main targets are cdk4 and cdk6. P14ARF binds to the MDM2 proto-oncogene and blocks MDM2-induced p53 degradation, resulting in an increase of p53 levels, and it induces a G1 and G2 arrest in a p53-dependent manner.

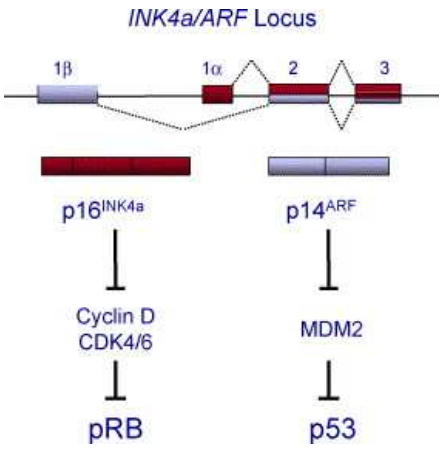


Figure 15. Splicing of the INK4a/ARF locus.

The Cip/Kip family is formed by p21 (Waf1, Cip1), p27 (Cip2), p57 (Kip2) which inactivate CDK-cyclin complexes. **P27** (12p13) regulates the G1 to S phase

transition of the cell cycle by binding to and inhibiting several CDK complexes in normal cells (Polyak et al., 1994). P27 also regulates cell migration as a cell-cycle independent function (Besson et al., 2004).

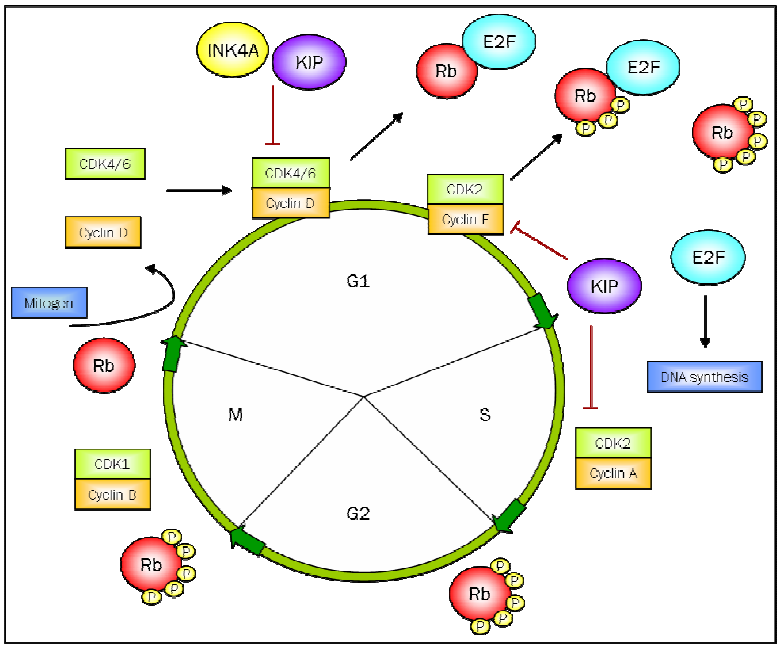


Figure 16. The cell cycle.

The main components responsible of the G1/S transition are two cell cycle kinases, cdk4/6-cyclin D and cdk2-cyclin E, the pocket proteins and the E2F transcription factors. During the G1 phase, the Rb-HDAC repressor complex binds to the E2F-DP1 transcription factors, inhibiting the transcription of target genes. Phosphorylation of Rb by cdk4/6 and cdk2 increases at the end of the G1 phase leading to the dissociation of the Rb-repressor complex from DNA-bound E2F. "Free" E2F is then active and ready to drive the transcription of S-phase genes encoding for proteins that switch the cell status from G1 to S phase and that are required for DNA replication (Tonini et al., 2002).

RB family. Rb (p105), p107 and p130 are members of a family of closely related proteins. They are referred to as pocket proteins because their main sequence similarity resides in the pocket domain which mediates interactions with viral oncoproteins as well as cellular proteins. Rb is a tumor suppressor gene which negatively regulates the G1-S phase transition in two ways. Either by interacting with the transactivation domain of E2F, in its hypophosphorylated form, and repressing the transcription of genes required for G1 to S phase transition or by active repression through the recruitment of histone deacetylases, polycomb group proteins or methyltransferases that act on the nearby surrounding nucleosome structure. Rb has also been implicated in regulating another phase of the cell cycle, directly involved in suppressing apoptosis, has shown to function as a coactivator in various differentiation processes such as that of the eye, brain, epidermis and is

involved in senescence (Du and Pogoriler, 2006). MDM2 has been shown to regulate pRb through a ubiquitin-dependent degradation. Activation of MDM2 might lower the levels of pRb facilitating progression from G1 to S phase and causing accelerated proliferation (Uchida et al., 2005).

Most genetic alterations in Rb1 such as point mutations or loss of heterozygosity lead to loss of pRb expression (Xu et al., 1993). Many studies examining the prognostic significance of pRb in cancer only determine whether the protein is expressed or not, however it has been shown that hyperphosphorylation can functionally inactivate pRb even when high levels of expression are described (Chatterjee et al., 2004b).

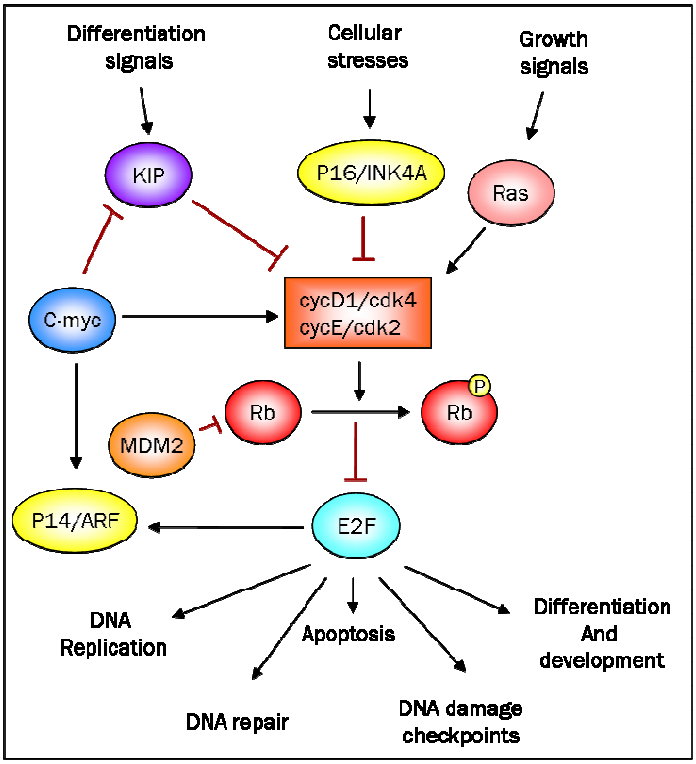


Figure 17. The pRb pathway.

pRb phosphorylation is a cell cycle dependent phenomenon and cyclin/cdk complexes are responsible for this regulation. CDK4/6 and Cyclin D1 phosphorylate Rb in G1 and Cyclin A and Cyclin B dependent kinases (Cdk2 and Cdk1) probably maintain Rb in its hyperphosphrylated form because pRb does not revert to the hypophosphorylated form until the end of mitosis.

E2F. The E2F family of transcription factors consists of at least 8 members. E2F1-3 are transcriptional activators, E2F4-5 are transcriptional repressors, E2F6 interacts with polycomb proteins and E2F7-8 are believed to repress specific promoters (Giacinti and Giordano, 2006). The E2F family plays a crucial role in the control of cell cycle and it regulates tumor suppressor proteins. E2F1-3 are negatively regulated by the pRb protein whilst E2F4-5 bind p130. Some of the target genes of E2Fs include cell cycle regulators such as cyclin E and A, and DNA replication factors such as PCNA, but they also induce the expression of multiple genes

involved in DNA repair and apoptosis (Stevaux and Dyson, 2002). E2F1 specifically, has been described to mediate p53-dependent/independent apoptosis (Roos and Kaina, 2006).

The Rb pathway in bladder cancer.

Cyclin D1 overexpression occurs frequently in bladder tumors and may be associated with the growth of low-grade papillary tumors (Lee et al., 1997a;Lee et al., 1997b). The expression of cyclin D1 has been associated with tumor stage and tumor grade (Mhawech et al., 2004). Cyclin D3 expression increases with stage in bladder cancer tumors (Lopez-Beltran et al., 2006) and results suggest expression is relevant to the progression-free survival of patients with Ta/T1 bladder carcinomas.

INK4a/ARF. The *INK4a/ARF* locus plays a central role in tumor suppression as reflected by the fact that a significant fraction (close to 50%) of all human cancers has inactivated this locus (Gonzalez and Serrano, 2006). mRNA levels of p16 and p14 proteins have been found to be absent from normal urothelium and to increase with stage and grade of bladder cancer (Frere-Belda et al., 2004). P16 loss of expression at the protein level has been associated to minimally invasive bladder cancer (Kruger et al., 2005). P16 and p14 methylation has been shown to be a useful biomarker for stage, clinical outcome and prognosis of patients with bladder cancer (Kawamoto et al., 2006) and loss of the 9p21 region containing the *INK4a/ARF* locus is deleted in a high number of bladder tumors (see genomic level).

Various studies have suggested that decreased protein levels of $p27^{Kip1}$ may be associated with transformation of bladder tumor cells into more aggressive cancers with early recurrence and shortened survival (Polyak *et al.*, 1994;Kamai et al., 2001). A recent study has found that low protein levels of $p27^{Kip1}$ in tumors are associated with a low risk of progression (OR of 0.3) (Schrier et al., 2006). However, this finding has not been confirmed in other studies (Santos *et al.*, 2003).

Mutations of the *Rb1* gene have been previously described in bladder cancer (Miyamoto et al., 1995). Santos et al (Santos et al., 2003) evaluated the prognostic value of the expression of pRb, together with other cell cycle regulator proteins, in bladder tumors and found no association with recurrence or progression, similar to other studies (Garcia, X et al., 2004). However, Shariat et al. evaluated the prognostic value of pRb expression and found an association with progression and death (Shariat *et al.*, 2004b). A small number of studies have analyzed mutations in the *Rb1* gene due to its large size, 27 exons, and the ample length of some of them.

E2F1 protein expression has been correlated with proliferation in urothelial carcinomas, showing it has a growth promoting effect (Zacharatos et al., 2004). E2F3 has been shown overexpressed in 33% of bladder tumors (Feber et al., 2004) and amplified in a number of bladder tumors (see Genomic level).

6.1.4 The p53 pathway. The p53 pathway is composed of a network of genes and their products that are targeted to respond to a variety of intrinsic and extrinsic stress signals that impact upon cellular homeostatic mechanisms that monitor DNA replication, chromosome segregation and cell division (Vogelstein et al., 2000). In response to a stress signal, the p53 protein is activated and this leads to either cell cycle arrest, cell senescence or cellular apoptosis. The p53 pathway in a cell can also communicate with neighboring cells by secreting a series of proteins altering the cellular environment and influencing angiogenic signals, and has a function in DNA repair.

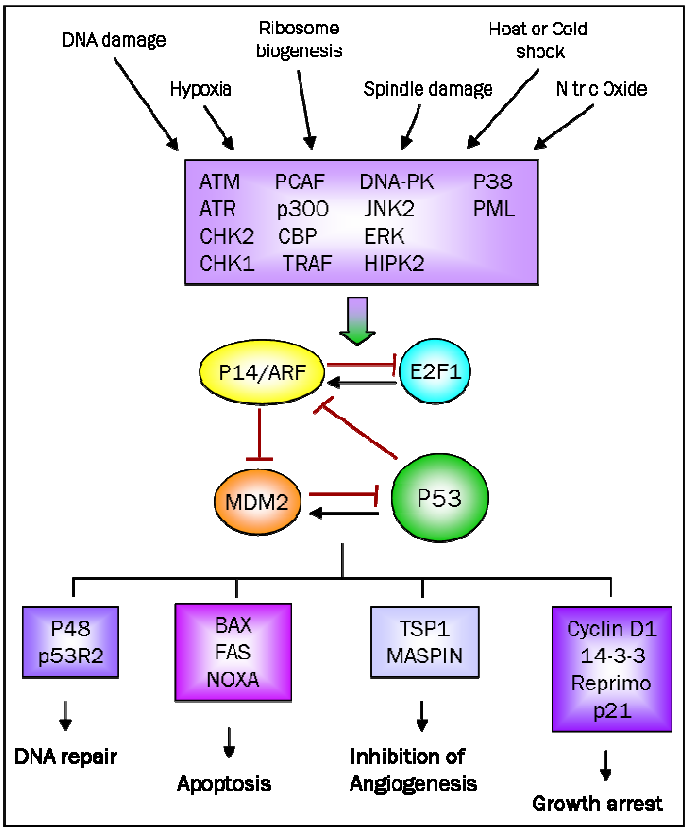


Figure 18. The p53 pathway.

Tp53. *p53* is a crucial tumor suppressor gene often referred to as the "guardian of the genome", and is found mutated or deleted in over 50% of human cancers (Soussi and Beroud, 2001).

Structure and function. The human *p53* gene spans 20 Kb of DNA and is located at 17p13. It is composed of 11 exons, the first of which is non-coding. The protein consists of 393 amino acids and contains 4 functional domains. At the N-terminus is a transcriptional activation domain, the central part is the sequence-specific DNA binding domain and the C-terminal portion contains an oligomerization domain and a regulatory domain. P53 protein forms tetramers, which are the functional forms.
P53 mainly functions as a DNA-binding transcription factor to regulate a number of genes which mediate cell-cycle arrest, apoptosis, senescence, differentiation, DNA repair and inhibition of angiogenesis and metastasis (Liu and Chen, 2006). P53 induces G1 arrest by upregulating p21 (el Deiry et al., 1993), G2 arrest by upregulating GADD45, 14-3-3σ and p21 (Hermeking et al., 1997;Bunz et al., 1998;Wang et al., 1999) and apoptosis by upregulating genes such as Bax, NOXA, and PUMA (Chipuk et al., 2004). In addition, it can repress genes such as myc to promote G1 arrest (Ho et al., 2005) and cyclin B1 to promote G2 arrest (Innocente et al., 1999).
In normal cells, p53 is maintained at very low levels and, in response to various intra or extracellular stresses, p53 is stabilized and activated (Vousden, 2002). Among the signals that activate the p53 protein are DNA damage, hypoxia, Heat and cold shock conditions and oncogenic stress. Different types of activating stimuli give rise to different p53 functions. It has been shown that oncogenic signalling, rather than DNA damage, is critical for p53 tumor suppression (Efeyan et al., 2006;Christophorou et al., 2006). DNA damage and oncogenic signalling interact with p53 through different pathways. DNA damage occurs through a p53-phosphorylation cascade involving ATM/Chk2/ATR/Chk1 and oncogenic signalling occurs through p53-stabilization pathway involving ARF and MDM2. Different causes of DNA damage response are: UV irradiation, alkylation of bases, depurination of DNA, Reactive oxygen species and different types of DNA damage activate different enzyme activities that modify the protein at different residues. For example, gamma-radiation activates the ATM kinase and the CHK2 kinase, both of which phosphorylate the p53 protein whilst UV-radiation activates ATR, CHK-1 and

casein Kinase-2 which result in modification of different amino acids of p53 (Appella and Anderson, 2001). p53 activity is controlled by: pos-translational modifications, the amount of p53, its DNA-binding ability, its sub-cellular location and its recruitment of transcriptional co-activators or co-repressors. In unstressed cells, p53 is diffusely distributed in the nucleus. Nuclear p53 binds to, and is ubiquitinated by, MDM2 and this promotes its export and degradation (Liang and Clarke, 2001). Production, export and degradation are a dynamic process, which keeps low levels of p53 in cells under normal conditions. Various proteins regulate p53 localization: Parc (Nikolaev et al., 2003), Glucocorticoid receptor (Sengupta et al., 2000), and GSK-3β (Qu et al., 2004). Parkin is an E3 ubiquitin ligase associated with Parkinsons disease. Parc shares the ubiquitin ligase activity domain which Parkin and directly interacts with and forms a complex with p53 in the cytoplasm in unstressed cells. Overexpression of Parc promotes cytoplasmic sequestration of ectopic p53. Glucocorticoid receptor forms a complex with p53 in vivo, resulting in cytoplasmic sequestration of both p53 and GR. GR antagonists result in nuclear accumulation of p53. Glycogen synthase kinase-3 β binds to p53 in the nucleus and enhances the cytoplasmic localization of p53 upon ER stress.

P53 is happlo-insuficient. P53 mRNA levels have been analyzed in a cell line p53 +/+, p53 +/-, p53 -/- and levels were approximately 4-fold lower in p53 +/- than p53 +/+ cells. Total protein levels were also approximately four fold lower in stressed or non-stressed conditions. These results indicate that p53 +/- cells are unable to compensate for subnormal p53 protein levels by translational or post-translational mechanisms which could mean mRNA levels are dominant over regulatory mechanisms for p53 protein levels in the cell. Loss of one p53 allele reduces the transactivation of p21 and MDM2, measured by p21 and MDM2 mRNA and protein level decrease; and the transrepression of survivin: survivin is repressed by p53 at the transcriptional level however, in +/- cells no repression was observed. Loss of one p53 allele also attenuates p53-dependent cell cycle regulation (Lynch and Milner, 2006). This suggests p53 mRNA levels are a critical determinant of p53 function.

P63 and *p73.* The *p53* tumor suppressor gene is a member of a family that includes two other genes termed *p63* and *p73*. *P63* (3q27-29) encodes multiple proteins, resulting from alternative splicing, that transactivate p53 reponsive elements or act

as a dominant negative towards *p53* and *p73*. *p73* can activate p53-regulated genes, suppress growth or induce apoptosis and is induced by DNA damage. Both *p63* and *p73* are important for regulation of normal development (Levrero et al., 2000).

P53-Hdm2 feedback loop. MDM2 can inactivate p53 activity in several ways: it can bind the p53 transactivation domain inhibiting p53-mediated transactivation (Momand et al., 1992), it can degrade p53 and it can translocate p53 to the cytoplasm. MDM2 is an E3 ubiquitin ligase that mediates p53 degradation (Bond et al., 2005b). MDM2 binds to the p53 N-terminus, and transfers monoubiquitin tags onto lysine residues mainly in the C-terminus of p53. The monoubiquitination is sufficient for p53 nuclear export to the cytoplasm, but not for degradation (Yang et al., 2004). P300 is involved in adding a polyubiquitin chain to p53 (Zhu et al., 2001). Other ubiquitin ligases able to mediate p53 ubiquitination and degradation are Pirh2, constitutively photomorphogenic 1, chaperone-associated uibiquitin ligase, topors and ARF-binding protein (Leng et al., 2003;Dornan et al., 2004;Chen et al., 2005;Esser et al., 2005).

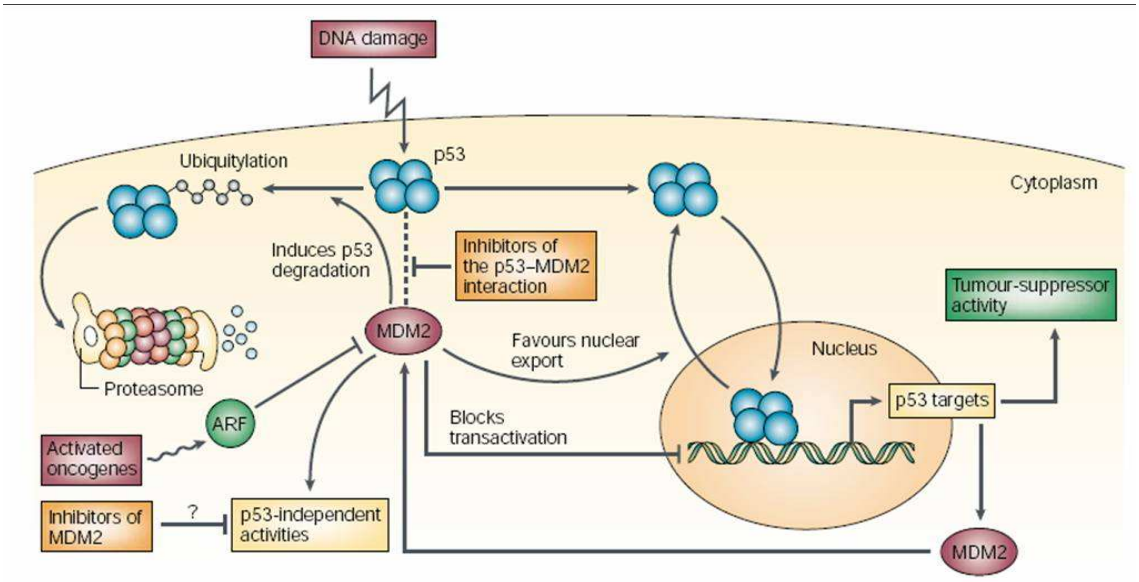


Figure 19. Regulation of p53 by mdm2 (Chene, 2003).

The p53-MDM2 interaction can be disrupted by phosphorylation. C-Abl phosphorylates MDM2 at Tyr394, impairing the inhibitory effect of MDM2 on p53 activity and stability, however phosphorylation can also have the opposite effect: AKT phosphorylates Ser166 of MDM2, enhancing its nuclear localization and consequently the inhibition and degradation of p53 (Haupt et al., 2002). *MDM2* gene itself is activated by p53, which gives the opportunity for feedback negative control of p53 activity. MDM2 works together with MDMX, that can also down-regulate the transcription function of p53 but cannot target it for degradation (Marine and Jochemsen, 2005).

Loss of MDM2 leads to an activation of p53 that is lethal during embryogenesis. However, stress-induced inhibition of MDM2 mediated by ARF is essential to activate p53's tumor suppressive activity (Iwakuma and Lozano, 2003;Efeyan *et al.*, 2006;Christophorou *et al.*, 2006). In response to oncogenic stress, ARF protein binds to MDM2, leading to the activation of p53 and cell cycle arrest. Failure of the mechanism that turns off MDM2 has been linked to cancer development (Michael and Oren, 2003). Increased expression of MDM2 may result from 4 mechanisms: gene amplification, increased expression activated by p53, stabilization by an aberrantly spliced form of HDMX (Giglio et al., 2005) or augmented translation. A T-to-G polymorphism at codon 309 has been described in the MDM2 promoter that also increases the levels of RNA and protein by creating an enhanced SP1 transcription factor binding site. Patients heterozygous for T/G or G/G homozygotes show an increase in cancer incidence and time of onset (Bond et al., 2005a). This suggests that elevated levels of MDM2 expression can dampen the efficiency of p53 tumor suppression. MDM2 overexpression has been suggested to also have effects, beyond disabling p53 (Ohkubo et al., 2006), on cell growth and survival (Ganguli and Wasylyk, 2003).

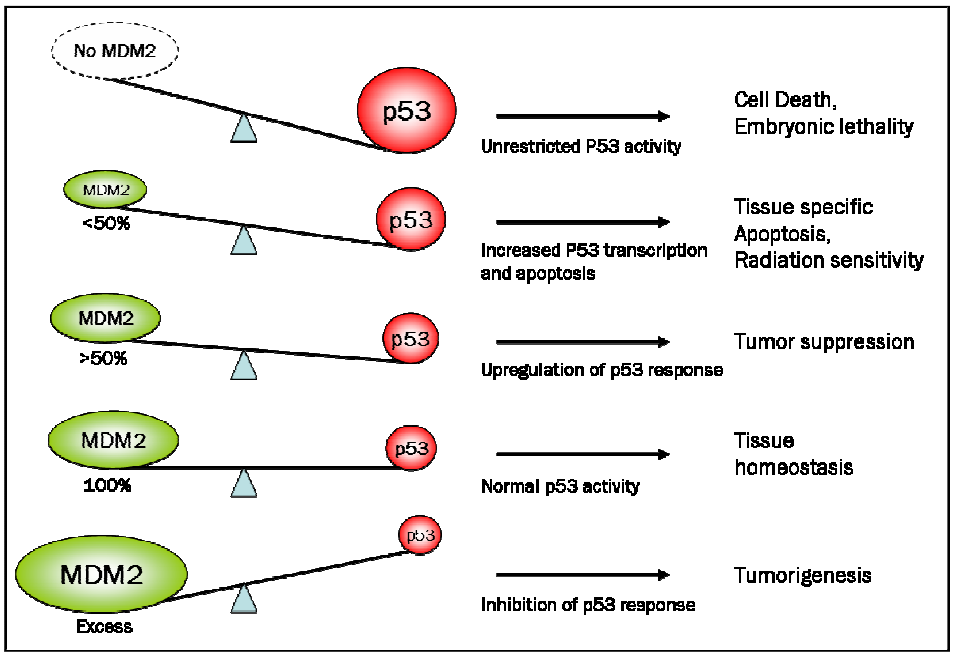


Figure 20. Tipping the p53-mdm2 balance (Poyurovsky and Prives, 2006).

Various feedback loops around p53 have been described. At least 7 negative feedback loops and 3 positive feedback loops. Of these, 6 act through MDM2 protein to regulate p53 activity (Harris and Levine, 2005).

Among others, two important genes in the p53 pathway are *Aurora A* kinase and *p21*. *Aurora-A* is a key regulatory component of the p53 pathway. Overexpression leads to increased degradation of p53, causing down-regulation of checkpoint-response pathways and facilitating oncogenic transformation of cells (Katayama et al., 2004). It is located in 20q13, amplification of which has been associated with an aggressive phenotype and aneuploidy. Aneuploidy is the most prevalent somatic alteration seen in solid tumors. It drives tumor progression by increasing gene instability, leading to further alteration of the tumor genome (Cahill et al., 1998). *P21* (6p21) is a cyclin-dependent kinase inhibitor. It binds and inhibits cyclin-CDK2 or CDK4 complexes, functioning as a regulator of cell cycle progression at G1. The expression of this gene is controlled by p53.

Alterations in Tp53. *p53* is the most extensively studied cancer gene in patient's tumors. The latest version of the Curie *p53* mutation database contains information on more than 1,300 different p53 mutations that have been detected in the tumors of more than 22,000 patients with various types of cancer (Soussi et al., 2005). Most mutations (80%) are located in the p53 DNA-binding domain which abrogates the p53 transcriptional activity (Olivier et al., 2002) (Figure 21 modified from IARC database, R10, July 2005) by changing the spectra and intensities of transactivation from response elements. This influence on target gene transactivation results in a variety of biological consequences which might influence tumor development and therapeutic efficacy (Menendez et al., 2006). The nature, type and location of mutations depend upon the tissue and particular carcinogens, responsible for the alterations. Six hotspots in the DNA binding domain account for 40% of all mutations described: codons 175, 245, 248, 249, 273, 282.

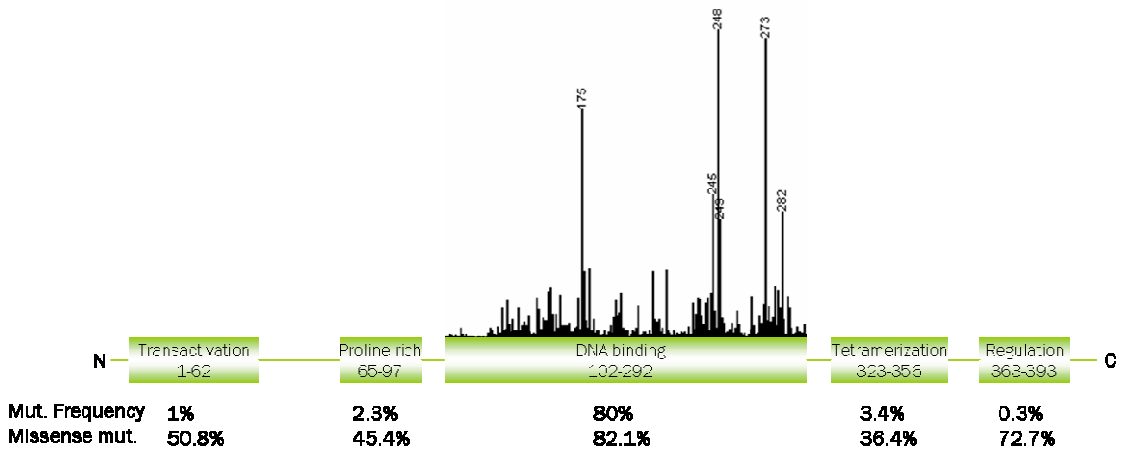


Figure 21. Mutation frequency by p53 domain and codon.

Most inactivating mutations (73.7%) are missense and can affect the conformation and specific interactions of p53 with DNA and other proteins leading to an increase in the half-life of the protein making mutant p53 detectable in tumors using immunohistochemistry. This has led for some time to the general assumption that positive immunostaining is a surrogate marker of *Tp53* mutations though there is extensive evidence that reality is more complex (Hall and McCluggage, 2006). Several studies have compared Tp53 status with p53 expression and found a high correlation (Cordon-Cardo et al., 1994;Erill *et al.*, 2004). However, some *p53* mutations do not lead to overexpression, normal p53 can accumulate in cells in

response to stress and alterations in other members of the p53 pathway can alter protein processing (Abdel-Fattah et al., 1998).

Normal p53 acts to suppress tumors, however, mutants rather than just relieve the suppression can promote tumor growth (van Dyke, 2005). In vivo studies suggest two possible explanations. First, many p53 mutant proteins can inhibit normal function by acting as dominant negative proteins competing with and blocking the activity of the endogenous wild type p53 protein. The mutated protein within the tetramer is thought to abolish the DNA-binding capacity of the entire complex (de Vries et al., 2002). Second, mutant proteins might acquire new abnormal functions (Lang et al., 2004;Olive et al., 2004). Mutant *p53* influences tumorigenesis in a manner distinct from deleting wild type *p53*. Mutant *p53* may have a direct effect on gene expression which is different from that of the wild type *p53* or a mutant *p53* may be able to prevent other p53 family members from functioning properly (Vousden and Prives, 2005).

P53 and bladder cancer

- P53 alterations and bladder cancer

Two hundred and sixty two mutations have been described in bladder cancer (IARC database) in the *Tp53* gene up to July 2005, 254 (96%) of which are located in exons 4 to 9. Missense mutations accounted for 72.1%, nonsense 12.5%, and silent 6%. The main hotspots are codons 285, 248, 280, 175 and 213, accounting for 28% of *p53* mutations. *P53* mutations have been described at higher frequencies in high grade and stage tumors (Spruck, III et al., 1994). Most studies on p53 mutations have been performed in tumors from relatively small groups of patients. Based on a review of 150 publications, the range of prevalence of mutations has been reported to be 0-48% among superficial tumors and 31-56% for invasive tumors. The prevalence of *p53* mutations is estimated to be between 14-52% (Malats et al., 2005). P53 overexpression has also been associated to high grade and stage (Kelsey et al., 2004).

- P53 alterations in relationship to carcinogen exposures

Tobacco. In some human cancers, *p53* mutations have been linked to environmental exposures, including tobacco (Hainaut et al., 2001). Tobacco smoke

contains benzo(a)pyrene and other PAH compounds which have been associated with the presence of transversions (Hussain and Harris, 1999). A study of the influence of cigarette smoking on *p53* mutations in bladder tumors, described the presence of G:C-T:A transversions only in smokers (Bernardini et al., 2001). Conflicting results have been obtained concerning the influence of smoking on p53 alterations. Some authors have reported no difference in the mutation spectrum between smokers and non-smokers (Kannio et al., 1996;Moore et al., 2003;Kelsey et al., 2005), whereas others found multiple *Tp53* mutations only in smokers (Spruck, III et al., 1993;Wallerand et al., 2005). In lung cancer, where 40% of tumors carry p53 mutations, alterations are generally more common in smokers than in non smokers, and a G to T transversion has been described as a molecular signature of tobacco smoke mutagens in smoking-associated lung cancer (Pfeifer et al., 2002).

Occupation. The number of studies focusing on p53-mutation-spectra in bladder cancer patients subject to specific occupations are less frequent, and because the range of occupations and etiologic factors to which individuals are subject to in each occupation is wide, the study of p53 alterations associated to occupation is complex. Sites and patterns of mutation in the *p53* gene have been studied in relation to etiologic factors. A study on occupational cancer showed that 70% of mutations occurred in exon 5, at hotspot codons 151 and 152 and the most common alterations were C to T transitions (Yasunaga et al., 1997). A higher frequency of p53 alterations or inactivation has been found among hair dye users and high risk occupations like tractor-trailer truck drivers and casting machine operators in men. No alterations among arsenic exposed individuals has been described (Kelsey *et al.*, 2005).

Susceptibility genes. The mutational spectrum of *p53* in bladder cancer may be influenced by the properties of metabolising enzymes. A study by Schroeder and colleagues has shown an association between GSTM1 null and NAT2 slow genotype with *p53* positive (mutated) vs. *p53* negative bladder cancer tumors (Schroeder et al., 2003), in agreement with two previous case:case studies (Brockmoller et al., 1996;Martone et al., 2000). However, this association was not pronounced. No significant difference in frequency of *p53* mutations and GST, NAT genotypes has

been recently described (Ryk et al., 2005), although among *p53* mutant patients transversions were more frequent in GSTM1 negative as compared to positive individuals.

- Prognostic implications

P53 mutations and expression status have been extensively analyzed in relation to the prognosis of bladder cancer patients. A clear consensus has been reported concerning the association of p53 alterations to high grade and stage (Lorenzo-Romero et al., 2003;Erill *et al.*, 2004;Shariat *et al.*, 2004b;Ryk *et al.*, 2005).

The association between p53 alterations and recurrence, progression, death or overall survival is not clear. Difficulties arise when comparing studies due to methodological issues of IHC (tissue preparation, antigen retrieval methods, type of antibody used, and assessment of positivity), different stages of bladder cancer in different studies and differences in treatments received by patients. Some studies have described p53 overexpression as an independent marker for disease progression (Sarkis et al., 1993;Esrig et al., 1994;Erill *et al.*, 2004;Schrier *et al.*, 2006) and death (Shariat et al., 2004b). Others have shown no association between p53 expression levels and recurrence, progression or overall survival in invasive tumors (Tiguert et al., 2001;Galmozzi et al., 2006).

A number of reviews (Schmitz-Drager et al., 2000;Slaton et al., 2001;Smith et al., 2003) highlight the discrepancies published of the value of p53 as an independent prognostic marker in bladder cancer. In agreement, a recent meta-analysis including 168 published studies (Malats et al., 2005) describes the controversies in the literature about the prognostic character of the *Tp53* gene and supports the notion that there is no conclusive evidence that p53 alterations represent an independent prognostic marker in bladder cancer.

Several studies have analyzed the prognostic value of p53 alterations in subgroups of patients treated homogeneously and results also remain controversial. Retrospective studies evaluating p53 expression in tumors from patients undergoing TUR have found that increased staining is a negative predictor for survival (Sarkis et al., 1994;Serth et al., 1995) whilst others have failed to identify the association (Gardiner et al., 1994;Tetu et al., 1996). The same inconsistencies appear when analyzing the prognostic value of p53 alterations in patients treated with cystectomy. Several studies describe p53 as an independent predictor of

prognosis (Esrig *et al.*, 1994;Shariat *et al.*, 2004b) whilst others do not (Nakopoulou et al., 1998;Hemal et al., 2003). Overall, two major problems of these studies are poor study design and retrospective collection of data.

- P53 status in bladder cancer therapy

Several reports have evaluated the response of patients treated with intravesical BCG in relationship to p53 status. A positive correlation has been described in various studies, supporting the notion of using the results of the analysis of p53 status to decide on BCG treatment (Sarkis *et al.*, 1994;Caliskan et al., 1997;Lee et al., 1997c;Saint et al., 2004). However, other studies have shown no relationship between response to BCG and p53 status (Lacombe et al., 1996;Okamura et al., 1998;Peyromaure et al., 2002).

Radiation therapy. It has been proposed that wildtype *p53* makes tumors more sensitive to radiation through the induction of apoptosis, and that *p53* inactivation might confer resistance to treatment. This is however the case in tumor cells capable of p53-dependent apoptosis. In those tumors that lose the ability of p53-dependent apoptosis, p53 leads to growth arrest and DNA repair, hence p53 inactivation will lead to DNA-damage induced mitotic catastrophe causing radiation sensitivity (Gudkov and Komarova, 2003). So, in the absence of apoptosis, p53 might act as a survival factor. The ability of p53 to follow apoptosis or growth arrest may be explained by: 1) the amount of activated p53 and the duration of the activation, 2) the spectrum of p53 responsive genes that are available for modulation, 3) the availability of p53 co-factors that differentially regulate the ability of p53 to bind to specific subsets of target genes, 4) tissue specificity. Controversial results have been observed regarding the relationship between response to radiotherapy and p53 alterations in several bladder tumors studies (Wu et al., 1996;Rotterud et al., 2001;Hinata et al., 2003).

Similarly, it has been proposed that innate resistance to **chemotherapy** may be a function of p53, although there are conflicting data on whether p53 mutations confer an increased resistance or responsiveness (Raghavan, 2003). *Tp53* mutated tumors may be less able to initiate apoptosis and therefore resistant to chemotherapeutic treatment. Some studies have shown that patients with mutated p53 tumors benefit from chemotherapy (Cote et al., 1997;Kielb et al., 2001), whereas others have shown that these tumors are resistant to chemotherapy

(Sarkis et al., 1995;Shiraishi et al., 2003a) or that there is no difference (Jankevicius et al., 2002;Shiraishi et al., 2003b).

- *P63* and *p73*

P63 is indispensable for the differentiation of a transitional urothelium and is expressed in normal bladder urothelium. CGH analysis of urothelial carcinomas has identified amplifications in the long arm of chromosome 3 (Richter et al., 1998). p63 is regulated in bladder carcinoma and has been described to be lost in most invasive cancers whereas superficial papillary tumors maintain the expression (Urist et al., 2002). The mechanism underlying the impaired p63 expression remain unrevealed. A decrease of the levels of p73 has also been associated to bladder cancer stage (Puig et al., 2003).

- *MDM2*

Immunohistochemical analysis have shown that certain bladder tumors have higher levels of MDM2 nuclear staining (Ioachim et al., 2000) and mRNA increased expression is associated to lower grade and stage (Schlott et al., 2004).

- *Aurora A kinase*

It is located in 20q13 where 35% of bladder tumors show amplification. Aurora A kinase gene amplification and associated increased expression of the mitotic kinase it encodes are associated with aneuploidy and aggressive clinical behavior in human bladder cancer (Sen et al., 2002).

- *P21*

Altered expression of p21 is associated with an increased risk of bladder cancer progression and death, but not with stage (Shariat *et al.*, 2004b).

The p53 and the pRb tumor suppressor pathways are connected. The checks and balances that exist between pRb and p53 involve the regulation of the G1/S transition and its checkpoints. Following DNA damage, the p53-dependent induction of p21 regulates the activity of cyclin E/Cdk2 and cyclin A/Cdk2 complexes both of which phosphorylate pRb leading to E2F-mediated activation of downstream targets. E2F1 dependent induction of ARF antagonizes the ability of

MDM2 to degrade p53, leading to p53 stabilization and potentially p53-mediated apoptosis or cell cycle arrest. Virtually all human tumors deregulate either the pRb or p53 pathway (Yamasaki, 2003).

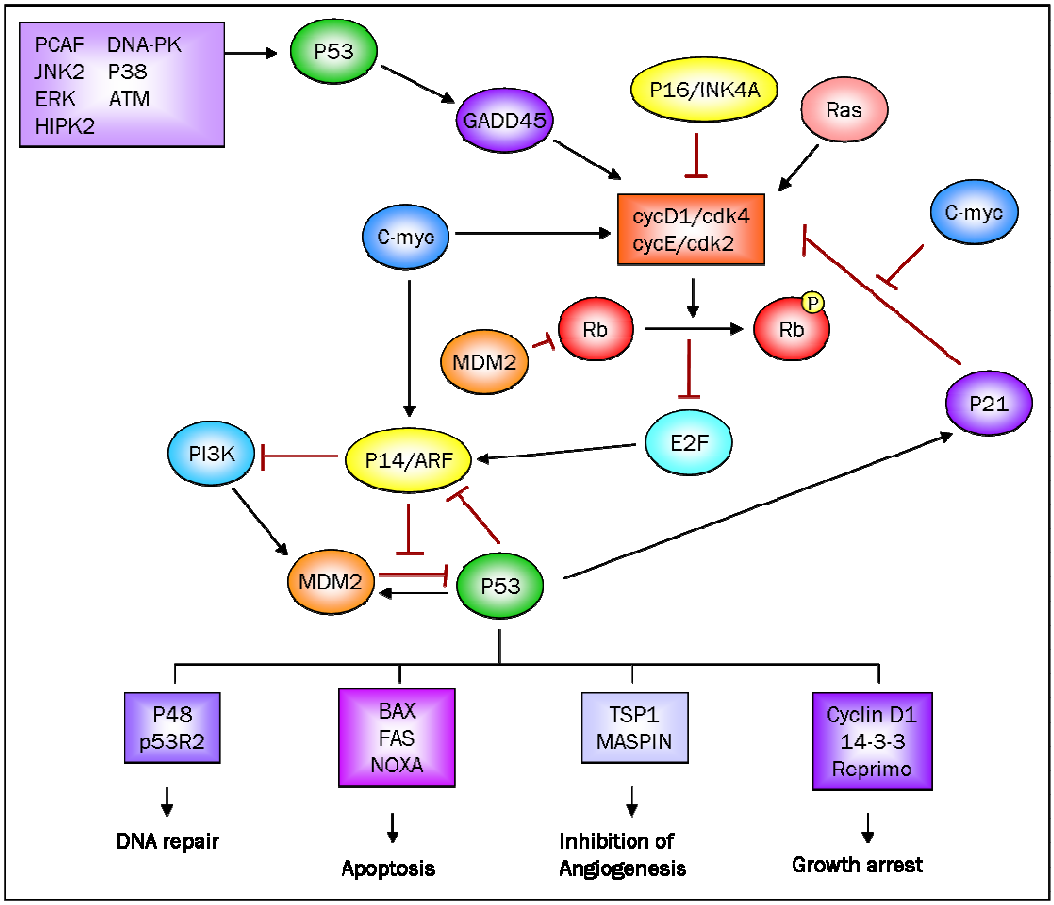


Figure 22. The Rb/P53 crosstalk pathway.

A few studies have combined the analysis of p53, p16, p21 and pRb expression in bladder tumors and described that they act in cooperative or synergistic ways to promote tumor progression: p53/p16 (Hitchings et al., 2004), p53/p21 (Garcia, X *et al.*, 2004;Shariat *et al.*, 2004b), p53/pRb (Cote et al., 1998) or p53/p21/pRb (Chatterjee et al., 2004a).

Although their pathways overlap there are additional influences that alter the function of these proteins.

6.1.5 Apoptosis. In the bladder, as in other tissues, failure of the regulatory genes involved at any point in the apoptotic pathway may result in prolonged survival of an abnormal genome, tumorigenesis and resistance to anticancer agents. Figure 23

gives an overview of the 2 pathways regulating apoptosis: extrinsic (green) and intrinsic (blue) pathways. Michael K. Rowe and De-Maw Chuang Vol. 6; Issue 21; 8 October 2004.

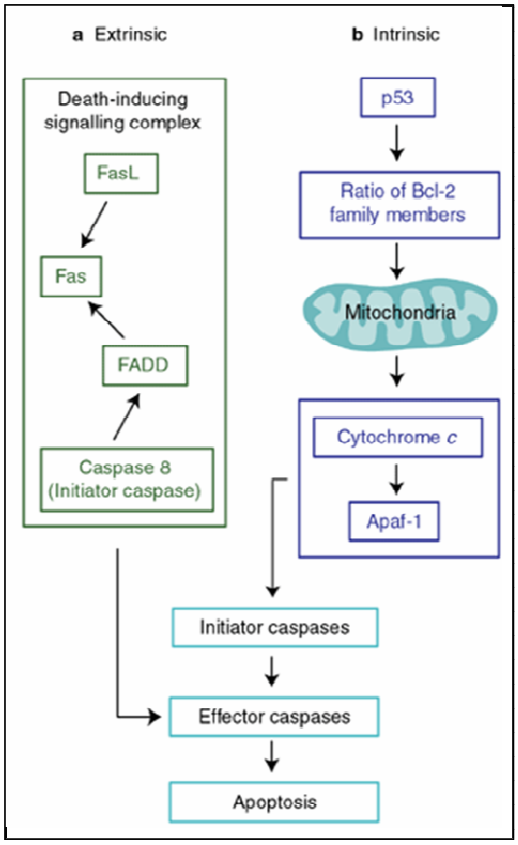


Figure 23. Apoptosis pathways.

(a) The extrinsic pathway centers around death receptors. Fas Ligand (FasL) binding to Fas receptor results in binding of the cytosolic death domain of the receptor to an adaptor protein (FADD) containing a death domain. The adaptor protein also has a death effector domain to which a caspase can bind. All four molecules (ligand, death receptor, adaptor protein, and caspase) form a death-inducing signalling complex that activates the effector caspases.

(b) The intrinsic pathway centers on the integrity of the mitochondria, which is maintained by the proteins of the Bcl-2 family. Some Bcl-2 family members increase membrane integrity (anti-apoptotic) and some decrease it (pro-apoptotic). The ratio of the pro- and anti-apoptotic Bcl-2 family members is tightly regulated by p53, among other molecules. If the ratio of the Bcl-2 family members tips towards the pro-apoptotic side, the mitochondria's membrane integrity decreases and the pro-apoptotic factors, such as cytochrome c, are released. Cytochrome c binds to Apaf-1, and this complex activates initiator caspases that in turn activate effector caspases.

FAS and FASL. Fas (10q24) and Fas ligand (1q23) relay important pro-apoptotic signals and play an important role in tumor development, progression and immune escape and therapeutic responses (Owen-Schaub, 2002a). FAS belongs to the tumor necrosis factor receptor superfamily and mediates cytotoxic immune responses. FAS and FASL have been implicated in bladder cancer (Owen-Schaub, 2002b). Low levels of FAS protein have been associated with higher grade, stage

and a poor prognosis (Yamana et al., 2005). Wild type *p53* is known to transcriptionally activate *TNFRSF6* encoding FAS. Since *p53* mutation is common in bladder cancer it could underlie downregulation of FAS but this has been shown not to be due to the mutations (Maas et al., 2004).

BCL-2. *BCL-2* (18q21) is a protooncogene member of the Bcl-2 family of proteins involved in mitochondrial permeabilization and it plays a potent role in suppressing apoptosis. It was initially suggested that BCL-2 mRNA and protein were not present in normal urothelium, but were present in 63% of low grade UC of the bladder (Gazzaniga et al., 1995). In a later report, BCL-2 protein was shown to be expressed in the basal layer of normal urothelium and in 24% of invasive UC (Shiina et al., 1996;Glick et al., 1996). An association between p53 and BCL-2 overexpression together with poor outcome has been shown in bladder tumors (Ong et al., 2001).

SURVIVIN. (17q25) *Survivin* belongs to the family of inhibitor of apoptosis proteins (IAPs). Inactivation of survivin leads to spontaneous cell death or mitotic aberrations. Survivin is expressed during the G2/M phase of the cell cycle, is associated with the microtubules of the mitotic spindle and directly inhibits caspase-3 and caspase-7 activity. Functionally, *BCL-2* and *SURVIVIN* have been positioned in non-overlapping anti-apoptotic pathways. Survivin expression is almost undetectable in many adult differentiated tissues (Ambrosini et al., 1997), however it increases upon malignant cell transformation. This overexpression can be caused by amplification of the 17q25 chromosomal arm, demethylation of survivin exon 1 and loss of p53, as survivin is transcriptionally repressed by *p53* (Schultz et al., 2006). Swana et al have reported that survivin expression is correlated with decreased time to recurrence of G1 bladder cancer (Swana et al., 1999). High expression of survivin has been associated with reduced disease-free survival (Ku et al., 2004). The fact that detectable levels of survivin in urine are associated with a positive urine cytology, make survivin a novel urine biomarker for the detection of bladder cancer (Smith et al., 2001;Shariat et al., 2004a).

FHIT. The fragile histidine triad (3p11) gene spans the most active common fragile site in the human genome, FRA3B. *FHIT* exerts its oncosuppressor activity through induction of FAS-dependent apoptotic cell death (Roz et al., 2004). FHIT inhibits

tumor growth by MDM2 inactivation, thus blocking MDM2 association with p53, leading to the stabilization of p53 (Nishizaki et al., 2004). Although *FHIT* mutations are rare, aberrations that lead to loss of expression have been described in lung, breast and bladder cancer (Sozzi et al., 1998;Skopelitou et al., 2001;Guler et al., 2004). Loss of FHIT expression is observed also in premalignant lesions, suggesting that FHIT is susceptible to damage caused by environmental carcinogens in initial steps of carciongenesis (Huebner and Croce, 2003). FHIT protein has been shown absent or reduced in 61% of bladder tumors and the expression correlated with pathological and clinical status (Baffa et al., 2000). On the basis of these observations a gene therapeutic approach has been tested in urothelial carcinoma cell lines. Reintroduction of the *FHIT* gene in FHIT negative bladder carcinoma cell lines caused an inhibition of cell growth and increased tumor cell death (Vecchione et al., 2004).

6.1.6 Telomerase activity. During normal cell division, telomere shortening leads to cell senescence and thus governs normal cell mortality. Telomere length is maintained by the enzyme telomerase. Lin and colleagues suggested that expression of telomerase activity could be a required event in tumorigenesis of human bladder cancer. They detected telomerase activity in almost all bladder tumors, having a clear association with grade and stage. Most tumors with high activity were of advanced grade and deep invasion (Lin et al., 1996;Okumura et al., 2004). The expression levels of human telomerase reverse transcriptase, the catalytic subunit of telomerase, have also been associated to stage and grade (Takihana et al., 2006). The measurement of telomerase activity in urine has been analysed in various studies and it has been proposed that it could be a useful diagnostic test to detect bladder cancer (Abd, I et al., 2005;Sanchini et al., 2005;Weikert et al., 2005).

6.1.7 Angiogenesis. Angiogenesis is the process by which tumours induce a blood supply from their surrounding tissues and it has been shown to be necessary for tumour growth (Streeter and Crew, 2001). Angiogenesis can be described in three steps: 1st) activation of endothelial cells, 2nd) migration and invasion of the endothelial cells following proteolytic degradation of the surrounding extracellular matrix, 3rd) maturation of the endothelial cells to coalesce and form water-tight

tubules that establish new blood flow (Folkman, 1992b). A histological measure of angiogenesis is microvessel density (MVD) and a variety of staining methods are also employed involving immunohistochemical techniques using antibodies against CD31, CD34 or factor VIII related antigen that recognize immature or new vascular endothelial cells. Increased angiogenesis occurs in and around urothelial tumors, seemingly because of an increased expression of angiogenesis inducers and a decreased expression of angiogenesis inhibitors. Inducers: vascular endothelial growth factor (Folkman, 1992a), fibroblast growth factors (FGF1 and 2), COX-2 (Li et al., 2004) and platelet-derived growth factor (Mizutani et al., 1997); and inhibitors: thrombospondin1 (Ioachim et al., 2006). Bladder tumors can stimulate more angiogenesis than normal urothelium, and increased microvessel density has been associated to prognosis. Dickinson et al described an association between increased MVD and mortality in invasive bladder tumors (Dickinson et al., 1994), and since then various studies have shown MVD as a significant independent prognostic indicator of recurrence and poor survival (Bochner et al., 1995;Ozer et al., 1999;Bartoletti et al., 2005b). In muscle-invasive tumors, microvessel density correlates with the presence of occult lymph-node metastases (Jaeger et al., 1995;Chaudhary et al., 1999). Angiogenesis thus has a key function in bladder cancer and is therefore attractive for therapeutic manipulation (Inoue et al., 2000).

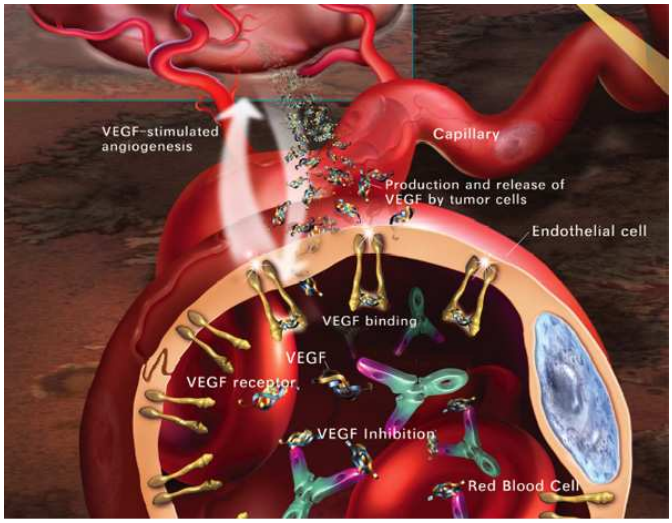


Figure 24. Angiogenesis steps.

6.1.8 Invasion and metastasis. Cadherins are the main mediators of cell-cell adhesion in epithelial tissues, being major components of both the adherens junctions and desmosomes. They are divided into 3 subtypes: N (neural), E (epithelial) and P (placental). Cadherins are expressed early in development, and changes in their subtype expression play an important role in determining the segregation of cells into distinct tissues and in maintaining tissue architecture

(Vleminckx and Kemler, 1999). **E-cadherin** (16q22) is one of the members of the Cadherin family and is present within epithelial cells. E-cadherin is a transmembrane protein with a surface glycoprotein that connects to neighboring cells and a cytoplasmic tail by which it mediates intracellular signaling through β-catenin. Loss or reduced expression of E-cadherin, which occurs primarily in invasive urothelial tumors, induces a defect in cell-cell adhesion. Loss of E-cadherin immunoreactivity has been described in 78% of high-grade invasive urothelial carcinomas, compared with only 28% of the low-grade non-invasive papillary tumors (Garcia, X et al., 2000). Several prospective studies indicate that the status of E-cadherin can serve as an independent prognostic indicator for disease progression (Popov et al., 2000;Shariat et al., 2001;Serdar et al., 2005). Hypermethylation of CpG dinucleotides in the CDH1 (which encodes E-cadherin) promoter occurs in 84% of urothelial carcinomas, providing evidence that the main mechanism of E-cadherin downregulation is transcriptional silencing (Ribeiro-Filho et al., 2002). **P-cadherin** (16q22) is another cadherin family member localized in the basal cell compartment of numerous normal epithelial tissues, in which E-cadherin is expressed throughout the mucosa. P-cadherin expression results in an improved ability for anchorage-independent growth. P-cadherin is expressed in the basal and parabasal layers of normal urothelium (Rieger-Christ et al., 2001). Expression increases significantly as grade and stage progress and this is associated with a worse survival.

6.2 Genomic level. Chromosomal aberrations can be primary, causally related to tumor development, or secondary, involved in progression. Aberrations such as deletions and copy number increases contribute to alterations in the expression of tumor suppressor genes and oncogenes, respectively. Detection and mapping of copy number abnormalities provide an approach for associating aberrations with disease phenotype and for localizing critical genes.

Comparative genomic hybridization is a method designed to identify chromosomal level alterations. Differentially labelled genomic DNA samples (a test and a reference) are competitively hybridized to chromosomal targets, and copy number is reflected by their signal intensity ratio (Lockwood et al., 2006). A limitation of CGH is that it cannot detect balanced chromosomal rearrangements. CGH provides

information on the relative copy number of sequences in the test genome compared with a normal diploid genome. Since its development in the early 1990s and its application on metaphase chromosomes, an important effort has been made to improve the resolution of the technology. The use of DNA targets immobilized in an array format, replacing the conventional metaphase spreads with a resolution of 20 Mb, represents a significant advance due to the higher resolution – around 1 Mb -, direct mapping of aberrations to the genome sequences and higher throughput (Pinkel et al., 1998). The different platforms vary in size of the genomic elements spotted and their coverage of the genome. Various microarray platforms have been developed to support array CGH studies. BAC-based platforms allow highly sensitive and reproducible detection of a wide range of copy number changes including single copy number gains and losses, homozygous deletions and high-level amplifications (Greshock et al., 2004). Other platforms include: single nucleotide polymorphism oligonucleotide arrays, which do not represent the entire genome but display lower nonspecific signal by decreasing the probability of crosshybridization to multiple short oligonucleotide targets on the arrray (Bignell et al., 2004); long oligonucleotide arrays which by elongating the targets aim to improve hybridization specificity (Brennan et al., 2004); and whole-genome tiling path arrays which are the only arrays covering the entire genome (Ishkanian et al., 2004). This submegabase resolution tiling array is comprised of 32433 overlapping BAC clones, it is very sensitive and allows the detection of microalterations that may fall between marker probes in other array platforms.

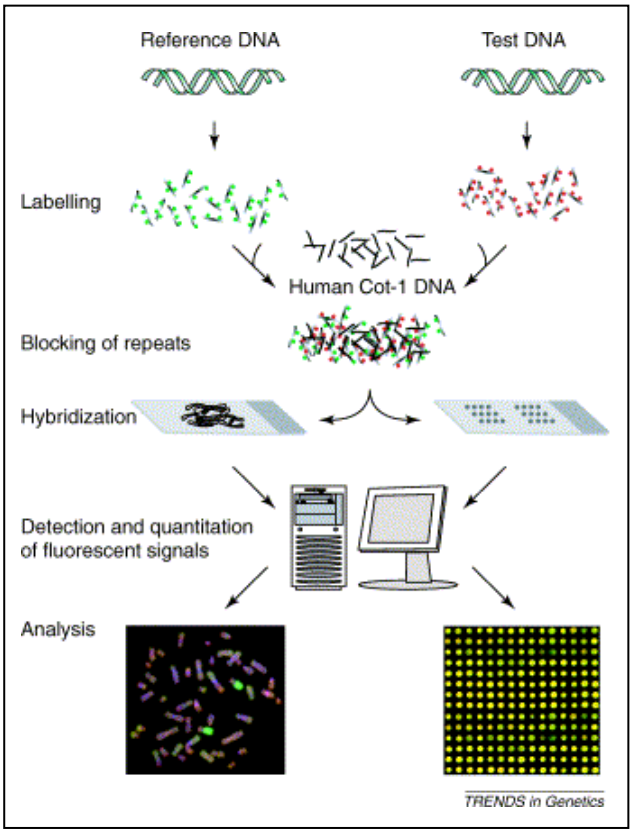


Figure 25. array CGH diagrammatic scheme (Mantripragada et al., 2004).

Few studies have used array CGH analysis based on a BAC platform to describe the chromosomal alterations associated to urothelial carcinoma. Veltman et al (Veltman *et al.*, 2003a) analyzed 41 bladder tumors. In addition to previously described alterations in large regions, a wide number of small genomic changes were detected. Hurst et al (Hurst *et al.*, 2004) analyzed 22 bladder cancer cell lines. Many genetic changes were identified, many of which were compatible with data available from previous conventional CGH studies. Blaveri et al (Blaveri *et al.*, 2005) have analyzed 98 bladder tumors of diverse stages and grades. The analysis showed significant increases in copy number alterations and genomic instability with increasing stage and with outcome. Recently, an interesting article has described analysis of bladder tumors by SNP arrays and found allelic imbalance was associated to stage (Primdahl et al., 2002;Koed et al., 2005).

There is general agreement on several broad concepts relating chromosomal alterations in urothelial carcinoma: higher rates of genetic alterations are seen in pT1 tumors compared with papillary pTa tumors (Richter et al., 1997); gains and amplifications predominate over deletions in advanced-stage bladder cancer (Richter *et al.*, 1998); the most consistently found alterations are gains of 1q, 8q and 20q and losses of 8p, 11pq and 9pq (Voorter et al., 1995;Simon *et al.*, 1998;Koo *et al.*, 1999;Zhao et al., 1999;Simon et al., 2000;Prat *et al.*, 2001;Yu et al., 2001;Obermann et al., 2003).
Here, we provide a summary of the major alterations reported in this tumor classified on the basis of their chromosomal location.

Chr. 9. The q arm of chromosome 9 is very frequently lost both in low and high grade tumors, suggesting it is a primary event in the genesis of bladder cancer (Hartmann et al., 2002). Losses at 9q cover 3 major deleted regions (9q22, 9q32-33, and 9q34), suggesting that 1 or several tumor suppressor genes may be located in them (Simon *et al.*, 1998). Candidate tumor suppressor genes in these regions include *NETRIN* (Amira et al., 2004), *TSC1* (Knowles *et al.*, 2003), *PTCH* (Aboulkassim et al., 2003) and *DBCCR1* (Wright et al., 2004). Allelic losses at 9q have been reported as an early lesion in the development of bladder cancer, but they have also been associated with invasive disease (Hirao et al., 2005) and with disease recurrence in superficial bladder tumors (Simoneau et al., 2000). The study

by Hirao et al analyzed p53 status and 9q LOH in bladder tumors and found no association between LOH and mutation or staining; thus, 9q loss may reflect genetic instability often associated with p53 inactivation in invasive urothelial carcinoma. Deletions in 9p, commonly found in bladder tumors, are mainly situated at 9p21 where the candidate genes are *p16 INK4A* and *p14 ARF* (Williamson et al., 1995).

Chr. 8. Alterations in chromosome 8 often involve loss of the p arm, with amplification of a small amplicon, and gain of the q arm. LOH in 8p is associated with a more aggressive tumor phenotype, indicating the possible presence of a tumor suppressor gene. Deletions in 8p have been identified by CGH (Richter et al., 1999) analysis and LOH in 23-52% of cases (Choi et al., 2000a). The 8p21-22 region (Choi et al., 2000b) contains candidate genes: *TRAIL-R2* (tumor necrosis factor-related apoptosis inducing ligand receptor 2) which induces death following TRAIL binding and has been found mutated in non-small cell lung cancer, head and neck cancer and breast cancer (Adams et al., 2005); *DBC2* (deleted in breast cancer 2) in which mutations have been identified in breast cancer and its expression in breast carcinoma cells suggests its role as a tumor suppressor gene; and *LZTS1* which shows reduced or absent protein expression in bladder tumor samples (Knowles et al., 2005). Amplification of 8p12 often involves *FGFR1* which is a member of the fibroblast growth factor receptor family involved in proliferation, migration, and differentiation of endothelial cells. Gain in 8q contains *CMYC* (Veltman *et al.*, 2003a).

Chr. 5. Alterations in chromosome 5 commonly involve deletion of 5q (Veltman et al., 2003b;Hurst *et al.*, 2004) and gain of 5p. In previous CGH studies, 5p amplification was found to be one of the few alterations occurring more frequently in muscle invasive tumors than in early invasive cancers (Richter *et al.*, 1998). The most common site of amplification contains, among others, the *TRIO* gene (5p12), which encodes a protein with a putative role in cell cycle regulation whose amplification is associated to high stage, grade and progression (Zheng et al., 2004). Loss at 5p13-12 has also been defined as a critical region involved in tumor progression in bladder carcinomas (Bohm et al., 2000) and a marker of adverse prognosis independent of tumor stage and grade (Bohm et al., 2002).

Chr. 6. Amplification of 6p22 is one of the most frequent genetic alterations in urinary bladder, affecting up to 20% of high grade, invasively growing tumors (Bruch et al., 2000;Veltman *et al.*, 2003a;Hurst *et al.*, 2004). This region has been narrowed down to 1.6 megabases at 6p22.3 which contains potential oncogenes such as *SOX4* (Aaboe et al., 2006), *CDKAL*, *E2F3* (Feber *et al.*, 2004), *DEK* (Evans et al., 2004) and *ID4* (Wu et al., 2005). *E2F3* is part of the E2F family involved in the Rb pathway. Amplification of this gene locus is associated with protein expression, invasive tumor growth and enhanced cell proliferation (Oeggerli et al., 2004;Oeggerli et al., 2006). E2F3 and DEK have been found overexpressed in bladder tumors when compared to normal bladder tissue (Wu *et al.*, 2005) which could be due to gene expression associated to a higher level of proliferation compared to normal tissue. However, ID4 overexpression can be described as ectopic in bladder cancer, since it is normally restricted to testes and brain (Riechmann et al., 1994b), and overexpression may more strictly depend on gene amplification.

Other common and consistently found alterations in bladder tumors are gains of 1q, and 20q and losses of 11p (Riechmann et al., 1994a;Prat *et al.*, 2001).

Table 6. Summary of common alterations detected by CGH (Mhawech-Fauceglia *et al.*, 2006b).

Alteration	Stage
Loss of 9 and Y	All stages and grades
Gains of 1p, 8pq, 20q Losses of 8p, 9pq, 11p	Most common genetic alterations in UC
Gain of 11q13 Loss of 9q	Papillary type Ta tumors
Losses of 9pq, 2q, 11pq, 10q, 17p Gains of 1q, 3pq, 5p, 6p, 8q, 10q	More frequent in T1 tumors
Gains of 3q, 5p, 7p, 10p, 18p, 20q Del 5q, 6q, 15q	More frequent in > T2 tumors
Gain of 1q Loss of 2q, 10q, 11p	More frequent in T1G2 than TaG2 tumors
Gain of 10p, 6p Loss of 5q, 6q, 18q	More frequent in TaG3 than TaG2 tumors

6.3 Microsatellite instability. It has been postulated that the number of somatic modifications in solid tumors is sufficiently high to necessarily involve the genesis of a genetically unstable phenotype within evolving clones (Loeb, 1991). One of the

mechanisms involved in genetic instability is the inactivation of DNA mismatch repair genes manifested by microsatellite instability. Gonzalez-Zulueta et al published the first systematic investigation into the frequency of MSI in urothelial carcinoma. They found that 3% had MSI, of which half had MSI at multiple loci (Gonzalez-Zulueta et al., 1993). Subsequently, Mao et al. analyzed 52 microsatellite markers in 32 urothelial carcinomas and found a 28% of MSI (Mao et al., 1994). To date, there have been around 20 studies of MSI and urothelial carcinoma (Catto et al., 2004), most focusing on the diagnostic role of microsatellite analysis. The main consensus is that the frequency of MSI at mono and dinucleotides in bladder cancer is low. There have been several reports suggesting a higher frequency of MSI at tetranucleotide repeats (Catto et al., 2003), which hints there are separate varieties of MSI. One group has shown very high rates of MSI in young patients (100% in patients 30-55 years old) (Christensen et al., 1998) and another group has found an association with tobacco smoking (Uchida et al., 1996). Overall, the association between MSI and clinicopathological data are contradictory. Bladder tumors with elevated microsatellite alterations at selected tetranucleotide repeats (EMAST) have been described to contain mutations in the *p53* gene (Danaee et al., 2002) but no association has been described with p53 overexpression (Burger et al., 2006). However, tumors with other MSI have a low rate of *p53* mutations. Instead, these tumors bypass apoptosis by developing mutations in the *BAX* gene (which has a microsatellite region within it), a downstream effector of p53. The wild-type *p53* is then able to perform its other cellular roles.

6.4 Aberrant methylation. As in other tumors, there is now extensive evidence that aberrant methylation is common in bladder cancer and may play an important role in tumor progression. Gene silencing by promoter hypermethylation has been the subject of many studies. Known examples of silencing of tumor suppressor genes by hypermethylation of the promoter region in bladder cancer are cadherin-1 (36% of tumors), RAS-associated domain family (RASSF1A) (35% of tumors), CDH13 (29% of tumors), secreted Frizzled-related protein 1 (sFRP1) (29% of tumors), FHIT (16% of tumors), retinoic acid β (15% of tumors), p16 (7% of tumors), and death-associated kinase (4% of tumors) (Maruyama et al., 2001). Hypermethylation of APC, $p14^{ARF}$ and RASSF1A have been described in the urine of bladder cancer patients (Dulaimi et al., 2004). Hypermethylation of various promoter regions shows

significant association with tumor grade and/or stage (Catto et al., 2005). A recent study showed that methylation of promoter regions of p16, p14, E-cadherin, RARβ2, RASSF1a and GSTP1 occurs in both normal and CIS samples from patients with urothelial carcinoma and increased with progression (Dhawan et al., 2006). sFRP gene silencing by methylation has been shown to be associated to invasive bladder cancer and to overall survival in these patients (Marsit et al., 2005).
Because methylation occurs early in carcinogenesis, it may be useful for the detection of recurrences (Skopelitou *et al.*, 2001).

7. Natural history of bladder tumors: dual track concept, clonal origin

Bladder cancer is a heterogeneous disease with a variable natural history. At one end of the spectrum, low-grade Ta tumors have a very low progression rate and require initial endoscopic treatment and surveillance but rarely present a threat to the patient. At the other extreme, high-grade tumors have a high malignant potential associated with significant progression and cancer death rates (Kirkali et al., 2005).

Dual track concept. It has now been 30 years since it was first proposed that there might be at least two separate pathways in the development of bladder cancer (Pugh, 1973). The dual track concept postulates that urothelial cancers arise via two distinct but partially overlapping pathways: papillary and nonpapillary (Czerniak and Herz F, 1995). At diagnosis, 80% of tumors are superficial at initial presentation arising from hyperplastic epithelium. Approximately 50-70% of these tumors will recur with 10-30% showing grade and stage progression. On the other hand 20% of patients present with muscle invasive or metastatic disease arising from severe dysplasia or CIS, of whom 50% will die within 2 to 3 years after diagnosis despite aggressive local therapy. The overlap between both pathways is reflected by the progression of superficial papillary tumors to high grade invasive carcinomas. Such progression is preceded by the development of CIS within a papillary lesion or in the adjacent areas of bladder mucosa and occurs in 10-15% of patients with a history of low grade superficial papillary tumor (Figure 26 (Wu, 2005)).
The sequence of mild, moderate and severe dysplasia does not fit well to urothelial precursor lesions in which severe dysplasia/CIS develops in a background of

urothelial hyperplasia. The classic cytoarchitectural features of mild to moderate dysplasia does not exist in bladder preneoplasia. Changes that mimic mild to moderate dysplasia of the urothelium are frequently reactive in nature. In contrast, hyperplasia is a well defined condition that can be diagnosed microscopically with a high degree of accuracy. Therefore, two major categories have been used to subdivide precursor intraurothelial lesions: low and high-grade intraurothelial neoplasia (LGIN and HGIN) (Czerniak et al., 2000). LGIN defines a flat hyperplastic change seen adjacent to low grade superficial papillary tumors; HGIN combines severe dysplasia and CIS and is seen adjacent to high-grade invasive bladder cancer (Spiess and Czerniak, 2006).

Losses in both arms of chromosome 9 occur commonly in both the papillary and the non-papillary pathways together with alterations in several gene clusters involved in the transcriptional regulation of proliferation, differentiation and programmed cell death (Kim et al., 2005). Papillary tumors are commonly associated with alterations in genes involved in the RAF/MEK/Erk and PI3K/PTEN/AKT pathway whereas they seldom harbour p53 or Rb pathway alterations. By contrast, alterations in the RAF/MEK/Erk and PI3K/PTEN/AKT pathway are less common among non-papillary invasive tumors which are – instead – commonly associated with Tp53 and Rb pathway alterations. It is generally thought that a small percentage of papillary tumors progress into invasive tumors although the genetic and genomic characteristics of this subgroup of tumors are unknown.

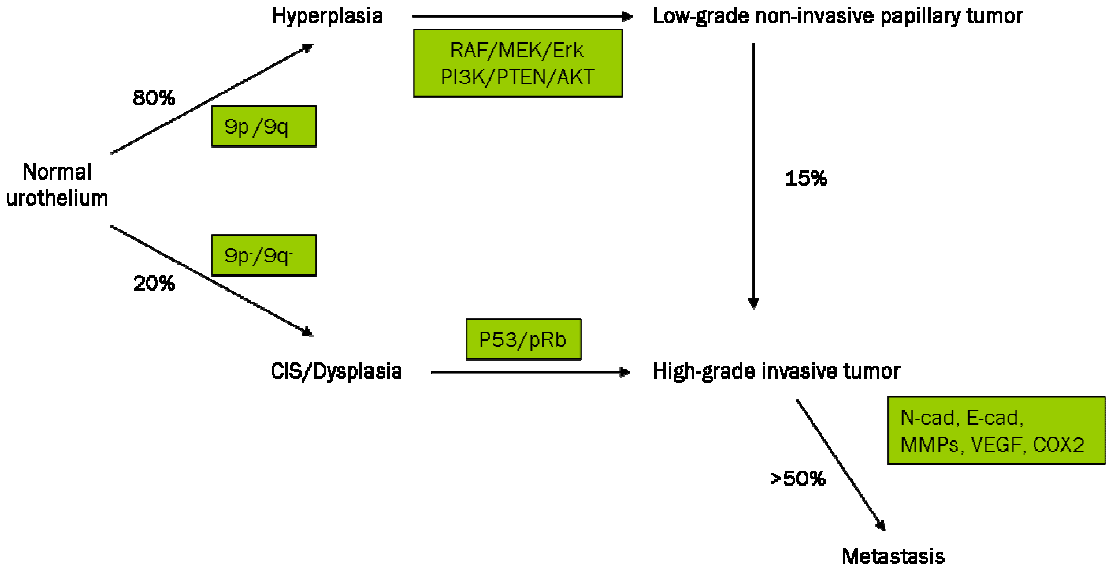


Figure 26. The dual track pathway.

Various articles have analyzed the characterization of these two distinct molecular pathways. H-ras overexpression in transgenic mice causes urothelial hyperplasia and superficial papillary non-invasive bladder tumors (Zhang et al., 2001). These results are in agreement with a study in which the HaSV ras transforming gene was introduced into transplants of normal urothelium, where cell proliferation was increased, leading to hyperplasia (Wagner et al., 1990). Thus suggesting, Ras has a role in generating hyperplasia in the normal mouse bladder. In striking contrast, Zhang and coworkers, previously targeted an SV40 large T oncogene to the urothelium, and showed that this oncogene induced bladder CIS which then advanced to become invasive and metastatic (Zhang et al., 1999). SV40 large T antigen, inactivates p53 and pRb, two genetic defects prevalent in human invasive bladder cancers, this suggests that the dysfunction of these two tumor suppressor proteins induces the formation of the aggressive form of urothelial carcinoma. Two studies (Bakkar et al., 2003;van Rhijn et al., 2004) showed that *FGFR3* mutations are exclusive of p53 alterations, defining these two genes separate pathways in bladder cancer. Bakkar et al analyzed *Tp53* mutations and van Rhijn et al analyzed p53 overexpression. Mhawech-Faauceglia et al. (Mhawech-Fauceglia *et al.*, 2006a) showed a similar relationship of *FGFR3* mutations and p53 expression. However, this has not been confirmed in other studies which find no difference in *Tp53* mutation frequency between *FGFR3* mutated or wild type samples (Zieger *et al.*, 2005). These discrepancies likely reflect patient selection biases since our group has found that the inverse relationship between *FGFR3* and *Tp53* mutations is lost in T1G3 tumors and likely reflects the wide spectrum of the disease and the various carcinogenesis pathways involved in these tumors (Hernandez et al., 2005).

Clonal origin. The development of multiple tumors in either a synchronous or metachronous manner in the same patient is a common characteristic of bladder cancer. There are two theories to explain these findings:

1) The “field defect” theory. A global change in the urothelium, consequence of continued exposure to both exogenous or endogenous carcinogenic compounds excreted in urine, gives rise to multiple clones of initiated cells evolving into tumors that are genetically unrelated.
2) Monoclonal theory. Postulates that multifocal tumors evolve from a single transformed cell, hence the progeny shares a number of identical genetic

alterations. Cells from the initiating clone spread either by intraluminal shedding with secondary implantation at distant sites or by intraepithelial migration.

The issue of monoclonal versus oligoclonal origin of multifocal urothelial carcinomas is clinically important due to its repercussions in treatment and surgical strategies, and in the genetic detection of recurrent or residual tumor cells (Duggan et al., 2004). If multifocal tumors are of a common clonal origin, molecular genetic findings specific to a patient´s urothelial carcinoma could be used to detect recurrent disease. However, if the tumors are polyclonal in origin it is conceivable that genetic diversity will occur and that the same molecular change will not be present in all tumors.

The validity of both theories has been studied by a varied number of techniques, mainly X chromosome inactivation, LOH, CGH and mutational analysis. All of these techniques have both advantages and pitfalls but they are still widely used: X chromosome inactivation is based on homogeneous methylation during development of a tumor, however hypomethylation has been described early in carcinogenesis and hypermethylation is associated to progression of tumors, it can only be tested in women whilst bladder cancer is more common in men and some inactivation might be tumor or tissue-specific; LOH analysis will inform on clonal origin only if concordant patterns with several genetic markers are demonstrated, interpretation must consider that monoclonal lesions retain the heterozygosity and that tumor heterogeneity and progressive cell selection can result in discordant microsatellite patterns in samples from different areas within a single tumor; certain mutations occur late in carcinogenesis hence a varied pattern of alterations could still mean monoclonality whilst specific mutations could be a consequence of a carcinogenic compound, hence finding the same alteration could still mean polyclonality. A summary of studies analyzing clonality in bladder tumors is shown in an article by Hafner et al. (Hafner et al., 2002). This work shows 14 studies which demonstrate monoclonality in the bladder tumors analyzed and 11 studies demonstrating polyclonality. However the two theories proposed are not strictly mutually exclusive. It has been suggested that oligoclonality is more common in early lesions with progression to higher stages leading to the overgrowth of one clone and pseudomonoclonality. Different clones might be present in early lesions

such as hyperplasia, dysplasia or CIS. Later a dominant clone might proliferate and spread throughout the urothelium, leading to secondary monoclonal tumors, which become clinically apparent. During progression to invasive stages multifocal tumors might accumulate further genetic alterations and develop into different but clonally related subclones (Figure 27 (Hafner *et al.*, 2002)). Molecular evidence supports the coexistence of both field defect and monoclonal tumors in one same patient (Jones et al., 2005).

A study by Zwarthoff and colleagues studied 11 patients for 17 years analyzing 104 recurrent bladder cancers. Tumor development was reconstructed by LOH and *FGFR3* mutation analysis, and the main conclusions were 1) chronology of tumor appearance did not run in parallel with the genetic evolution, 2) evolutionary genetic trees or pedigrees could be described for each patient as different bladder tumors had some identical alterations and the number of alterations increased between tumors of the same patient, 3) results suggested that extensive LOH was due to random genetic instability and this LOH was hypothesized to be caused by mitotic recombination (van Tilborg et al., 2000).

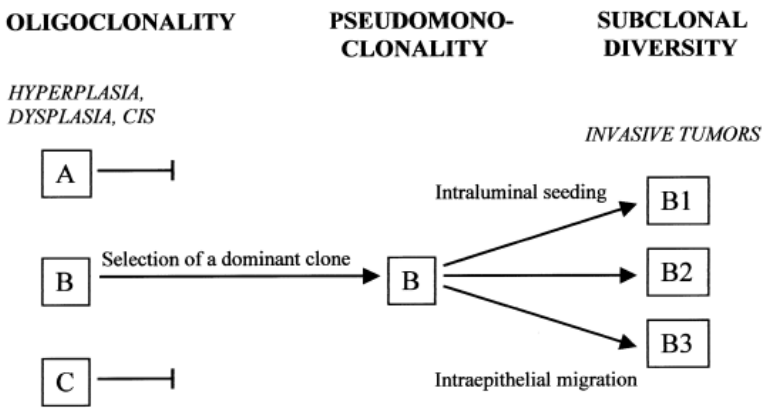


Figure 27. Possible clonal evolution (Hafner et al., 2002).

8. T1G3 bladder tumors: an important clinical challenge

Stage T1 grade 3 bladder carcinomas represent approximately 10% of all superficial bladder tumors (Lopez-Beltran et al., 2004). T1G3 tumors are associated with an aggressive course, having the highest risk of progression among superficial tumors (Masters et al., 2003). Transurethral resection followed by intravesical instillations of bacillus Calmette-Guérin represent the treatment of choice, and has been shown to be effective (Peyromaure and Zerbib, 2004). Studies dealing with T1G3 tumor treatment have reached diverse conclusions, probably due to the incorrect histopathological diagnosis, the use of different protocols of BCG therapy and different lengths of follow-up. Radical cystectomy has been reported to be a therapeutic option for patients with T1G3 tumors (Herr *et al.*, 2001). However, the rate of complications after cystectomy is relatively high (Chang et al., 2002).

Therefore, this tumor group poses important clinical challenges and of utmost interest is the identification of factors that allow to discriminate patients who require aggressive treatment vs. those who do not.

The EPICURO study, which our group has designed and coordinated, is an excellent framework in which to analyze this subgroup of tumors because the low incidence in the population has rendered studies with low number of samples with small statistical power for the findings described. The EPICURO study is a multicenter prospective study comprising 1356 incident cases and 1294 controls compiled between 1997 and 2001. Information on environmental exposures (smoking habits, occupation, diet, drinking water contaminants), clinical data (age, sex, treatment) and tumor characteristics (appearance of tumor in endocopy, multifocality, size, location) have been compiled for a high percentage of patients. The material obtained from patients includes paraffin embedded samples from primary and secondary tumors, blood and fresh tumors in a small percentage of cases, and the prospective nature of the study allows for a follow up period of 62 months which allows the analysis of biological markers and their association with prognosis in a subgroup of 136 T1G3 tumors. Tumors are all evaluated by the same pathologist which gives a homogeneous criteria for the classification into this subgroup of high risk tumors.

As mentioned previously, the prognosis of these tumors is challenging because around 50% progress to invasive tumors and no marker up till now aids in choosing

the right treatment for the patient and the difference between a TUR+BCG or a cystectomy is life-changing for a patients lifestyle after the surgery. The dilemma is therefore to identify those patients whose tumors are lethal and treat them aggressively, whilst not overtreating those whose tumors will not progress. Very few prospective studies have concentrated on T1G3 tumors alone and the prognostic factors implicated in survival. The reason is that it is difficult to compile a large series of patients within this category of tumors in a short period of time. López-Beltrán (Lopez-Beltran *et al.*, 2004) have observed that down-regulation of p27Kip1 and overexpression of Cyclin D1 and Cyclin D3 might be relevant predictors of survival in a group of 51 T1G3 tumors. Kim et al (Kim et al., 2002) have shown expression of COX-2 correlated with recurrence and progression in a homogeneous group of 37 T1G3 tumors. In the case of p53 as a prognostic marker in this group of tumors, there are only a few retrospective series on T1G3 bladder cancer patients. Steiner et al (Steiner et al., 2000) analyzed p53 immunoreactivity as a prognostic marker in 38 T1G3 tumors and described p53 not to be a helpful marker for decision making about treatment to the patients, similar to a study by Peyomaure et al (Peyromaure *et al.*, 2002) who analyzed 29 tumors and found no predictive value for recurrence and progression for p53 overexpression. Wolf and colleagues described a significant difference in tumor-free survival between p53 positive and negative (protein expression) tumors. And thus, it remains unclear whether p53 may be helpful to guide treatment decision in this subgroup of bladder tumors.

- **To analyze the p53 pathway in T1G3 bladder tumors**

  The p53 pathway and outcome among patients with T1G3 bladder tumors

  1)To determine the status of the p53 pathway in T1G3 bladder tumors.

  2) To examine whether alterations in this pathway are associated with prognosis.

- **To characterize T1G3 tumors at the genomic level and to evaluate the association between molecular alterations and outcome**

  Genomic analysis of T1G3 bladder tumors by aCGH: Association with alterations in *FGFR3* and *Tp53* and with patient outcome

  1) To characterize T1G3 tumors at the genomic level.

  2) To determine whether the number and type of genomic changes are associated with alterations in *FGFR3 or Tp53.*

  3) To examine the association of global genomic changes and patient outcome.

- **To identify new molecular alterations involved in bladder tumors**

  PIK3CA mutations are an early genetic alteration associated with *FGFR3* mutations in superficial papillary bladder tumors

  1) To evaluate the mutational status of PIK3CA in bladder cancer

  2) To examine whether PIK3CA mutations are associated with alterations in *FGFR3 or Tp53.*

The p53 pathway and outcome among patients with T1G3 bladder tumors

Elena López-Knowles, Silvia Hernández, Manolis Kogevinas, Josep Lloreta, Alex Amorós, Adonina Tardón, Alfredo Carrato, Sirish Kishore, Consol Serra, Núria Malats, and Francisco X. Real on behalf of the EPICURO Study Investigators. Clinical Cancer Research (In press)

T1G3 cases represent 10% of bladder tumors diagnosed. They are categorized as superficial disease, although their risk of progression is much higher than that of stage Ta/T1 with lower grade tumors, also included in the same category. No molecular markers have been described to predict the outcome of patients with these tumors, partly due to the small number of cases studied and to the variability in the methodological procedures. The *Tp53* gene has been widely studied in bladder cancer but no consensus as to the prognostic value of its alterations has been reached. The EPICURO Study allows the analysis of a high number of T1G3 tumors, in a prospective setting with long term follow-up, using homogeneous methodology. The aim of this work was to analyze two central members of the p53 pathway, *Tp53* and *HDM2*, and 4 downstream targets of p53 in 119 T1G3 tumors and to determine their alterations in these tumors and their prognostic value. P53 mutations were evaluated by sequencing of PCR products of exons 4-9, containing over 80% of published gene mutations; HDM2 copy number was measured by quantitative PCR, and expression of both proteins was evaluated by immunohistochemistry (IHC) on tissue sections; the expression of the products of 4 target genes of the p53 pathway (COX2, Survivin, 14-3-3σ and IGF1-R) was assessed by IHC using a T1G3 tumor tissue microarray.

P53 alterations were frequently observed in T1G3 tumors: 58% of tumors had inactivating mutations and 66% protein overexpression. These frequencies are similar to those described in muscle-invasive bladder tumors. Overall, HDM2 alterations were less frequent and they were more common in wild type *p53* tumors: 9% of tumors had *HDM2* amplification and 6% positive staining. The 119 tumors were classified into 3 categories according to these results: group 1, comprising tumors with inactivating *Tp53* mutations or *HDM2* gains (65%); group 2, comprising tumors overexpressing p53 in the absence of mutations (20%); and group 3 with no alterations in any of these assays (15%). This classification, in which groups 1 and 2 would be considered to have an inactive p53 pathway, was substantiated by the fact that expression of IGF1-R, 14-3-3σ, and COX-2 was similar in groups 1 and 2, and significantly different from group 3.

Our findings suggest that tumors in group 3 arise through molecular mechanisms different from p53 pathway alterations. Patient outcome was similar for patients in the 3 groups.

Description of the work carried out by the candidate

Elena López-Knowles isolated tumor DNA, performed *Tp53* mutational status together wih Silvia Hernández. She performed HDM2 immunohistochemical analysis and quantitative PCR, as well as most of the immunohistochemical analyses of the p53 target gene products. She participated in the statistical analyses of the data. She wrote the first draft of the manuscript and participated in the discussion of the results and in the preparation of the final version of the manuscript.

Lopez-Knowles E, Hernandez S, Kogevinas M, Lloreta J, Amoros A, Tardon A, Carrato A, Kishore S, Serra C, Malats N, Real FX; EPICURO Study Investigators
The p53 pathway and outcome among patients with T1G3 bladder tumors.
Clinical cancer research 2006 Oct 15;12(20 Pt 1):6029-36.

Genomic analysis of T1G3 bladder tumors by array comparative genomic hybridization: Association with alterations in FGFR3 and p53 and wih patient outcome.

Elena López-Knowles, Núria Malats, Ritu Roydasgupta, Alex Amorós, Juan José Lozano, Jane Fridlyand, Fred Waldman, and Francisco X. Real. Cancer Research (submitted)

T1G3 bladder tumors represent a high risk bladder cancer subgroup located at the crossroads between superficial and invasive urothelial carcinomas. The aim of the study was to characterize these tumors at the genomic level, analyze the relationship with *FGFR3* and *p53* status, and assess the association with outcome. Fifty two T1G3 and 14 TaG1 tumors from the EPICURO study were analyzed using an array CGH platform containing 2464 BAC clones used to examine gains and losses in tumor DNA.

T1G3 tumors were characterized by a high frequency of alterations. Among the regions frequently altered, we found amplifications at chromosome 20, 6p22.3, 12q14, and 3q26 and deletions at 9p21, 2q21, 8p12, and 8p21-23. Comparison of T1G3 vs. TaG1 tumors identified a high level of similarity between T1G3 tumors and muscle invasive tumors both regarding the fraction of the genome altered (FGA) (47% vs. 3% respectively) and at the individual clone level. 9q21 losses were significantly more common among *FGFR3* mutant tumors and 10q23-26 losses were significantly more common among *Tp53* mutants. Quantitative PCR analysis of 6 genes mapping to 10q23-26 pointed to 10q23-24 as the region with more losses in *Tp53* mutant tumors. FGA was similar regardless of the *Tp53* and *FGFR3* mutational status. High levels of FGA were an independent predictor of outcome: tumor recurrence (HR=3.69, 95%CI 1.42-9.55) (p=0.007), progression (HR=2.98, 95%CI 0.94-9.52) (p=0.065) and cancer-specific death (HR=3.27, 95%CI 0.86-12.34) (p=0.081).

Conclusions: 1) T1G3 tumors display a high number of genomic changes, similar to muscle-invasive tumors; 2) genomic instability is unrelated to p53 pathway status, suggesting the involvement of other pathways/genes in this phenotype; and 3) FGA may be a valuable predictor of clinical outcome in these patients.

Description of the work carried out by the candidate

Elena López-Knowles isolated tumor DNA, performed array CGH experiments and carried out the array CGH analyses. In addition, she carried out *Tp53* and *FGFR3* mutational status together wih Silvia Hernández, performed quantitative PCR analyses and PTEN immunohistochemistry, and participated in the statistical analyses of the data. She wrote the fist draft of the manuscript and participated in the discussion of the results and in the preparation of the final version of the manuscript.

Array comparative genomic hybridization analysis of T1G3 bladder tumors reveals their similarity to muscle-invasive cancers and genomic changes associated with *FGFR3* and p53 alterations

Elena López-Knowles [1], Núria Malats [2], Ritu Roydasgupta [3], Alex Amorós [2], Juan José Lozano [4], Jane Fridlyand [3], Fred Waldman [5,*], and Francisco X. Real [1,6,*]

[1] Unitat de Biologia Cel.lular i Molecular, Institut Municipal d'Investigació Mèdica, Barcelona, Spain;
[2] CREAL, Institut Municipal d'Investigació Mèdica, Barcelona, Spain;
[3] Center for Bioinformatics and Molecular Biostatistics, UCSF Comprehensive Cancer Center, San Francisco;
[4] Centre de Regulació Genòmica, Barcelona;
[5] Departments of Laboratory Medicine, Cancer Center, and Urology, University of California San Francisco, San Francisco;
[6] Universitat Pompeu Fabra, Barcelona, Spain

* F.W. and F.X.R. have made equivalent contributions and share senior authorship

Correspondence should be addressed to Francisco X. Real, Institut Municipal d'Investigació Mèdica, Universitat Pompeu Fabra, Carrer del Dr. Aiguader 80, 08003-Barcelona, Spain. Phone 34-93-2211009, FAX 34-93-2216448 (e-mail: preal@imim.es) or to Fred Waldman, at Department of Laboratory Medicine, University of California San Francisco Comprehensive Cancer Center, San Francisco, CA 94143-0808 (e-mail waldman@cc.ucsf.edu).

Grant support: This study was supported, in part, by grants C03/010, G03/174, 00/0745, and PI051436 from Instituto de Salud Carlos III, Ministerio de Sanidad, Madrid, and grant 089715 from NIH. E. L. was supported by a Predoctoral Fellowship from the Ramón Areces Foundation.

Key words: array CGH, bladder cancer, FGFR3, genomic instability, p53, prognosis, T1G3 bladder tumors

Running title: Array CGH analysis of T1G3 bladder tumors

Abbreviations: aCGH, array comparative genomic hybridization; BCG, Bacillus Calmette Guérin; CGH, comparative genomic hybridization; FDR, false discovery rate; FGA, fraction of the genome altered; FGFR3, fibroblast growth factor receptor 3; MAD, mean absolute deviation; mut, mutant; TUR, transurethral resection; UCC, urothelial cell carcinoma; wt, wild type

Abstract

T1G3 bladder tumors are at the cross-roads between superficial and muscle-invasive urothelial carcinomas. Fifty-two cases were drawn from a multicenter prospective study. Arrays containing 2464 BAC clones were used to examine gains and losses in tumor DNA. TaqMan assays were used to quantify gene dosage at 10q23-26. Association with outcome was assessed using multivariable Cox analysis. The fraction of the genome altered (FGA) in T1G3 tumors (47%) was much higher than in TaG1 tumors (3%), as is characteristic of muscle-invasive cancers. FGA was similar in *Tp53* wild type and mutant tumors. Among the regions frequently altered, we found amplifications at chromosome 20, 6p22.3, 12q14, and 3q26 and deletions at 9p21, 2q21, 8p12, and 8p21-23. Losses at 9q21 and 10q23-26 were significantly higher among *FGFR3* and *Tp53* mutant than among wild type tumors, respectively. Quantitative PCR analysis identified 10q23-24 as the region with more losses in *Tp53* mutant tumors; immunohistochemistry points to a new gene, different from *PTEN*. High levels of FGA were an independent predictor of outcome: tumor recurrence (HR=3.69, 95%CI 1.42-9.55) (p=0.007), progression (HR=2.98, 95%CI 0.94-9.52) (p=0.065), cancer-specific death (HR=3.27, 95%CI 0.86-12.34) (p=0.081). We show that T1G3 tumors display a high number of genomic changes, similar to that of muscle-invasive tumors and identify novel regions of gains/losses. FGA is unrelated to p53 pathway status, suggesting the involvement of other pathways/genes in acquisition of genomic instability, and it is a predictor of clinical outcome. On the basis of their biological features, T1G3 tumors are muscle-invasive cancers that were diagnosed early.

INTRODUCTION

In the Western world, more than 90% of bladder cancer patients present with urothelial cell carcinoma (UCC). Patient prognosis varies substantially according to the stage and grade of the tumor at diagnosis (1). Approximately 70% of UCCs are papillary, superficial (Ta or T1) and of low grade at the time of presentation while most of the remaining cases are muscle-invasive (≥T2). Tumors classified as T1G3 constitute approximately 10% of all incident bladder cancers and represent a particularly important clinical challenge: long-term follow-up indicates that 50% of them progress to become muscle-invasive, while only 14% of TaG1 tumors do (2). The risk of death from bladder cancer is 10-fold higher for patients with T1G3 than for patients with low grade Ta and T1 tumors (3). Clinical or pathological characteristics fail to distinguish patients with T1G3 tumors who will progress from those who will not.

There is wide acceptance that bladder carcinogenesis can proceed along two distinct, but overlapping, pathways. Low grade, papillary, superficial tumors are commonly associated with chromosome 9 losses and with activating *FGFR3* mutations (4). In contrast, p53 alterations are frequently associated with high grade UCC, flat carcinoma in situ, and muscle invasion (5). Overall, *FGFR3* and *Tp53* mutations display a mutually exclusive distribution in bladder tumors (6,7). However, our recent work has shown the lack of an inverse association between *FGFR3* and *Tp53* mutations in patients with T1G3 tumors as well as no association of gene mutations and patient outcome (8). These findings place T1G3 bladder tumors at the cross-roads between superficial and muscle-invasive urothelial cancers.

A hallmark of high grade UCC is genomic instability associated with aneuploidy. Global genomic alterations have been analyzed by fluorescence in situ hybridization, metaphase comparative genomic hybridization (CGH), and by array CGH (aCGH) (9). The latter provides a greater level of resolution in mapping chromosomal regions that are gained or lost during neoplastic progression. Up to now, few studies have used aCGH, or similar single nucleotide polymorphism-based strategies, to study bladder cancer at the whole genome level. Furthermore, the number of tumors analyzed for each stage and grade category has been small (10-13). Because T1G3 represent only approximately 10% of all UCC, few of these tumors have been analyzed.

Here, we focus on the molecular analysis of T1G3 tumors with three specific aims: 1) to examine the level and pattern of genomic alterations present in this group of tumors using aCGH; 2) to examine whether the number and type of genomic changes are associated with alterations in *FGFR3 or Tp53* in an attempt to get more insight into the role of these molecular pathways in bladder carcinogenesis; and 3) to determine the association of global genomic changes and patient outcome.

MATERIALS AND METHODS

Patients and tumors. Cases (n=52) were drawn from the EPICURO study which comprises 1356 consecutive patients with incident bladder cancer recruited prospectively in 18 general hospitals in Spain between 1997-2001 (14,15). The following sociodemographic and clinical information was retrieved from hospital records using a computer-assisted questionnaire: age, sex, number of tumors at time of diagnosis, tumor size, localization in the bladder, treatment, and area of residence, among others. Tumor stage and grade were defined according to the TNM system and the ISUP-WHO classification (16) with three grade categories as described in more detail elsewhere (15). A panel of expert pathologists examined sections from all blocks retrieved from the hospital where the patients were diagnosed; stage and grade classification used here corresponds to that of the panel of experts. Cases were prospectively followed-up both through hospital records and by telephone interviews, either to the patient or a next-of-kin, when the former was not reachable or was deceased as reported in detail elsewhere (15). Only 1% of patients were lost to follow-up as of December 2005. The median follow-up period for patients with nonmuscle invasive tumors who were alive and free of disease was 62 months. Progression was defined as the appearance of a muscle-invasive tumor or distant metastasis; all other tumor events were classified as recurrences. Written informed consent was obtained from all individuals. The study was approved by the Ethics Committees of all participating institutions.

DNA preparation. H-E-stained sections were used to select areas containing $\geq$50% tumor cells for manual microdissection. Consecutive sections were used for DNA extraction: scraped material was digested by adding proteinase K on three consecutive days followed by purification using Amicon Microcon YM-30 columns

(Millipore) for the aCGH analysis and using the Dneasy Tissue Kit (Qiagen GmbH, Hilden, Germany) for mutation analysis. DNA from 64 T1G3, 17 TaG1 and 11 ≥T2 formalin-fixed, paraffin embedded primary bladder tumors was extracted from archival blocks of the EPICURO study. The DNA was quantitated by Taqman real time polymerase chain reaction amplification of sequences containing CA repeats at: D1S2868 (1p22), D2S385 (2q31), D4S1605 (4p16), D5S643 (5q32), D10S586 (10p12), and D11S1315 (11p15) (17).

Array CGH. Human 2.0 arrays were obtained from the University of California, San Francisco Cancer Center array core. The array consisted of 2464 BAC clones spotted in triplicate and distributed approximately uniformly across the genome with an average genome-wide resolution of 1.4 Mb (18). Tumor DNA (100 ng) and reference DNA (50 ng) were amplified and labelled by random priming (Invitrogen, Carlsbad, CA) with Cy3-dUTP and Cy5-dUTP respectively (Amersham Biosciences, Piscataway, NJ). Unincorporated fluorescent nucleotides were removed using Sephadex G-50 spin columns. Labelled tumor and reference DNA were mixed with 100 μg Cot-1 DNA (Life technologies, Inc., Gaithersburg, MD), precipitated and resuspended in 60 μl of a hybridization solution containing 50% formamide, 10% dextran sulphate, 2xSSC, 4% SDS and 100μg yeast tRNA. The hybridization solution was heated to 72 ºC for 10 min to denature the DNA and then incubated for 1 h at 37 ºC to allow blocking of the repetitive sequences. Hybridization was performed for 48 h on a slow rocking table followed by one wash in 50% formamide/2xSSC at 45 ºC, one wash in 2xSSC/0.1% SDS at 45 ºC, and two washes in phosphate buffer at room temperature. Slides were mounted in 90% glycerol in phosphate buffer containing 4'-6-diamidino-2-phenylindole at a concentration of 0.3 μg/ml. Arrays were imaged using a charged coupled device camera (Sensys, Photometric, equipped with a Kodak KAF 1400 chip) coupled to a 1x optical system.

The quality of the CGH array and intrinsic variability of the method was tested by performing a sex-mismatched normal versus normal hybridization simultaneously with the tumor analysis, using the same batch of arrays, with identical conditions. The average replicate standard deviation of these clones was 7%.

Array CGH data analysis. The methods used have been reported in detail elsewhere (11,19-22). The acquired microarray images were analyzed using Spot and Sproc

software (23). This software was used to calculate a single centered log2 ratio of test intensity over reference intensity of each clone. Clones for which a signal was obtained only in ≤1 of the 3 spots corresponding to each BAC clone in the array, those yielding a signal in <80% of samples, and those with a standard deviation of the replicates >0.33% were excluded from further analysis. A total of 228 clones (9.2%) were excluded. To assess chromosomal-scale genomic changes, an alteration frequency >40% was considered. To measure the amount of the genome altered, each clone was assigned a genomic distance equal to the sum of one half the distance between its center and that of its neighbouring clones. The genomic distances of clones that are gained or lost are added and the resulting value represents the FGA.

***FGFR3 and* p53 genetic status**. *FGFR3* (exons 7, 10, and 15) and *Tp53* (exons 4-9) sequencing and definition of mutational status were performed as described in detail elsewhere (8). For *Tp53*, all point mutations except for those located at introns outside of splice regions and those that occurred at exons but were synonymous, were considered to be pathogenic. In addition, *HDM2* gene copy number estimation, p53 nuclear overexpression assessed using immunohistochemistry, and p53 pathway status classification were performed as reported elsewhere (24).

Quantitative PCR analysis. To assess in greater detail the region of chromosome 10q in which DNA losses occurred, TaqMan assays were performed with tumor DNA and primers specific for 6 genes spanning 10q23-26 (*DNTT*, *FAS*, *DMBT1*, MXI1, *BUB3*, and *FGFR2*) and *CDK5* (7q36) as an endogenous control. Assays were performed using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probes were designed by the Custom Assay Gene expression facility of Applied Biosystems or Primer Express software (Applied Biosystems) and synthesized by The Oligo Factory (Applied Biosystems). TaqMan probes were labeled with 6-carboxyfluorescein. Primer and probe sequences are shown in Supplementary Table 1. Five ng of DNA were used per reaction. For *CDK5*, each reaction (20 μl) consisted of 900 nM primers, 10 nM probe, and DNA in 1x TaqMan Universal Master Mix (Applied Biosystems). For the test genes, each reaction consisted of 1 μl TaqMan primer and probe mix and DNA

in 1x TaqMan Universal Master Mix. All assays were performed in duplicate. PCR parameters were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles at 95 °C for 10 sec, and 1 min at 60 °C. TaqMan software was used to calculate a *Ct* value for each reaction, where the *Ct* value is the point in the extension phase of the PCR reaction at which the product is distinguishable from the background. To quantify gene copy number in each sample and calculate fold changes, a calibrator was used. This consisted of a pool of DNA isolated from paraffin-embedded normal tonsil (n=9), liver (n=4), kidney (n=4), and spleen (n=1). Taqman results were classified as gain, when the gene dose was >1.3 compared to DNA from normal tissue, and as loss when it was <0.4.

PTEN immunohistochemistry. Expression of PTEN was assessed using a tissue microarray containing two cores of each tumor sample. Antigen retrieval was carried out with 0.1 M citrate pH 6.0 and pressure cooker for 20 min. A Tech-Mate 500 instrument (Ventana Medical System, Tucson, AZ) was used for immunostaining with mouse monoclonal antibody 17A (1:2 dilution of hybridoma supernatant) (25). As secondary antibody, the Envision+ anti-mouse reagent was applied (DAKO, Copenhagen, Denmark). Reactions were developed using diaminobenzidine. Sections were counterstained, dehydrated, and mounted. Scoring was performed by two independent investigators as a dichotomous variable (positive or negative); in cases of discrepancy, a consensus was reached.

Statistical analyses. To assess the representativity of the tumors analyzed, the clinico-pathological characteristics of the 52 cases and the remaining 84 from the EPICURO study were compared using Chi square, except when there were less than 5 expected subjects in one of the cells, in which case Fisher's exact test was used. The association of FGA with *Tp53* and *FGFR3* molecular status and tumor stage was analyzed using Student's T test. Median gene copy number and proportion of cases with gene losses were compared using Student's T test and Chi square/Fisher´s, respectively, as appropriate. Receiver Operation Curves were built to establish the optimal cut-off to assess association of FGA with outcome. To compare the survival functions on the basis of FGA, a Kaplan-Meier curve was used and to test for equality of survivor functions the Log-rank and Wilcoxon test were applied.

aCGH data were analysed using the freely available R language using circular binary segmentation (CBS) with default parameters to translate experimental intensity measurements into regions of equal copy number as implemented in the DNAcopy R/Bioconductor package (19). Missing values for clones mapping within segmented regions of equal copy number were imputed by using the value of the corresponding segment. Thus, each clone was assigned a segment value referred to as its "smoothed" value. The scaled median absolute deviation (MAD) of the difference between the observed and smoothed values was used to estimate the tumor-specific experimental variation. All of the tumors had MAD less than 0.25. The gain and loss status for each probe was assigned using the mergeLevel procedure (20).

The frequency of alterations at each clone was computed as the proportion of samples showing an alteration at that locus. The extent of the genome assigned to each clone was computed by assigning a genomic distance equal to half the distance to the two neighboring clones or to the end of a chromosome for clones with only one neighbor. The number of chromosomal break points was computed based on the copy number levels. Finally, to identify single technical or biological outliers such as high level amplifications, the presence of the outliers within a segment was allowed by assigning the original observed log2ratio to the clones for which the observed values were more than four tumor-specific MAD away from the smoothed values. The amplification status for a clone was then determined as described (26). The clones are declared amplified if they were high relative to the surrounding clones with the required difference becoming larger as the value of the clone gets smaller.

Whole chromosome changes were assigned to chromosomes without identified breakpoints and when the chromosomal segment mapped to the gain or loss level and at least 90% of the clones in the chromosome were above or below the level of no change. A hard threshold of -.75 was used to declare a clone to be a homozygous deletion.

Clone-wise comparison of phenotypic groups was made using moderated t-statistics (21), based on empirical Bayes method of shrinkage of standard errors towards a common value, on smoothed data and adjusting for multiple testing by controlling for false discovery rate (FDR) (22). An FDR adjusted p-value of $\leq$0.05 was used to declare a clone significant. Kruskal-Wallis rank sum test was used to

analyze association of phenotypes with genomic events such as number of break points, number of chromosomes with break points, number of amplifications, number of chromosomes with amplifications and number of whole chromosome changes and also fraction of genome altered. A two-sided p-value cut-off of 0.05 was used to declare significance.

Analyses were performed using SSPS and R (www.r-project.org) software.

RESULTS

Patients and tumor material. Sixty-four T1G3 tumors, drawn from a total of 136 T1G3 tumors recruited to the EPICURO study, were characterized by aCGH; the results obtained for 52 of them were considered suitable for further analysis (81.2%). The remaining samples were deemed unsuitable because of excessive noise in the profile, presumably due to the low quality of the DNA isolated. The characteristics of cases whose tumors were analyzed (n=52) were similar to those of cases whose tumors were not analyzed (n=84) (Supplementary Table 2). In addition, a small series of 14 TaG1 tumors processed identically for aCGH are included in this report, mainly for comparison with the T1G3 tumors. The median age of patients whose tumors were analyzed was 66.1 (41-78) years. Multiple tumors were present at diagnosis in 31.9% of cases. Patients had received the following treatment for the primary tumor: transurethral resection (TUR) (n=9), TUR+BCG (n=24), TUR+intravesical chemotherapy (n=5), TUR+BCG+intravesical chemotherapy (n=4), cystectomy (n=8), other treatments (n=2). Of the 52 patients included in the study, 21 recurred, 14 progressed, and 11 died from bladder cancer.

Genomic profile of T1G3 tumors. The most frequent chromosomal-scale gains and losses identified in T1G3 tumors are shown in Figure 1A. Some chromosomal changes tended to be associated: loss of one chromosomal arm and gain of the other arm, possibly resulting from isochromosome formation, was observed for chromosomes 5, 6, 8, 10, 17, and 18. Whole chromosome loss (9, 15, 16) or gain (20, 21) in 40% of tumors were also observed, presumably as a result of chromosome missegregation.

Analysis of individual clones showed that copy number changes involved widely varying lengths of each chromosome, in some cases affecting single clones.

The proportion of T1G3 tumors showing a given gain or loss was very high for certain clones, involving up to 71% of tumors (Figure 1B). Although copy number alterations were observed throughout the genome, their distribution was not uniform. The most common regions of alteration are shown in Table 1; tables with the clones that showed gain/loss or amplification/deletion are available upon request. In general, regions showing highest frequency gains also showed a high frequency of amplification. Chromosome 20 had the highest rate of gains, with an average frequency of 60%. The regions showing highest frequency of amplification were 6p22.3, 17q11.2-q12, 15q11-q13, 12q14, 8q22.2, 11q13, and 3q26.3. Table 1 shows some of the candidate genes present therein that are potentially involved in bladder cancer progression.

The highest frequency loss was at 8pter-p11 between clones GS1-77L23 and RP11-140K14 (71%); this region contains *MYOM2* and *CSMD1* (27). The highest frequency of deletions was at 2q14, 2q21-q24, 5q31-q35, 8p12, 8p21-23, 9p21, 9q31-q34, 10q21-q26, and 11p15. Table 1 shows some of the candidate genes present in these regions that may be involved in bladder cancer progression.

Comparison of the genomic profile of T1G3 and TaG1 tumors. The genomic profiles of T1G3 and TaG1 tumors were dramatically different (Figure 1B). The region most frequently gained in TaG1 tumors was 4q32-34 (21%). Chromosome 9 showed the highest frequency of losses, at 9p13, between clones RP11-17J8 and RP11-61G7 (29%). Among the genes proposed to be involved in cancer mapping to this region are *GALT*, *CNTFR*, and *NPR2*.

To assess the global genomic changes, the fraction of genome altered (FGA) was estimated for both tumor groups (Figure 2A): the FGA of T1G3 tumors was much higher than that of TaG1 tumors (47.7% vs. 3.5%; $p<0.001$). Similarly, the median number of specific structural or numerical alterations per tumor was higher among T1G3 than among TaG1 samples (Supplementary Table 3).

At the individual clone level, 399 clones showed a significantly different copy number in both tumor groups ($p<0.05$, FDR-adjusted p value), as indicated in the lower panel in Figure 1B. These clones were distributed throughout the genome. T1G3 tumors displayed a significantly higher frequency of gains (1q22-23, 3q22-26, 8q21-24, and 20) and losses (2q34-36, 4p16, 4q31-tel, 5q31-35, 8ptel-p12, 10q22-26, 11p15-11, and 17p12) than TaG1 tumors. Similar observations have

been made in an independent study for 8q21-24, chromosome 20, and 2q34-36 (11).

***FGFR3* and p53 genetic status and genomic alterations in T1G3 tumors.** Forty-five tumors were *FGFR3* wild type and 7 tumors were mutant. The FGA was similar in both groups (47% vs. 49%, p=0.856) (Figure 2B). Comparison at the individual clone level showed a pattern of gains and losses that was similar in both groups of tumors except for 56 individual clones (FDR adjusted p value <0.05) that were confined to 9q13-32. This region spans from clone CTD-2029L20 to Rp11-46P18 and contains, among others, *PTCH* and *NTRK2*. Cases harbouring mutant *FGFR3* had more losses at these sites than those that were wild type (Figure 3). While the number of tumors that were *FGFR3* mutant is small, it is remarkable that losses at 9p21 and in a broad region of 9q have also been found to be associated with *FGFR3* mutant tumors, but not with *FGFR3* wild type tumors, in another study (28). The median number of specific structural or numerical alterations per tumor is shown in Supplementary Table 3.

Regarding p53 genetic status, two types of analysis were performed. First, we compared *Tp53* wild type (n=24) vs. *Tp53* mutant (n=28) tumors: FGA was similar in both groups (49% vs. 46%, p=0.579) (Figure 2C). Based on a more exhaustive analysis of the p53 pathway including *Tp53* sequencing, *HDM2* gene copy number, p53 nuclear overexpression, and expression of p53 target genes (24), we compared tumors with (n=44) and without (n=8) p53 pathway alterations. Both groups showed a similar FGA (wild type=41% vs. altered=49%, Student's T test p=0.31).

At the individual clone level, the pattern of gains and losses was similar except for 72 individual clones (FDR adjusted p value <0.05) confined to 10q11 and 10q21-26. Cases harbouring mutant *Tp53* had more losses than those that were wild type (Figure 3). The median number of specific structural or numerical alterations per tumor is shown in Supplementary Table 3.

Tumors with an intact p53 pathway had a higher prevalence of losses at 8p12-23, although the difference was not statistically significant (p=0.073).

The 10q23-24 region is involved in bladder cancer progression. Because it has also been proposed that 10q23 losses may be associated with bladder cancer

progression (29,30), we performed a search of smaller regions that are deleted in *Tp53* mutant T1G3 tumors using TaqMan assays to determine the dose of 6 genes covering 10q23-10q26.

First, using the 52 tumors analyzed by aCGH, we validated the results with quantitative PCR for 2 of the genes mapping to 10q23-10q26 (*DNTT* and *BUB3*); 15 of these samples were also tested using an additional 4 genes mapping to this region. The location and function of these genes is shown in Supplementary Table 4. The proportion of mutant and wild type cases showing losses at each of the loci were: *FAS* (29% vs. 0%), *DNTT* (22% vs. 0%), *MXI1* (43% vs. 13%), *FGFR2* (no losses in either group), *DMBT1* (29% vs. 0%), and *BUB3* (7% vs. 0%). These results confirmed *DNTT, FAS*, *MXI1*, and *DMBT1* as candidates in this region as they are more commonly lost in mutated samples. We then extended this analysis to include a higher number of independent T1G3 tumor samples from the EPICURO study for which *Tp53* mutational status is known (24). The proportion of cases displaying losses at 10q23-q26 was higher among *Tp53* mutant cases for all loci but *MXI1*; however, the differences were not statistically significant (Figure 4A). When the mean copy number of each gene was compared, a significantly lower dose of *DNTT1* was found among *Tp53* mutant tumors (p=0.008, Student's T test).

Since losses at 10q23 may be a marker of bladder cancer progression (30), we also analyzed two of these genes, *BUB3* and *DNTT*, in TaG1 (n=28) and $\geq$T2 (n=26) tumors from the EPICURO study. Figure 4B shows that the proportion of cases showing losses at *DNTT* was significantly higher among higher stage tumors ($p<0.001$ Fishers' exact test). By contrast, there was no significant difference for *BUB3* (p=0.320, Fishers' exact test). Altogether, these results support the notion that a gene located in the vicinity of *DNTT* may play an important role in bladder cancer.

PTEN, a well known tumor suppressor gene, also maps to 10q23.3. We examined the expression of PTEN in T1G3 bladder tumors using immunohistochemistry and compared expression in *Tp53* wild type and mutant tumors. PTEN expression was similar in both groups of tumors (data not shown), suggesting that *PTEN* is not the relevant gene whose loss is associated with the pathway implicating *Tp53* mutations.

Association of aCGH results and prognosis in patients with T1G3 tumors. Because FGA provides an overall estimate of the genomic alterations, we analyzed its association with clinical outcome. In a multivariable analysis, none of the classical socio-demogrpahical or clinico-pathological variables (age, sex, number of tumors, tumor size, localization in bladder, treatment, and area of catchment) independently predicted any of the outcomes measured. When a 47.4% cut-off was used in the multivariable analysis, chosen on the basis of receiver operating curve (ROC) analysis, genomic alterations were found to be associated with clinical outcome: recurrence (HR=3.69, 95%CI 1.42-9.55) (p=0.007), progression (HR=2.98, 95%CI 0.94-9.52) (p=0.065), cancer-specific death (HR=3.27, 95%CI 0.86-12.34) (p=0.081) (Figure 5). Similar results were obtained when association with outcome was assessed using mean and median FGA as cut-off values (Supplementary Table 5).

DISCUSSION

Overall, superficial bladder tumors, comprising the Ta and T1 categories, are characterized by a relatively low risk of progression to muscle invasion. However, T1 tumors have already crossed the basement membrane, the major barrier against invasion, and there is debate as to how they should be classified (4). Genomic instability characterizes a subset of bladder tumors (31) and p53 pathway alterations have been proposed to play a major role. Here, we hypothesized that the amount of global genomic changes, quantitated as the FGA, might be associated with inactivation of the p53 pathway and distinguish T1G3 tumors with distinct prognosis.

At the genetic level, superficial bladder tumors are characterized mainly by the loss of chromosome 9, activating mutations in *FGFR3*, and a low frequency of *Tp53* alterations (6,7), whilst invasive tumors often display a larger number of alterations affecting multiple chromosomes, including loss of 9, 2q, 8p, 11p, 17p, gain of 8q, 17q and 20, and a high frequency of *Tp53* alterations (32). Loss of 9q has been observed in low and high grade tumors, suggesting that it occurs early on in the course of bladder carcinogenesis. In this work we have used aCGH to explore global genomic changes and their relationship with *FGFR3* and p53 pathway alterations.

Genomic changes in T1G3 tumors. It has been proposed that an increasing number of genomic aberrations is linked to tumor progression, and poor prognosis, in several tumor types. As expected, our findings indicate that T1G3 tumors harbour a number of genomic alterations that is much higher than that of TaG1 tumors and similar to that found in muscle-invasive cancers (11). This observation supports the notion that T1 tumors are biologically similar to those that invade muscle (≥T2) but they have been diagnosed earlier. This notion is also supported by the lower prevalence of *FGFR3* mutations in this subgroup (15).

An analysis at the individual clone level confirms the observations made with FGA: losses and gains occurred at a higher frequency in T1G3 than in TaG1 tumors in wide regions of chromosomes 1, 2, 3, 4, 5, 8, 10, 11, 17, and 20. By contrast, losses of a few clones in 1p and most clones covering chromosome 9 were not significantly more common in the former than in the latter. This finding is in agreement with the notion that chromosome 9 alterations are an almost universal and very early event in bladder cancer development (33).

We have identified here several regions that are gained/amplified in T1G3 bladder tumors (i.e. 8q22, 3q26, 12q14, 15q11, 6p22, and 17q11-12), including some that have not received attention earlier. Among the latter are 3q26 and 15q11. Several recent studies have shown amplification of *PIK3CA* – a gene located at 3q26 - in a variety of human tumors (34). In addition, activating *PIK3CA* mutations have also been reported in a wide variety of human tumors. The frequent amplification of this region in UCC has recently led us to search for somatic *PIK3CA* mutations. Indeed, we have found that mutations occur in approximately 20% of UCC and are mainly associated with low grade/stage tumors harbouring *FGFR3* mutations (35).

Regarding losses/deletions, our findings extend prior information indicating a role for genes located in the following regions: 10q11, where *ERCC6* is located; 12q23 containing *p63*, a member of the p53 gene family (36); 8p12 containing *BNIPL3*, a candidate tumor suppressor gene involved in apoptosis; 8p21 containing *DBC2*, a candidate bladder tumor suppressor gene (37); 8p23.1 containing *CSMD1* found to be deleted in head and neck cancer (27); 11p11.2 containing *KAI1* (38), and 11p15.5 containing *p57* (39). Several regions in chromosome 9 were also found to be frequently lost, in agreement with prior studies. As discussed below, we

have focused on the analysis of the 10q23-26 region given the association of losses therein with *Tp53* alterations.

The p53 pathway and genomic instability in UCC. Despite the well established role of *Tp53* in the maintenance of genome integrity, *Tp53* wild type and mutant tumors displayed a similar level of FGA. Therefore, our study indicates that genomic instability is an almost universal hallmark of T1G3 tumors.

Because our analysis of *Tp53* mutations was restricted to exons 4-9, we cannot completely rule out that alterations in other exons may account for its inactivation, though this possibility is rather unlikely taking into account the massive knowledge gathered on *Tp53* mutations over the last 10 years (40). We have also explored the possibility that tumors harbouring *Tp53* exon 4-9 wild type sequences display other alterations leading to p53 pathway inactivation (i.e. HDM2 amplification). In this regard, we have shown that 85% of T1G3 tumors display alterations either in the sequence of *Tp53*, its nuclear overexpression, or *HDM2* gain/amplification. These tumors also differ from the remaining 15% in which there is no evidence for p53 pathway inactivation regarding the expression of p53 target genes (24). Altogether, our findings suggest that other genes/pathways lead to genomic instability in the latter tumor group. To get some insight into the genomic differences beween *Tp53* wild type and *Tp53* mutant tumors at the individual clone level, we have applied stringent statistical criteria in order to avoid false positive findings due to multiple testing. The most significant region identified corresponds to 10q21-26, previously associated to progression in bladder cancer ($p<0.05$). This region was more frequently lost in *Tp53* mutated tumors than in those wild type. *PTEN*, a gene coding for a lipid phosphatase that is a direct target of p53, maps to this region and has previously been shown to be inactivated in advanced bladder tumors (41). Therefore, we first analyzed PTEN expression in the panel of tumors studied by aCGH and found that PTEN expression is similar regardless of *Tp53* mutational status. These findings do not support *PTEN* as the candidate gene being differentially altered in *Tp53* wild type and mutant tumors. To further explore the role of other genes present in this region, we chose 6 candidates - based on their position, function, or previous associations - for a detailed confirmation of changes in gene dose using quantitative PCR. Quantitative PCR results indicate that *FAS*, *DNTT*, *FGFR2* and *DMBT1* show the same tendency observed with aCGH: losses

were more common among *Tp53* mutant samples than among wild type tumors. However, the difference was only significant for *DNTT*. Because previous studies have associated 10q loss to progression we examined two of the genes of interest in this area in a subset of tumors from different stages: DNTT losses were strongly associated with tumor progression. While our study did not aim at determining the minimal deleted region, several genes in 10q23 have been involved in bladder cancer, including *SFRP5*. Interestingly, *SFRP* methylation and *Tp53* alterations have recently been reported to be independently associated with invasive bladder (42).

Regarding the 15% of tumors for which there is no evidence of p53 pathway alterations, our analysis suggests that the 8p12-23 region is more frequently lost in this group of tumors. This result requires validation in a different set of tumors given the small number of tumors present in this subgroup.

FGA and patient outcome. Finally, we assessed whether the level of genomic alterations is predictive of patient outcome. Our analysis of progression and cancer-specific death is limited by the relatively low number of events recorded, despite a median follow-up of 62 months, due to the fact that this patient cohort was prospectively recruited (15). However, high level genomic alterations were an independent predictor of recurrence in this group of patients. Similar HR were observed for progression and cancer death. The lack of statistical significance likely results from the small number of events. Because patients who undergo recurrence are more likely to suffer progression – and cancer death – than those who do not, an association with these endpoints may be of value in predicting the prognosis of patients with T1G3. These findings are in general agreement with our observation that FGA is also an independent risk factor for prediction of outcome among patients with muscle-invasive tumors (11). The association of FGA with outcome is particulary important because there are no established factors with predictive value. Recent work from our group has shown that *FGFR3* mutations, *Tp53* mutations, p53 nuclear overexpression, *HDM2* gene alterations, and expression of p53 target genes are not associated with outcome in patients with T1G3 tumors (15), thus highlighting the potential importance of the findings reported here.

In conclusion, our results indicate that: 1) T1G3 tumors universally display a high number of genomic changes, similar at the qualitative and quantitative levels,

to those of muscle-invasive tumors; 2) genomic instability is unrelated to p53 pathway status, suggesting the involvement of other genes in a subset of T1G3 tumors; and 3) FGA is a promising marker of outcome in patients with T1G3 bladder tumors.

ACKNOWLEDGEMENTS

We thank Manolis Kogevinas, Silvia Hernández, Francisco Fernández, Josep Lloreta, and Lauro Sumoy for valuable contributions, Lars Dyrskjot for valuable discussions and sharing of unpublished data, Rafael Pulido por providing anti-PTEN monoclonal antibodies, and the investigators, monitors, secretaries and patients of the EPICURO study for continued support.

Reference List

1. Kirkali Z, Chan T, Manoharan M et al. Bladder cancer: epidemiology, staging and grading, and diagnosis. Urology 2005; 66: 4-34

2. Chopin DK and Gattegno B. Superficial bladder tumors. Eur Urol 2002; 42: 533-541

3. Smith JA, Jr. Surgical management of superficial bladder cancer (stages Ta/T1/CIS). Semin Surg Oncol 1997; 13: 328-334

4. Knowles MA. Molecular subtypes of bladder cancer: Jekyll and Hyde or chalk and cheese? Carcinogenesis 2006; 27: 361-373

5. Malats N, Bustos A, Nascimento CM et al. P53 as a prognostic marker for bladder cancer: a meta-analysis and review. Lancet Oncol 2005; 6: 678-686

6. van Rhijn BW, van der Kwast TH, Vis AN et al. FGFR3 and P53 characterize alternative genetic pathways in the pathogenesis of urothelial cell carcinoma. Cancer Res 2004; 64: 1911-1914

7. Bakkar AA, Wallerand H, Radvanyi F et al. FGFR3 and TP53 gene mutations define two distinct pathways in urothelial cell carcinoma of the bladder. Cancer Res 2003; 63: 8108-8112

8. Hernandez S, Lopez-Knowles E, Lloreta J et al. FGFR3 and Tp53 mutations in T1G3 transitional bladder carcinomas: independent distribution and lack of association with prognosis. Clin Cancer Res 2005; 11: 5444-5450

9. Greshock J, Naylor TL, Margolin A et al. 1-Mb resolution array-based comparative genomic hybridization using a BAC clone set optimized for cancer gene analysis. Genome Res 2004; 14: 179-187

10. Hurst CD, Fiegler H, Carr P, Williams S, Carter NP, Knowles MA. High-resolution analysis of genomic copy number alterations in bladder cancer by microarray-based comparative genomic hybridization. Oncogene 2004; 23: 2250-2263

11. Blaveri E, Brewer JL, Roydasgupta R et al. Bladder cancer stage and outcome by array-based comparative genomic hybridization. Clin Cancer Res 2005; 11: 7012-7022

12. Veltman JA, Fridlyand J, Pejavar S et al. Array-based comparative genomic hybridization for genome-wide screening of DNA copy number in bladder tumors. Cancer Res 2003; 63: 2872-2880

13. Primdahl H, Wikman FP, von der MH, Zhou XG, Wolf H, Orntoft TF. Allelic imbalances in human bladder cancer: genome-wide detection with high-density single-nucleotide polymorphism arrays. J Natl Cancer Inst 2002; 94: 216-223

14. Garcia-Closas M, Malats N, Silverman D et al. NAT2 slow acetylation, GSTM1 null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses. Lancet 2005; 366: 649-659

15. Hernandez S, Lopez-Knowles E, Lloreta J et al. Prospective study of FGFR3 mutations as a prognostic factor in nonmuscle invasive urothelial bladder carcinomas. J Clin Oncol 2006; 24: 3664-3671

16. Epstein JI. The new World Health Organization/International Society of Urological Pathology (WHO/ISUP) classification for TA, T1 bladder tumors: is it an improvement? Crit Rev Oncol Hematol 2003; 47: 83-89

17. Ginzinger DG, Godfrey TE, Nigro J et al. Measurement of DNA copy number at microsatellite loci using quantitative PCR analysis. Cancer Res 2000; 60: 5405-5409

18. Snijders AM, Nowak N, Segraves R et al. Assembly of microarrays for genome-wide measurement of DNA copy number. Nat Genet 2001; 29: 263-264

19. Olshen AB, Venkatraman ES, Lucito R, Wigler M. Circular binary segmentation for the analysis of array-based DNA copy number data. Biostatistics 2004; 5: 557-572

20. Willenbrock H and Fridlyand J. A comparison study: applying segmentation to array CGH data for downstream analyses. Bioinformatics 2005; 21: 4084-4091

21. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004; 3: Article3

22. Benjamini Y and Hochberg Y. Controlling the False Discovery Rate - A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B-Methodological 1995; 57: 289-300

23. Jain AN, Tokuyasu TA, Snijders AM, Segraves R, Albertson DG, Pinkel D. Fully automatic quantification of microarray image data. Genome Res 2002; 12: 325-332

24. López-Knowles E, Hernandez S, Kogevinas M et al. The p53 pathway and outcome among patients with T1G3 bladder tumors. Clin Cancer Res 2006 (in press)

25. Torres J, Navarro S, Rogla I et al. Heterogeneous lack of expression of the tumour suppressor PTEN protein in human neoplastic tissues. Eur J Cancer 2001; 37: 114-121

26. Fridlyand J, Snijders AM, Ylstra B et al. Breast tumor copy number aberration phenotypes and genomic instability. BMC Cancer 2006; 6: 96

27. Scholnick SB and Richter TM. The role of CSMD1 in head and neck carcinogenesis. Genes Chromosomes Cancer 2003; 38: 281-283

28. Zieger K, Dyrskjot L, Wiuf C et al. Role of activating fibroblast growth factor receptor 3 mutations in the development of bladder tumors. Clin Cancer Res 2005; 11: 7709-7719

29. Simon R, Burger H, Brinkschmidt C, Bocker W, Hertle L, Terpe HJ. Chromosomal aberrations associated with invasion in papillary superficial bladder cancer. J Pathol 1998; 185: 345-351

30. Cappellen D, Gil Diez dM, Chopin D, Thiery JP, Radvanyi F. Frequent loss of heterozygosity on chromosome 10q in muscle-invasive transitional cell carcinomas of the bladder. Oncogene 1997; 14: 3059-3066

31. Yamamoto Y, Matsuyama H, Kawauchi S et al. Biological characteristics in bladder cancer depend on the type of genetic instability. Clin Cancer Res 2006; 12: 2752-2758

32. Richter J, Beffa L, Wagner U et al. Patterns of chromosomal imbalances in advanced urinary bladder cancer detected by comparative genomic hybridization. Am J Pathol 1998; 153: 1615-1621

33. Knowles MA. The genetics of transitional cell carcinoma: progress and potential clinical application. BJU Int 1999; 84: 412-427

34. Cully M, You H, Levine AJ, Mak TW. Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. Nat Rev Cancer 2006; 6: 184-192

35. Lopez-Knowles E, Hernandez S, Malats N et al. PIK3CA mutations are an early genetic alteration associated with FGFR3 mutations in superficial papillary bladder tumors. Cancer Res 2006; 66: 7401-7404

36. Puig P, Capodieci P, Drobnjak M et al. p73 Expression in human normal and tumor tissues: loss of p73alpha expression is associated with tumor progression in bladder cancer. Clin Cancer Res 2003; 9: 5642-5651

37. Knowles MA, Aveyard JS, Taylor CF, Harnden P, Bass S. Mutation analysis of the 8p candidate tumour suppressor genes DBC2 (RHOBTB2) and LZTS1 in bladder cancer. Cancer Lett 2005; 225: 121-130

38. Su JS, Arima K, Hasegawa M et al. Decreased expression of KAI1 metastasis suppressor gene is a recurrence predictor in primary pTa and pT1 urothelial bladder carcinoma. Int J Urol 2004; 11: 74-82

39. Oya M and Schulz WA. Decreased expression of p57(KIP2)mRNA in human bladder cancer. Br J Cancer 2000; 83: 626-631

40. Soussi T and Beroud C. Assessing TP53 status in human tumours to evaluate clinical outcome. Nat Rev Cancer 2001; 1: 233-240

41. Koksal IT, Yasar D, Dirice E et al. Differential PTEN protein expression profiles in superficial versus invasive bladder cancers. Urol Int 2005; 75: 102-106

42. Marsit CJ, Karagas MR, Andrew A et al. Epigenetic inactivation of SFRP genes and TP53 alteration act jointly as markers of invasive bladder cancer. Cancer Res 2005; 65: 7081-7085

FIGURE LEGENDS

Figure 1. Genomic changes in T1G3 tumors using array CGH. *Panel A:* Global-scale genomic changes at the chromosomal arm level (n=52). The proportion of cases showing gains or losses at each arm is shown. *Panel B:* Comparison of T1G3 (n=52) vs. TaG1 (n=14) tumors. The proportion of cases showing gain or loss of each individual clone is shown. The lower panel displays the value of the statistic corresponding to the comparison of alterations in each clone in T1G3 vs. TaG1 tumors at 0.05 (black line) and 0.1 (grey line) thresholds.

Figure 2. Comparison of the fraction genome altered (FGA) in different tumor subgroups. Panel A shows the comparison of gains and losses in T1G3 vs. TaG1 tumors. Panel B shows the comparison of T1G3 tumors that are *FGFR3* wt vs. mutant. Panel C shows the comparison of T1G3 tumors that are *Tp53* wt vs. mutant.

Figure 3. Association of molecular alterations in *FGFR3* and *Tp53* with specific genomic gains and losses assessed by aCGH. *Panels A and C*: Median of Log2 ratios for BAC clones corresponding to chromosome 9 according to *FGFR3* status. There are 45 *FGFR3* wild type tumors (A) and 7 mutated tumors (C). Clones are ordered on the basis of the UCSC mapping position. *Panels B and D*: Median of Log2 ratios for BAC clones corresponding to chromosome 10 according to *Tp53* status. There are 24 *Tp53* wild type tumors (B) and 28 mutated tumors (D). Clones are ordered on the basis of the UCSC mapping position. Grey squares at top indicate clones differentially expressed significantly between wild type and mutant tumors with a $p<0.05$ as indicated in Materials and Methods.

Figure 4. TaqMan analysis of selected genes located in the 10q23-26 region. *Panel A:* findings according to *Tp53* mutational status. *Panel B:* findings according to stage and grade. For each locus, the proportion of tumors displaying loss, or

gain/no change is shown. Asterisks indicate comparisons for which the p value is <0.05.

Figure 5. Kaplan-Meier analysis of the association of FGA and recurrence (A), progression (B), and cancer-specific survival (C) among patients with T1G3 tumors. FGA is an independent predictor of recurrence.

Table 1. Most commonly gained/lost and amplified/deleted regions in T1G3 tumors. The percentage shown indicates the range of gains/losses (A/B) and amplifications/deletions (C/D) for the BAC clones corresponding to each specific chromosomal location, computed as indicated in Materials and Methods.

A

Gains

Location	Clones (n)	% gained
1q11-23	15	51-65
3q23-tel	28	51-54
5p13-15	14	48-52
6p22	5	52-56
8q11-13	26	50-56
8q21-24	32	50-63
17q23	7	48-50
20p11-13	38	54-60
20q	58	58-67

B

Losses

Location	Clones (n)	% lost
1p35-33	11	47-54
4p16	9	53-54
5q31-qtel	41	54-60
8ptel-p11	56	50-71
9p22-qtel	91	46-62
10q11-tel	80	48-71
11q23-tel	13	51-54
11ptel-p11	78	46-63
16p13-qtel	63	48-62

C

Amplifications

Location	Clones (n)	% amplified	Candidates
1q21-22	10	4-10	*PIK4CB, SLAM*
3q26.3	1	10	*PIK3CA*
6p22.3	6	8-21	*E2F3*
8q22	5	12	*SPAG1*
11q13	7	8-10	*Cyclin D1*
12q14	4	8-13	*MDM2*
15q11-13	1	13	*SNRPN*
17q11-12	1	17	*ERBB2*
20p11	1	12	
20q13	1	12	

D

Deletions

Location	Clones (n)	% deleted	Candidates
1q25	1	13	
2q14	1	17	
2q21-24	3	8-13	
5q31-35	7	6-12	*NOLA2*
8p21-23	17	6-21	*CSMD1, TNFRSF10*
8p12	2	6-10	
9p21	4	6-15	*INK4A/ARF*
9q31-34	9	6-10	*VAV2, SARDH*
10q21-26	10	6-10	*DNTT*
11p15	9	6-12	*p57/KIP2*

Figure 1 Panel A

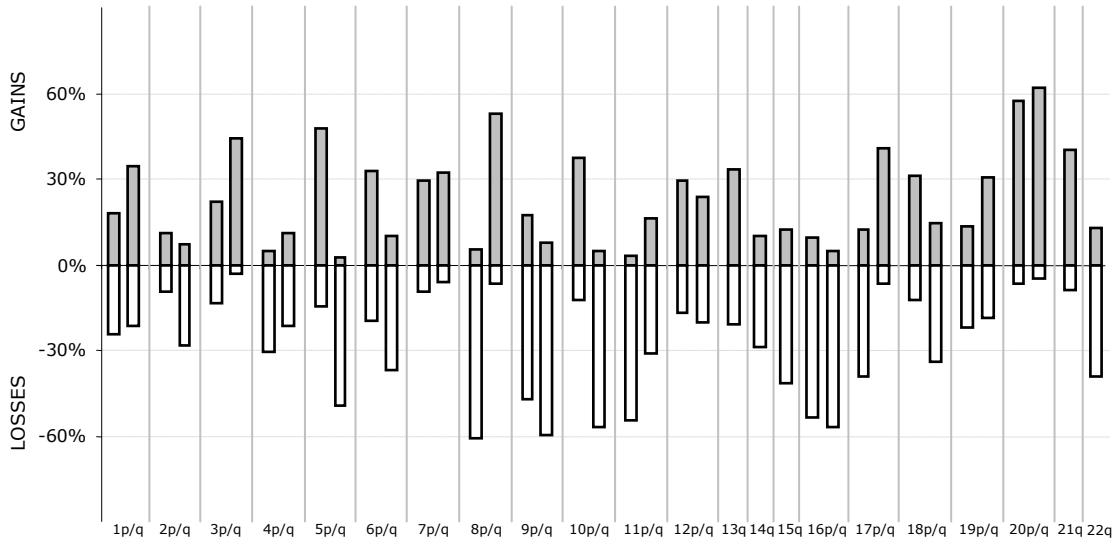


Figure 1 Panel B

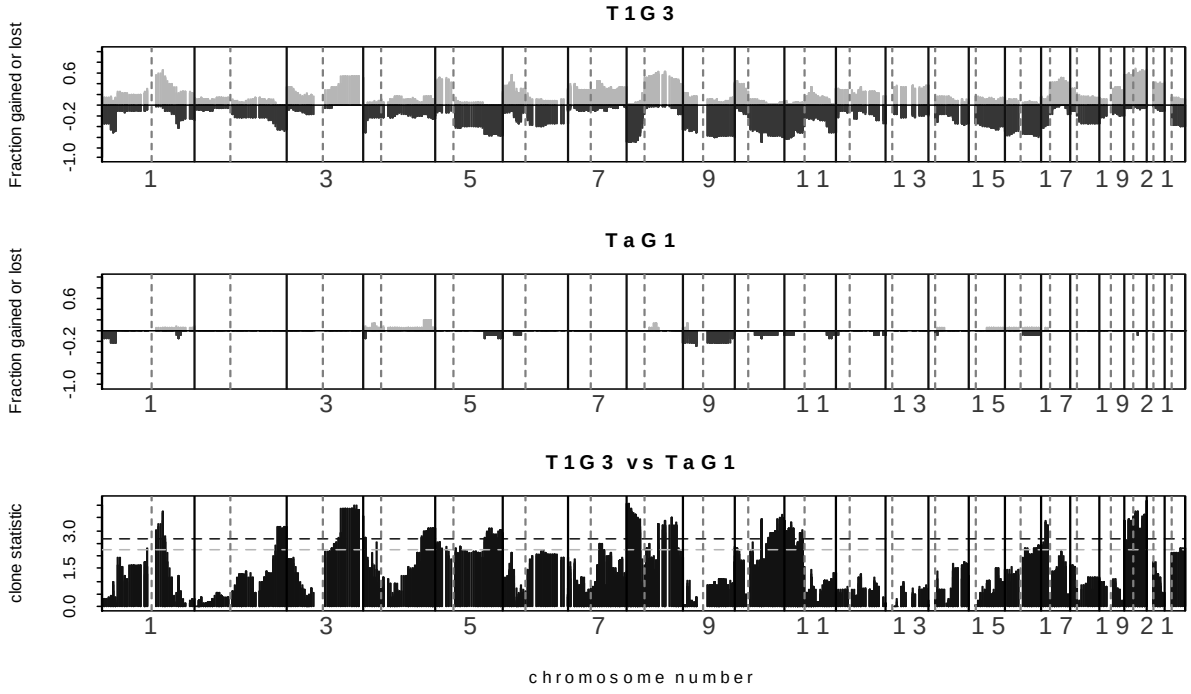


Figure 2

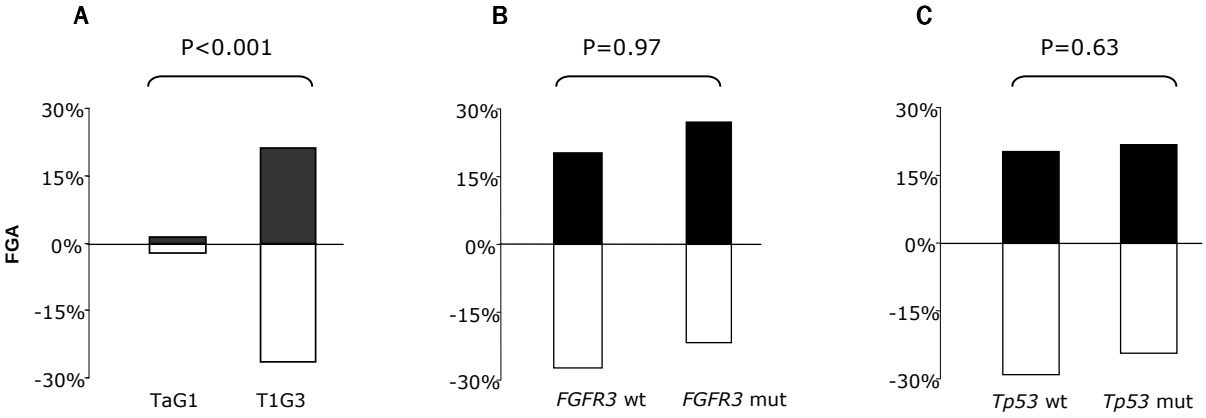
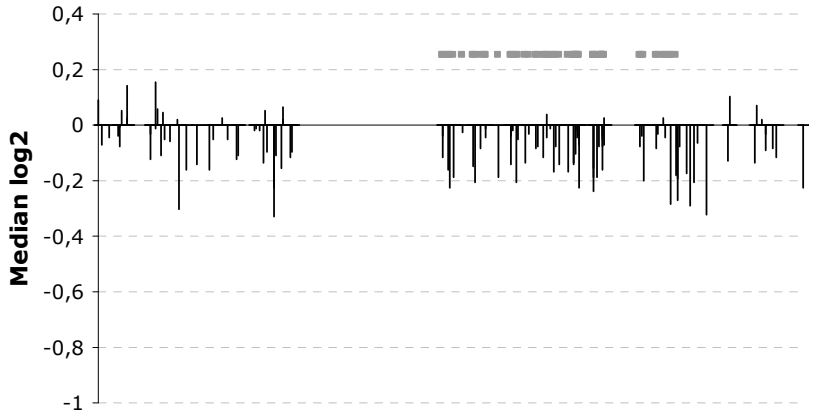
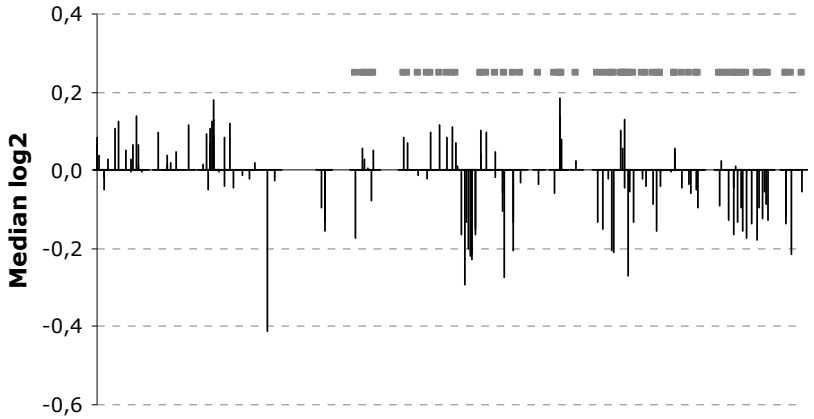

A
P<0.001
30%
15%
0%
-15%
-30%
FGA
TaG1
T1G3
B
P=0.97
30%
15%
0%
-15%
-30%
FGFR3 wt
FGFR3 mut
C
P=0.63
30%
15%
0%
-15%
-30%
Tp53 wt
Tp53 mut

Figure 3

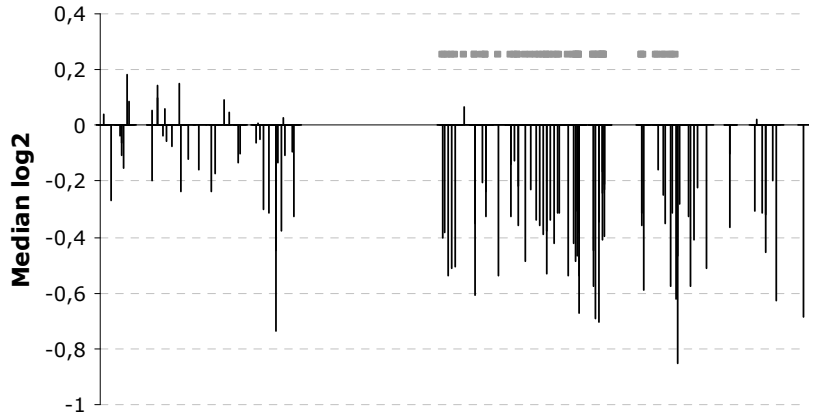
A



B



C



D

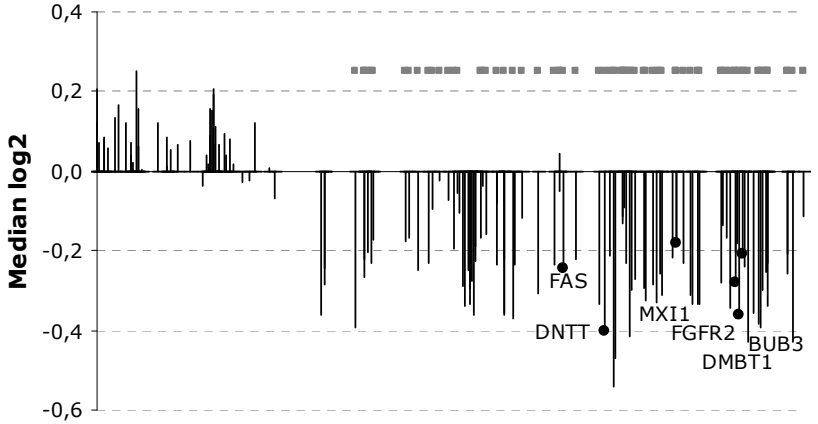


Figure 4[a]

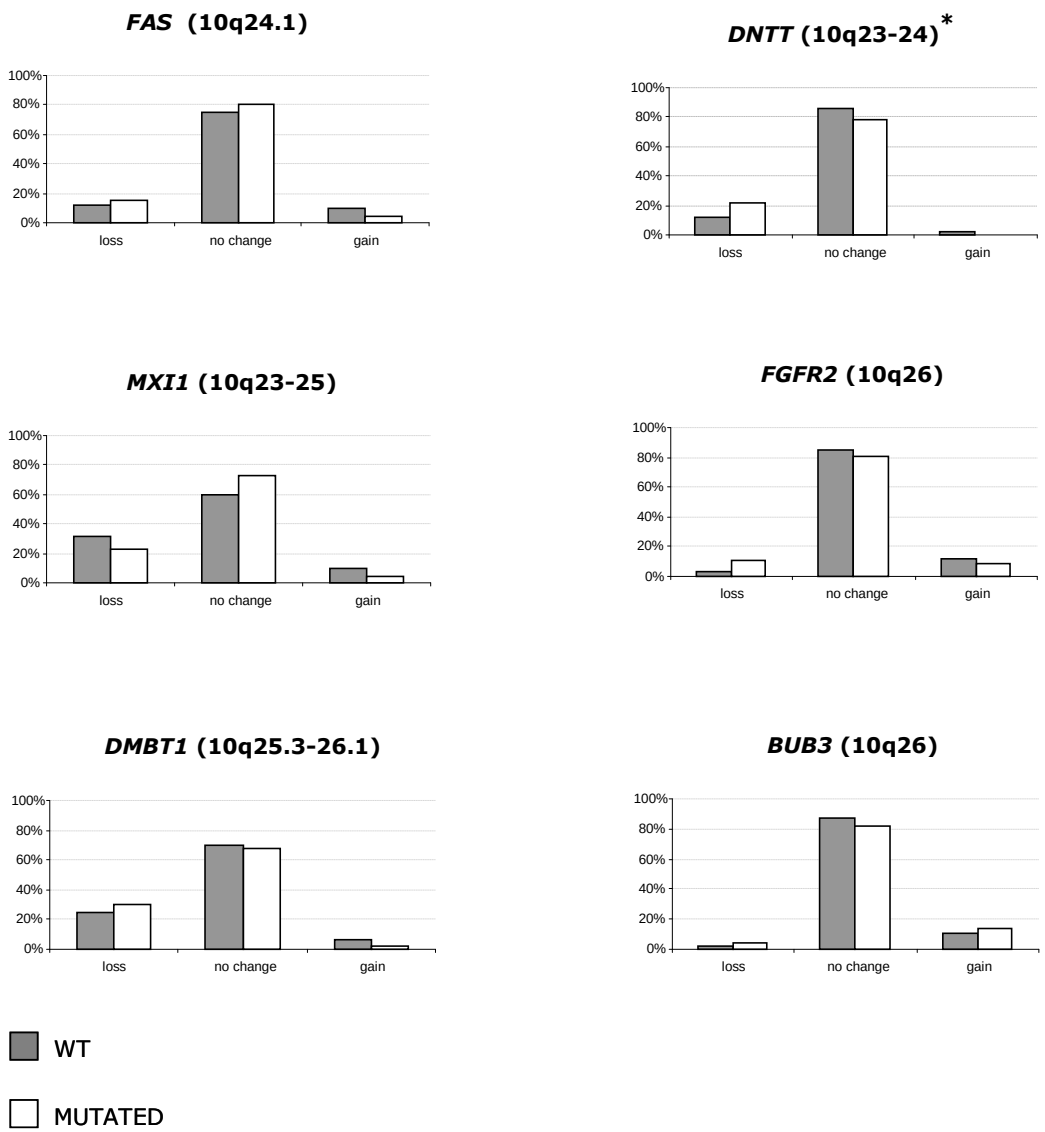
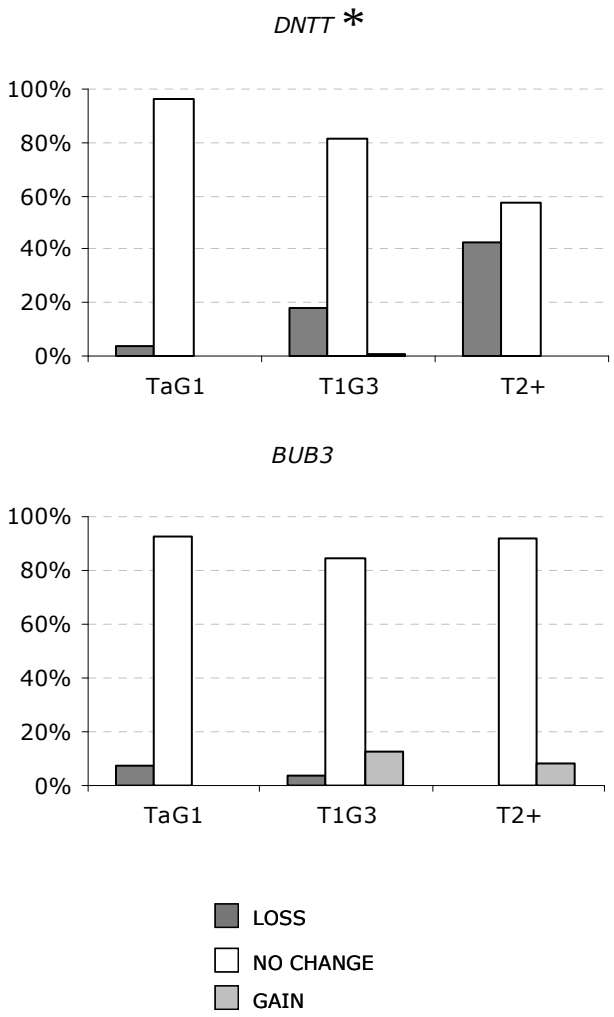


Figure 4B



PIK3CA Mutations Are an Early Genetic Alteration Associated with FGFR3 Mutations in Superficial Papillary Bladder Tumors

Elena López-Knowles, Silvia Hernández, Núria Malats, Manolis Kogevinas, Josep Lloreta, Alfredo Carrato, Adonina Tardón, Consol Serra, and Francisco X. Real on behalf of the EPICURO Study Investigators.
Published in Cancer Research. 2006; 66: (15). August 1, 2006

Bladder tumors can be classified, at the molecular level, following two distinct but slightly overlapping pathways. Superficial papillary tumors are characterized by *FGFR3* mutations and chromosome 9 losses whereas invasive tumors are characterized by p53 and pRb pathway alterations. A genomic characterization of T1G3 tumors, using array comparative genomic hybridization, identified amplifications in 3q26 including a gene not previously associated to bladder cancer, *PIK3CA*. *PIK3CA* encodes the p110α subunit of PI3K involved mainly in cell survival. *PIK3CA* activating mutations and copy number gains had been identified in other tumors types. Thus the aim was to undertake a mutational analysis of exons 9 and 20 of this gene in a panel of bladder cell lines and tumors (n=130) covering the whole disease spectrum. The analysis was performed using DNA extracted from paraffin-embedded tissues, which was amplified by PCR, and sequencing.

Overall, 20% of bladder tumors had alterations in *PIK3CA* at the previously identified hotspots: codons 542, 545 and 1047. The distribution of alterations decreased with stage and grade. The distribution according to stage was as follows: papillary urothelial neoplasms of low malignant potential (PUNLMP; 11 of 43, 25.6%), Ta (9 of 57, 16%), T1 (2 of 10, 20%), and muscle-invasive tumors (0 of 20, 0%; P = 0.019). According to grade the distribution was: grade 1 (6 of 27, 22.2%), grade 2 (3 of 23, 13%), and grade 3 (2 of 37, 5.4%; P = 0.047). Mutations in *PIK3CA* were significantly more common among *FGFR3* mutant tumors.

Our study has identified mutations in *PIK3CA* as a common event in early bladder carcinogenesis, and its association with *FGFR3* mutations indicates it is characteristic of the papillary pathway. PIK3CA may constitute a new diagnostic and prognostic marker, as well as a therapeutic target, in bladder cancer.

Description of the work carried out by the candidate

Elena López-Knowles isolated tumor DNA and performed *PIK3CA* mutational analyses with the help of a technician, carried out *FGFR3* mutational status studies together wih Silvia Hernández, and participated in the statistical analyses of the data. She participated in the discussion of the results and in the preparation of the final version of the manuscript.

Lopez-Knowles E, Hernandez S, Malats N, Kogevinas M, Lloreta J, Carrato A, Tardon A, Serra C, Real FX.
PIK3CA mutations are an early genetic alteration associated with FGFR3 mutations in superficial papillary bladder tumors.
Cancer Research. 2006 Aug 1;66(15):7401-4.

T1G3 tumors are a clinical challenge. T1G3 bladder cancer is a subgroup of tumors which represent 10% of diagnosed bladder cancers and are considered a high risk group because although the level of recurrences is similar to other superficial tumors, the progression rate is much higher, increasing to around 50% of patients. Treatment is dependent on the risk the tumor poses for the patient. There are no applicable molecular markers that predict this risk; therefore, identifying new predictors of disease progression is a clinical challenge.

The following are several aspects that render the study of T1G3 tumors particularly difficult: 1) variability in stage classification and use of homogeneous criteria for classification, 2) sample size, 3) heterogeneity in patient treatment, 4) DNA quality, and 5) the retrospective or prospective nature of most studies. We have made an attempt to overcome several of these difficulties in the framework of the EPICURO study: 1) a reference pathologist reviewed all blocks received from all cases diagnosed in the different hospitals, thus warranting homogeneous criteria for classification; 2) we have collected a high number of incident bladder cancer patients leading to one of the largest groups of T1G3 tumors (n=136); 3) whenever possible, results have been stratified according to treatment regimes; 4) DNA was extracted by manual microdissection and several purification methods were used to ensure the best results for each type of study; and 5) the prospective nature of the study and the very high rate of follow-up achieved eliminates biases and confounding present in a retrospective study. However, our study is not a clinical trial and also suffers from certain limitations, as discussed below.

Molecular characterization of T1G3 tumors. The dual pathway describing the development and progression of bladder cancer classifies tumors into papillary superficial tumors and non-papillary invasive tumors. Superficial papillary tumors (Ta, T1) are characterized by somatic mutations in *H-Ras* and *FGFR3*, and losses of chromosome 9; non-papillary invasive tumors ($\geq$T2) are characterized by alterations in the *Tp53* and *pRb* pathways and losses and gains of many chromosomes. However, the inclusion of both Ta and T1 tumors together within the category of superficial bladder tumors is imprecise and misleading (Richter et al., 1997;Bakkar et al., 2003). An important aim of this work was to characterize the T1G3 subgroup

of tumors from a genetic and genomic point of view in order to provide further clues as to how should T1G3 tumors be classified.

FGFR3 and Tp53 pathway characterization. Two important genes that differentiate the two pathways of bladder cancer, *FGFR3* and *Tp53*, have been analyzed in the T1G3 tumor subgroup. *FGFR3* alterations are common in subgroups of superficial tumors (TaG1, TaG2) and occur in approximately 15% of muscle-invasive tumors. By contrast, *Tp53* mutations are common in invasive tumors. Various studies have shown that FGFR3 alterations are almost mutually exclusive of Tp53 alterations when analyzing a broad spectrum of bladder tumors (Bakkar *et al.*, 2003;van Rhijn *et al.*, 2004;Mhawech-Fauceglia *et al.*, 2006a), thus defining these two genes separate pathways in bladder cancer. Bakkar et al showed only 3% of the tumors analyzed harboured mutations in both genes, the concordance of mutations was only present in 1 T1G3 tumor; van Rhijn et al showed 5.7% alteration in both markers but the stage and grade of the tumors harboring this concordance is not described and Mhawech-Fauceglia et al identified no association between p53 and FGFR3 protein expression but did not describe the number or percentage of cases with alterations of both markers. Zieger et al (Zieger *et al.*, 2005) have described alterations in both genes in 5.8% of bladder tumors, and more specifically 10% of concordance in T1G3 tumors. These observations are in general agreement with our own recent findings, in the largest series of T1G3 tumors reported so far, indicating that 9.2% (11/119) harbour mutations in both genes (Hernandez et al., 2005). These results likely reflect patient selection biases in different studies and the wide spectrum of the disease and the various carcinogenesis pathways involved in these tumors.

FGFR3 mutations in human disease constitute a fascinating area of research. Germline mutations are a common feature of skeletal disorders associated with short limbs. More recently, somatic mutations occurring in mosaicism have been identified in epidermal nevus (Hafner et al., 2006b). Somatic mutations have also been reported both in bona fide neoplasms, such as bladder cancer and cervix carcinomas (Cappellen et al., 1999) and in benign proliferative lesions with no malignant potential such as seborrheic keratoses (Hafner et al., 2006a). In bladder cancer, mutations occur in a stage and grade dependent manner. The most

common mutations described in hotspots 248, 249, 372 and 375, lead to increased and prolonged activation of the receptor. In a large study of superficial bladder cancer (n=772) carried out by our group, the frequency of mutations were shown to decrease with stage and grade (Hernandez et al., 2006): PUNLMP had 77%, TaG1 had 61.5%, TaG2 had 58.1%, TaG3 had 34.1% and T1G2 had 26.9%. There was no difference in the distribution of specific mutations according to stage and/or grade, except for the A393E mutation that was more commonly found in PUNLMP. The decrease of mutations with stage and grade has been proposed to be due to the proliferation of cells following a different molecular pathway with no FGFR3 mutations, as it has been shown that FGFR3-mutated superficial tumors progress and retain their mutation (Zieger *et al.*, 2005). In our study, were analyzed in 119 T1G3 tumors and 17% of mutations were identified (Hernandez et al., 2005). These results are in agreement with work of Van Rhijn et al (van Rhijn et al., 2003) who found 19% of mutations in a small group of T1G3 tumors (n=27).

TP53 mutations are rare in low-grade non-invasive papillary tumors, but frequent in high-grade invasive urothelial tumors. T*P53* mutations were analyzed in 119 T1G3 samples and 65.5% of tumors had inactivating mutations. This is in agreement with the level of mutations identified in the studies of muscle-invasive tumors by, among others, Lorenzo-Romero et al (Lorenzo-Romero et al., 2003) who identified 41% of mutations in superficial tumors and 61% in invasive tumors. However, it is in disagreement with the study by Kelsey et al (Kelsey et al., 2004) who identified 4.6% of mutations in low grade superficial tumors, 19.4% in high grade superficial tumors and only 15% in invasive tumors. Although different studies show variability in the frequency of alterations a recent review by Malats et al (Malats et al., 2005) has shown that the frequency of mutations ranges from 0-48% in superficial tumors and 31-56% of invasive tumors, being more frequent in more aggresive tumors. Figure 1 shows the distribution of mutations described in the IARC database in 3 distinct group of bladder tumors: papillary, CIS and TCC (Olivier et al., 2002). And outlines the number of mutations that have been identified in each subgroup of bladder cancer.

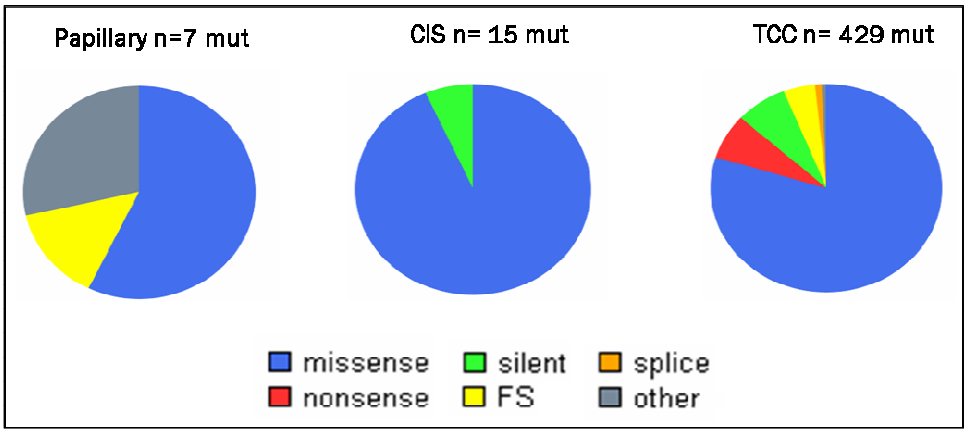


Figure 1. IARC *TP53* Mutation Database, R10 release, July 2005

P53 protein expression was also evaluated in the study and overexpression was found in 66% of tumors, at levels comparative to muscle-invasive tumors. These levels are higher than those described by Steiner (Steiner et al., 2000) who identified 37% overexpression and Shariat et al (Shariat *et al.*, 2004b) who described 28% in Ta/T1 tumors, but similar to the 62% described by Peyromaure (Peyromaure et al., 2002) and Kelsey who described 45% in homogeneous populations of T1G3 cases. Again variability is observed between studies due to the use of different antibodies, different methodology of immunostaining, and different cutoff levels to define positivity. In our study, p53 overexpression was defined as >20% reactive cells, and to take into consideration both staining intensity and proportion of reactive cells, we evaluated data according to a histoscore. The same results were obtained using both classifications. The DO7 antibody employed in the study, is the most frequently used in p53 staining protocols, and further analysis was also done with 1801 antibody which yielded similar results. Because immunohistochemistry may not be a robust technique yielding substantial interlaboratory variability, we placed special emphasis in standardizing our assays by using an automated immunostainer.

Overall, we conclude that - regarding p53 alterations - T1G3 tumors are similar genetically to muscle-invasive tumors.

Because p53 analyses only provides a partial view of the status of the p53 pathway in tumors, we analysed alterations in another major gene involved in this pathway, *HDM2*. *HDM2* is a negative regulator of *Tp53*, but at the same time *Tp53* activates *HDM2*, hence forming a feedback loop. Based on our data, we have found indications that the p53 pathway is inactivated at least in 85% of the T1G3 tumors.

This includes tumors that harbour mutations or alterations in either *Tp53* or *HDM2*, and a group of tumors with high levels of p53 immunostaining without detectable mutations. Of course, we cannot rule out that this latter group may harbour genetic alterations in these genes that were not detected in our assays; alternatively, they may have alterations in other proteins acting in the p53 pathway that could lead to the stabilization of the protein. In the remaining 15% of tumors, we have failed to identify alterations in p53 or HDM2. We have hypothesized that other genetic pathays may be involved in the development of these tumors. To test this notion, we examined the expression of 4 p53 target genes (Survivin, IGF1-R, 14-3-3 σ and COX2) using immunohistochemistry and a tissue microarray. Other well characterized p53 target genes, such as P21, were not included because we felt that they would not be informative: the control of p21 expression is complex and can be mediated by p53-independent pathways (Zeng and el Deiry, 1996). Survivin is an apoptosis inhibitor repressed by wt p53 (Xia and Altieri, 2006). Levels of this protein were elevated in all 3 groups and while they were slightly lower in the wt *Tp53* subgroup, the differences were not statistically significant. IGF1-R is a member of the receptor tyrosine kinase family of proteins and signals through the Ras/Raf/ERK and PI3K/AKT pathway. It is expressed at high levels in most malignant tumors and has been reported to be repressed by wild type p53 (Werner et al., 1996). In our study, the levels of IGF1-R were higher among wild type tumors than among p53 pathway-inactive tumors, however the difference in expression was marginally significant. *14-3-3 σ* harbors a p53-binding site which mediates induction after DNA damage, this induction is required for stable cell cycle arrest. In tumors, p53 mutation results in downregulation of 14-3-3 σ expression (Hermeking et al., 1997). Our results show this decrease in expression, in groups 1 and 2 vs. group 3, in a statistically significant manner. COX-2 has a major role in inflammation and there is evidence associating COX-2 overexpression and p53 accumulation (Shigemasa et al., 2003). Our results indicate the same tendency in a statistically significant manner.

Altogether, the results obtained support the notion that groups 1+2 and group 3 have different molecular characteristics. Since a proportion of the T1G3 tumors included in this study were also analyzed by array CGH, we compared the gains and losses at the genomic level in the 3 subgroups of tumors according to the p53 pathway status. Twenty-eight clones were differentially represented though the

differences failed to reach statistical significance ($p \leq 0.1$). This interesting finding points to the region 8p12-22 to contain candidate genes associated with these alternative molecular pathways; more work is necessary to confirm and extend these findings.

Identification of new molecular alterations in T1G3 tumors. The genomic analysis undertaken by array CGH allowed us to identify a region of amplification at 3q26 in a high proportion (9.7%) of T1G3 tumors. The *PIK3CA* gene, which encodes de p110α protein, maps to this region. This protein belongs to the class 1A PI3K family, involved mainly in cell survival. This gene has been shown to contain activating mutations and to suffer amplification in a variety of cancer types (Karakas et al., 2006). Mutations are restricted to a few, common, hotspots and lead to activation of the PI3K kinase pathway giving rise to cell survival and invasion (Bader et al., 2006). We hypothesized that in bladder cancer, some tumors might also harbour gene mutations. To explore this issue in detail, we undertook a mutational analysis of a panel of tumors representing the whole spectrum of the disease. Unexepectedly, the frequency of mutations was higher in superficial low stage and low grade tumors and decreased with stage and grade: PUNLMP had 25.6%, Ta had 16%, T1 had 10.2% and T2+ had 0% of mutations; grade 1 had 22.2%, grade 2 had 13% and grade 3 had 5.4% of mutations. Twenty percent of T1G3 tumors were mutated for PIK3CA. These findings raise the issue of why does the mutation frequency decrease as tumor stage and grade increases in bladder cancer. In various tumor types, such as breast, hepatocellular carcinoma and gastric cancer, mutations have been described both in early and late lesions (Lee et al., 2005) whilst in brain, colorectal and thyroid cancer, mutations are described at late stages of tumor progression and are thought to occur in the step previous to invasion. *PIK3CA* mutations are frequent at late stages of glioma (Broderick *et al.*, 2004), colon cancer (Samuels and Velculescu, 2004) and thyroid cancer progression (Garcia-Rostan *et al.*, 2005). Therefore, mutations may confer a tumor advantage at different stages in a tissue-dependent manner. Regarding bladder cancer, we have put forward several hypotheses: 1) one should consider the role of mutation separately among papillary and non-papillary tumors as these may be two different diseases; 2) it is possible that mutations in *PIK3CA* may have deleterious effects on tumor cells in a specific context; 3) the PIK3CA pathway could remain

active throughout bladder cancer stages and grades by mechanisms other than mutation. In reference to the second hypothesis, *PIK3CA* mutations may lead to high levels of *AKT* activation which can give rise to suppresion of metastasis and invasion in breast cancer cell lines. This suppression is mediated by the AKT1 isoform through NFAT. This could explain a negative selection of cells containing *PIK3CA* mutations. However, the dominant negative version of *AKT* in bladder cancer cell lines has been described to have the same effect of suppression of invasion (Wu et al., 2004). In reference to the third hypothesis, high grade and stage bladder tumors are characterized by overexpression of EGFR and downregulation of PTEN, both of which lead to activation of the PI3K pathway. Preliminary results in our study of a range of bladder tumors show PTEN expression is lost in 62% of invasive tumors, and the loss is less frequent in lower grade/stage and when compared to *PIK3CA* status overall, PTEN loss is more common in *PIK3CA* wild type tumors, although not in a statistically significant manner. This study however is limited due to its size and other genes in the pathway in a larger group should be analyzed.

Moreover, amplification, which has been described as a mechanism of activation, could be present in higher grade tumors, as overexpression of the protein has been described in bladder tumors of all stages and grades compared to normal urothelium (Benistant et al., 2000). Studies in ovarian and breast tumors, have described amplification and mutation to be mutually exclusive events but the relationship between these two types of molecular alterations in bladder cancer has not yet been analyzed. Altogether, our findings further support the role of the PI3K pathway in bladder through a variety of mechanisms and provide the basis or novel studies. Therefore, because *PIK3CA* mutations seem to be a common alteration in bladder cancer and the status of other members of the pathway in previous bladder studies suggest PI3K pathway is inactivated in most bladder tumors through different mechanisms, the pathway may be altered in all bladder tumors.

Genomic characterization. To determine the frequency and type of genomic alterations in T1G3 tumors, we have used array CGH. A total of 52 T1G3 tumors were analyzed, together with a small group of TaG1 and muscle-invasive tumors, for comparison purposes. T1G3 tumors were characterized by a high number of genomic alterations including gains, losses, high level amplifications and deletions

in most chromosomes, although not distributed uniformly across the genome, pointing to specific regions harbouring oncogenes and tumor suppressor genes. Some of these regions have been previously identified in bladder cancer and others provide new candidates. The confirmation of previously described genes validates the analysis and the identification of new candidate genes is a first step for further analysis. When comparing the FGA (fraction of genome altered) of T1G3 (47.7%) and TaG1 (3.51%) tumors, a highly significant difference was observed. T1G3 tumors had a level of alterations similar to that of muscle-invasive tumors (60.9% in our study, data not shown). The FGA in our muscle invasive series was higher than that reported by the group of our collaborators using the same array platform, (16%) (Blaveri et al., 2005). The number of tumors studied by Blaveri were n=55, whilst in our study we only analyzed n=10. This difference may be accounted for by the procedures used for manual microdissection and tumor area selection. The spectrum of aberrations in muscle-invasive tumors is similar to that observed in T1G3 tumors however the aberrations are less frequent, therefore it is possible to suggest that the heterogeneity of aberrations present in T2+ tumors becomes diluted as the number of cases analyzed increases. Results are comparable because the protocol followed, the array used and the analysis was the same for both studies. At the individual clone level there were also more differences between T1G3 and TaG1 tumors than comparing with invasive tumors. TaG1 tumors were characterized mainly by losses of chromosome 9, hence T1G3 tumors had differential copy number changes in most chromosomes compared to TaG1 tumors in a statistically significant manner. The T1G3 group had alterations in most chromosomes and only two very small regions in chromosomes 11 and 12 had clones with differential copy number changes statistically significant compared to invasive tumors included in our study. However, this comparison has limited value due to the low number of invasive tumors studied since this was not our primary goal. When comparing our array CGH data to Blaveri's study, certain frequent alterations were found in common in T1 tumors: amplifications at 6p22, 8q22, 11q13 and 12q14 and deletions at 2q14, 8p23, 9q31-34, 10q23-24 and 11p15. Hence at the clone and global alteration level T1G3 tumors show a higher similarity to higher stage tumors.

Our array CGH suffers from some limitations. First, we used DNA extracted from paraffin embedded material as test and commercial normal DNA as reference. The

selection of the reference DNA was based on previous studies in which comparison of two DNAs extracted from paraffin embedded material gave very poor results. Second, some tumors had to be eliminated from the study due to the low quality of the results. The use of DNA from fresh frozen tissue would have allowed the analysis of all cases because the quality of the material is higher.

Mechanisms involved in genomic instability. The fraction of the genome altered is a measure of the instability seen in these tumors. The array CGH analysis has shown that T1G3 tumors are characterized by high levels of instability, contrary to that observed in lower stage and grade superficial tumors, and similar to that of muscle-invasive cancers. This genomic instability, or aneuploidy, can be caused by a variety of mechanisms, including defective DNA repair, loss of DNA damage checkpoints, defective recombination or other rearrangements of the DNA sequence, misregulation of the cell cycle, disruption of the mitotic spindle apparatus, and centrosomal duplication or amplification (Saunders et al., 2000). Because p53 is involved in many processes related to the DNA damage response and it regulates cell cycle, apoptosis and maintenance of the genetic integrity, we hypothesized that tumors with p53 alterations might have a greater level of genomic alterations. When T1G3 tumors were categorized according to *Tp53* mutational status, there was no difference in FGA level between *Tp53* mutated and wild type tumors. *TP53* mutational inactivation has been associated to centrosome amplification in cultured mouse cells. However, in human cells, additional mutations seem to be required for the generation of instability (Bunz et al., 2002). Human cells are likely to have additional regulatory mechanisms for centrosome number control. Our more detailed study of p53 pathway alterations also led to a comparison of FGA according to this classification. Similarly, we found no difference at the FGA level between seemingly p53-competent and p53-inactivated tumors. Therefore, it is likely that other alterations – possibly unrelated to the p53 pathway - cause genomic instability. A few candidates for this role are discussed below.

Cyclin E, a regulatory subunit for CDK2, plays a key role in centrosome duplication. Bladder tumors harbouring *TP53* mutations associated with overexpression of Cyclin E have been shown to be associated to higher chromosome instability (Kawamura et al., 2004). This finding is consistent with our observation that *Tp53* mutated tumors with Cyclin E gains (mean FGA=55%) had slightly higher FGA than

Tp53 mutated tumors with Cyclin E normal copy number (mean FGA=43%) (P=0.14). However, the association between gains of Cyclin E and higher FGA was independent on p53 status, suggesting an independent role of cyclin E. This issue merits further study in our enlarged case series of T1G3 tumors, using quantitative gene copy number analysis as well as protein overexpression.

Genomic instability has also been significantly associated to aurora A kinase and Rb alterations (Iovino et al., 2006). Aurora A kinase is essential for mitotic progression and its overexpression leads to an abnormally high number of centrosomes due to abortive cell cycles; also leading to aneuploidy. Increased Aurora A gene copy number is associated to higher levels of FGA in our series. Tumors with Aurora Kinase normal copy number or loss had an FGA of 39%, whilst tumors with Aurora Kinase gains had an FGA of 52% (P=0.05), providing support to previous studies in which amplification and/or overexpression of Aurora is related to aneuploidy and aggressive clinical behavior in human bladder cancer (Sen et al., 2002). Therefore this instability may be caused by several mechanisms which involve p53, Cyclin E gain, Aurora Kinase A gain and other genes involved in chromosomal segregation.

Making progress in establishing the prognosis of patients with T1G3 tumors. Prognosis of bladder cancer patients relies nowadays on clinical-pathological markers such as grade, stage, multiplicity, size and presence of CIS as primary prognostic variables. These parameters classify tumors into several risk subgroups. However, heterogeneity exists within the groups. Molecular markers have been widely used to study their value in establishing the prognosis of patients with bladder cancer. One aim of our study, and one of the major reasons for focusing on T1G3 tumors, was the identification of novel markers predictive of patient outcome.

FGFR3. Activating *FGFR3* mutations have been suggested to have a regulatory role in proliferation and differentiation in chondrocytes. Premature exit from the cell cycle and defective differentiation seem to be the main cause of longitudinal bone growth retardation in FGFR3-related human chondrodysplasias (Legeai-Mallet et al., 2004). This unusual biological response is mediated by Stat1 and may also be caused through AKT downregulation (Priore et al., 2006). In other cell types, *FGFR3* activation leads to proliferation and transformation (Kimura et al., 2001). These observations suggest, *FGFR3* mutations may play opposite roles depending on the

cell type. In nude mice, FGFR3 activating mutations on the skin, lead to proliferation and formation of benign lesions. In the study of bladder cancer, introduction of activating mutations in the bladder of nude mice gives rise hyperplasia. In cell lines, *FGFR3* activating mutations are suggested to reduce the ability of tumors to become invasive, having little effect on differentiation (Kimura *et al.*, 2001;Bakkar *et al.*, 2003). It has been described that proliferation decreases in normal urothelium when subjected to MAPK inhibitors. However, high grade or stage disease, which have massive alterations in cell cycle regulation, are less sensitive to these inhibitors because they are less dependent on MAPK signalling for proliferation (Swiatkowski et al., 2003). Therefore in low grade and stage tumors it would be hypothesized that FGFR3 alterations give rise to proliferation but not progression or invasion, and in high grade and stage tumors FGFR3 alterations to be less important for proliferation and might not have an effect due to other mutations overtaking the function of FGFR3 alterations.

In the context of the EPICURO study, we have carried out the largest study of the prognostic value of *FGFR3* mutations in 764 patients with superficial bladder cancer. Unlike most of the published reports, our study was prospective. In agreement with prior reports, as described above, we found that *FGFR3* mutations were significantly more common among papillary grade 1 and grade 2 tumors than among grade 3 tumors, regardless of the level of invasion in the bladder wall (i.e. Ta or T1). In the univariate analysis, *FGFR3* mutations were associated with higher levels of recurrence, lower level of progression and lower rate of death from bladder cancer. In the multivariable analysis, when the whole group of superficial tumors was considered, mutations were also associated to a higher risk of recurrence. Importantly, when the results were stratified by stage and grade, the independent predictive value only remained significant for the TaG1 group of tumors (HR 2.12, 95% CI, P= 0.004); the association was almost significant for TaG2 tumors (HR 1.46; 95% CI, P= 0.07). By contrast, there was no association with any of the outcome parameters in patients with higher stage and/or grade tumors. In T1G3 tumors FGFR3 status did not predict recurrence or survival. The lack of significant association with progression or death could be due to the small number of events identified in the follow up of these patients, and could reflect a limitation of the study. Therefore, our findings confirm the hypothesis that FGFR3 mutations have a prognostic value mainly in low grade low stage tumors. A longer follow-up will be

required to determine whether FGFR3 mutations are also a significant predictor of progression or bladder cancer-related death.

P53. Despite the fact that p53 abnormalities have been widely studied in relation with outcome in bladder cancer, few studies have focused on T1G3 tumors, and all of them have focused on p53 overexpression rather than gene mutation. Peyromaure et al studied p53 overexpression in 29 T1G3 tumors and found that it was not predictive of recurrence and progression (Peyromaure *et al.*, 2002); Steiner et al (Steiner *et al.*, 2000) also analyzed 31 T1G3 tumors for p53 overexpression and found that immunostaining did not add relevant information to stage and grade; Queipo Zaragoza et al (Queipo Zaragoza et al., 2005) found p53 immunostaining to be an independent predictor of progression in 83 T1G3 tumors. When analysing the p53 pathway (mutations, immunohistochemistry and global pathway status) and T1G3 tumors in our study, no association with outcome was identified.

This variability in the results of published studies has been recently reviewed (Malats *et al.*, 2005). Among others, the following reasons have been put forward to account for the different results: immunohistochemical technique (i.e. antigen retrieval, secondary antibody), antibody used, threshold used to define positive/negative, the length of follow up, and the nature of the study (i.e. prospective or retrospective). We believe that our study fulfills many of the recommendations that have recently been made for the assessment of prognostic markers in cancer (ReMARK). While it would be desirable that our study is replicated in an independent prospective series with sufficient power, we conclude that at the present time there is not sufficient evidence to support the use of Tp53 mutation detection of p53 nuclear overexpression to aid in the establishment of the prognosis of patients with T1G3 bladder tumors. Our findings support the notion that p53 inactivation is crucial for the development of T1G3 tumors.

An interesting finding of our study was that the group of tumors for which we found no evidence of *p53* and *HDM2* alterations (group 3), seemed to fare the worst of all in outcome. This analysis is limited by the fact that there were few patients in each arm and we plan to carry out similar studies in a larger case series. However, the findings are of interest and we propose two hypotheses to account for them. First, it

has been described that the ATM-Chk2-p53 cascade is activated in human bladder tumors before the occurrence of p53 mutations and/or defects in DNA damage signalling. This activation occurs early in tumorigenesis and may be lost in more advanced tumors (Bartkova et al., 2005). Second, as previously mentioned, the clones covering the region 8p12-22 were shown to be lost more frequently among tumors in group 3 than among tumors in the other two groups. This region has previously been highlighted in bladder cancer (Richter *et al.*, 1999;Choi *et al.*, 2000a) and could contain genes putatively associated to worse outcome (Fornari et al., 2006). *BNIP3L* (8p12-21), is a propapoptotic transcriptional target of *p53*. *BNIP3L* silencing by siRNA blocks apoptosis in wild type p53-expressing cells under hypoxia and promotes tumor growth in vitro through reduced sensitivity to hypoxia and increased proliferation. The knockdown of *BNIP3L* promotes tumorigenicity of wild type versus mutant p53 expressing tumor (Fei et al., 2004). *DBC2*, deleted in breast cancer 2, located at 8p21, was cloned from a region homozygously deleted in breast cancer. This gene contains a protein-protein interaction domain; and mutations have been identified in a primary breast tumor and in a breast cancer cell line. The expression of wild type and mutant *DBC2* (the breast tumor mutation generated in a breast tumor cell line) indicated a potent anti-proliferative effect in vitro of the wild type protein and not the mutated, confirming its potential role as a tumor suppressor (Hamaguchi et al., 2002). Only 1 somatic mutation in this gene has been described in bladder cancer. It is a missense mutation whose function is unknown (Knowles et al., 2005). *TRAIL-R2/DR5* (8p21) induces death following *TRAIL* binding. It is a p53 target that can signal apoptotic death. Its activity is consistent with that of a dosage-dependant tumor suppressor gene and mutations have been identified at low frequency in several cancers (Rubio-Moscardo et al., 2005). No association between mutation and LOH in bladder cancer has been however described (Adams et al., 2005). This could mean its not the tumor suppressor target in this region or that the inactivation of the remaining allele occurs by a mechanism other than mutation or that haploinsufficiency generates a phenotypic effect.

PIK3CA. Alterations have been associated to aggressive type tumors in breast (Buttitta et al., 2006;Li et al., 2006b), brain (Broderick et al., 2004) and thyroid cancer (Garcia-Rostan et al., 2005). The presence of PIK3CA mutations in a subset

of patients with FGFR3 mutations, including T1G3 tumors may imply that PIK3CA mutations could enhance the malignant behavior of *FGFR3* mutant tumors. This hypothesis is currently being tested in our laboratory. In T1G3 tumors, due to the infrequent mutations of *FGFR3* and *PIK3CA* no concordance was observed. Because the number of bladder tumors studied was small in each category, no relationship with outcome has been assessed. However, the prognostic value of mutations in *PIK3CA*, and other members of the pathway, and of overall pathway activation remains an important goal for the future.

Genomic instability. From the array CGH analysis, two types of data are obtained: a value of the fraction of the genome altered per tumor sample and information on gains and losses of each of 2464 individual clones. When the association of FGA with outcome was assessed in a multivariable analysis, this parameter emerged as an independent predictor of recurrence. Overall, these findings suggest that the level of "molecular aneuploidy" is associated with outcome. The HR for progression and cancer-related death were close to significance but a larger series and follow-up will be required to more firmly establish the association. Previously, centrosome hyperamplification has been shown to be an independent predictor of recurrence in non-muscle invasive bladder cancers, including stages Ta and T1 tumors (Yamamoto et al., 2004) and - in muscle invasive tumors - high levels of FGA have been associated to overall survival (Blaveri *et al.*, 2005). Further work is warranted in this area and the aim should be to reduce the complexity of the array analysis in a manner that may be more cost-effective. While the sudy of the association of alterations at the individual clone level with outcome is an important aim, we have – so far - stayed away from this analysis due to the small number of events of each outcome category and the risk of false positive findings.

None of the "gene level" alterations analyzed in this study are independent predictors of outcome for T1G3 tumors. Probably there is not one or few genes that can predict the outcome in these patients due the high number of alterations present in the tumor. Only the amount of instability is associated with outcome. Significant correspondence has been observed between copy number alterations and gene expression (Pollack et al., 2002). Recent work by Carter and colleagues have shown that chromosomal instability, inferred from gene expression data,

predicts clinical outcome in several cancers (Carter et al., 2006), which could also apply to bladder cancer. A signature of 25 genes has been described to associate with outcome in grade 1 and 2 breast tumors, however grade 3 tumors could not be classified using this measure. These genes have functions in mitosis, cell cycle and DNA replication and repair, all of which might underlie chromosome instability. So maybe, as described in the study by Carter et al, this instability may reveal regions of common alterations which underscore genes implicated in the progression or recurrence of this disease such as those observed in the study by Blaveri et al. with array CGH analysis in invasive tumors. The analysis of alterations in these genes may add to the clinical prognostic factors used for patient management.

Several studies are classifying bladder tumors by SNP arrays, CGH arrays, expression arrays, LOH marker analysis but the more robust and patient-applicable classifiers are clinical markers. A combination between clinical markers and robust molecular markers in a nomogram should hopefully soon be able to predict the natural history of the disease.

An important factor and possible restriction in the analysis of prognostic markers in bladder tumors comes from the study by Zwarthoff et al that analyzed 104 tumors from 11 patients through 17 years. They found that when a tumor is ressected, other bladder tumors of lower stage and grade and with lower level of alterations are still present in the bladder and are not detected until later follow ups of the patient. In most cases the first tumor extracted was the last tumor in the chronology of the disease, containing the highest amount of instability and alterations. This means the molecular analysis of this tumor, which is used as a prognostic classifier for the patient, cannot be associated to the recurrences observed later on, as the tumors observed of the same stage and grade and similar alterations are not recurrences but previous tumors of the same pathway. This is not the case however for progressions, as they generally will contain higher level of alterations and therefore will probably be detected after the primary tumor diagnosis.

A biological model of T1G3 tumors. The genetic characterization of bladder tumors has indicated that *FGFR3* and *PIK3CA* are involved in the same bladder cancer pathway whereas *p53* is characteristic of a different pathway. Admittedly, however, these two pathways are not completely independent and indeed display some overlap. T1G3 tumors are characterized by a low frequency of *FGFR3* and *PIK3CA*

mutations, an inactive p53 pathway in 85% of the tumors analyzed, and a high level of instability being at the crossroads between both the superficial and invasive pathways. Studies analyzing p53 alterations at the protein and gene level in CIS, have described 72% of mutations and 60-75% overexpression (Hartmann et al., 2002;Hopman et al., 2002;Shariat et al., 2003) and aneuploidy in CIS lesions not associated to papillary tumors (Hopman *et al.*, 2002). It could be suggested that bladder cancer arise via two distinct pathways. One gives rise to superficial low-grade low-stage tumors characterized by mutations of *FGFR3* and *PIK3CA* and the other gives rise to CIS lesions characterized by p53 alterations and aneuploidy. T1G3 tumors would converge from both pathways harboring an inactive p53 pathway in most tumors and a low frequency of FGFR3 and PIK3CA mutations (Figure 2). TaG3 tumors would also converse at the same point because the risk of progression and death from bladder cancer for patiens with these tumors is similar to that of patients with T1G3 tumors (Herr, 2000;Lebret et al., 2000). TaG3 tumors showed a lower frequency of *FGFR3* and *PIK3CA* mutations than low grade non-invasive tumors, intermediate with that of T1G3 tumors.

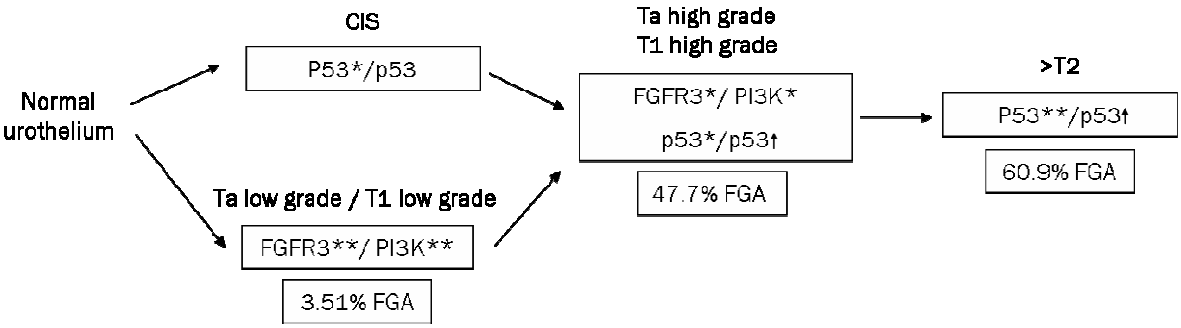


Figure 2. Bladder cancer model

Are these genes target of therapies for bladder cancer? Defining the genetic pathways of urothelial tumorigenesis provides a framework for pathway-based therapies that will aid current standard of care. For example, the majority of low grade, non-invasive tumors have *H-RAS*, *FGFR3* or *PIK3CA* mutations, therefore, it is conceivable that inhibiting one or more components of these signalling pathways could have a therapeutic effect. Because high grade superficial and invasive tumors have deficiencies in *p53* or *pRb* pathways, restoring these pathway could curb tumor growth.

FGFR3: in vitro screening assays have recently identified several small molecules, such as SU5402, SU6668, PD173074 and CHIR-258 that inhibit FGFR3 (Mohammadi et al., 1997;Laird et al., 2000;Grand et al., 2004). CHIR-258, for example, has been used on multiple myeloma cells and produces an apoptotic response (Trudel et al., 2005). Besides the small molecule approach, monoclonal antibodies have been raised against FGFR3 that show inhibitory effects. An anti-FGFR3 antibody inhibited growth of RT112 urothelial tumor cell line in vivo (Gomez-Roman et al., 2005). In bladder cancer patients, it could be hypothesized that FGFR3 inhibitors have a potential role in superficial low grade low stage tumors that are characterized by activating mutations or overexpression of FGFR3. Only a subset of T1G3 tumors may benefit from these inhibitors, as the levels of overexpression and mutations is lower, however other alterations may override the activation of FGFR3.

Table 1. FGFR3 therapy

Agent	Mechanism of action	Effects in vitro
SU5402, SU6668	Binds to catalytic domain and competes with ATP binding	Inhibits phosphorylation of FGFR3, ERK1/2, STAT3. Reduces tumor growth and induces apoptosis
PD173074	Competes with ATP binding	Induces growth arrest, differentiation and apoptosis
CHIR- 258	Binds broadly to the kinase domain of various RTKs	Inhibit cell growth and phosphorylation of FGFR3, ERK1/2
FGFR3 (B-9)	Blocks ligand-receptor interaction	Inhibits RT-112 urothelial tumor cell proliferation
HuCAL- Fab1	Blocks ligand-receptor interaction	Inhibits FGFR3-mediated cell proliferation

PI3K: various drugs have had FDA approval upstream of the PI3K pathway, such as Iressa (gefitinib) and Herceptin (trastuzumab) which have an effect on *EGFR* and *ErbB2* respectively. Regarding PIK3CA inhibitors, the best characterized are LY294002 and wortmannin. These small molecules sensitize tumor cells to chemotherapy and radiation and despite their antitumor efficacy, their poor solubility and high toxicity have limited their clinical application. Also, they are both ATP-binding competitors which give them low specificity, since ATP pockets of different kinases are structurally similar. Because of this lack of specificity, other inhibitors that work through mechanisms other than ATP binding inhibition are being studied. An example would be an inhibitor of the association between p85 and growth factor receptor or with p110. Derivatives of LY294002 and wormannin

compounds are in late preclinical development: BEC235 (Novartis), PX-866 (ProlX) and SF1126 (Semafore). These new inhibitors have greater solubility and less protein binding. Other inhibitors of PI3Ks are also being developed: IC4856068 is an isoform-specific inhibitor of p110δ catalytic subunit (Geng et al., 2004); halenaquinone which inhibits PI3K kinase activity (Fujiwara et al., 2001) and deguelin (Udeani et al., 1997). Stress is required for efficient inhibition of the pathway and, because tumors are stressed in vivo, PI3K blockade may be sufficient to inhibit growth (Powis et al., 2006). PIK3CA inhibitors are hypothesized to have a potential role in superficial low-grade low-stage tumors characterized by activating mutations. Overall in bladder cancer the pathway is suggested to be activated through other mechanisms which would also make PIK3CA inhibitors a potential for treatment however, this has to be confirmed by analyzing other mechanisms of activation of the gene. Only a small subset of T1G3 tumors may benefit from these inhibitors if mutations are taken under consideration, however the pathway may be inactive in most tumors and/or other pathways, such as the p53 pathway, might be a better target in this group of tumors.

Table 2. PI3K therapy

Agent	Mechanism of action	Effects in vitro
LY294002	Inhibits p110 ATP binding site	Inhibit tumor cell growth
Wortmannin	Binds p110 subunit	Inhibit tumor cell growth
IC486068	Inhibits p110δ	Inhibits radiation-induced AKT phosphorylation
Halenaquinone	Inhibits p110δ	Induce apoptosis in cancer cells
Deguelin	Inhibits PI3K	Downregulates AKT phosphorylation
PX-866	Inhibits p110 ATP binding site	Inhibits PI3K signaling

P53: the story of p53 functional restoration is already a long one. Several groups have attempted to use viral-vector-based gene therapy approaches to deliver functional p53 to urothelial tumor cells. One of the most tested vectors is a replication-deficient adenoviral vector bearing the wild type *p53* gene, which shows growth inhibition in tumor cell lines and xenograft and orthotopic urothelial tumor models (Pagliaro, 2000). Another method to exploit p53 deficiency is the use of an E1B-55K deleted adenovirus that was designed to replicate and lyse tumor cells containing mutant *p53;* this virus cannot replicate in cells with wild type p53 (McNeish et al., 2004). A different strategy to restore p53 function is by small molecules that activate p53 reporter activity, increase expression of p53 target

genes and induce apoptosis in p53 deficient cells (Wang et al., 2006). A number of different mechanisms for p53 pathway activation have been identified depending on the target alteration to restore. When homozygous mutations are present, chaperone-like small molecules and small peptides may restore wild type conformation on some mutant p53 proteins. When heterozygous mutations are identified, siRNA specific to a point mutation can eliminate the dominant negative effect it exerts on the wild type *p53*. When *MDM2* is amplified, small molecules (nutlins) can inhibit the *MDM2-p53* interaction, giving rise to an apoptotic response in tumor cells. When p53 is sequestered in the cytoplasm by some protein (example mot-2), inhibitors that release p53 from their anchors allow its translocation to the nucleus (Resnick-Silverman and Manfredi, 2006). Inhibitors of both *Tp53* mutation and MDM2 amplification could be beneficial in T1G3 and invasive tumors.

Table 3. p53 therapy

Agent	Mechanism of action	Effects in vitro
Ad5CMV-Tp53	Restore p53 function	Inhibit urothelial tumor cell growth, apoptosis and synergizes with cisplatin in tumor inhibition
VV-TK-Tp53	Restore p53 function	P53-induced apoptosis and lytic effects of the virus
Nutlin	Inhibit p53-mdm2 interaction	Activate the p53 pathway
CP-31398	Restores p53 conformation and DNA binding ability to mutant p53	Upregulates p53 responsive genes; induces cell cycle arrest and apoptosis
PRIMA 1	Restores transcriptional activity to mutant p53	Cytotoxic tu tumor cells that express p53 mutant

Making progress in bladder cancer. To reduce the morbidity, mortality, and health costs due to bladder cancer, several areas need to be tackled simultaneously. From an epidemiological standpoint, a major aim should be to reduce the population exposure to bladder cancer carcinogens, including a decrease in tobacco smoking, in occupational exposures associated with risk, and – possibly – in other exposures such as water disinfection byproducts. From a clinical standpoint, an early diagnosis and correct classification of the tumor is mandatory. The development of screening strategies for subjects at risk, the identification of methods for improved detection of recurrences, the development of better methods to treat high grade superficial tumors with intravesical therapy, and to take advantage of molecular methods to better assess patient's outcome. From a therapeutic standpoint, the identification of non-toxic therapies that may decrease recurrences, the development of better and more selective treatments for high grade disease, and the improvement of current treatment for muscle-invasive and metastatic disease.

- The p53 pathway is inactivated, through a variety of mechanisms, in the vast majority of T1G3 bladder tumors.

- T1G3 tumors display high levels of genomic instability, regardless of the p53 pathway status of the tumor.

- *PIK3CA* alterations are common events in bladder tumors: mutations occur more commonly in low-grade low-stage bladder cancer whereas gene copy number gain/amplifications occur in high grade tumors.

- Among all molecular parameters analyzed so far, the fraction of the genome altered is the only one that predicts outcome among patients with T1G3 tumors.

- T1G3 tumors should be classified together with muscle-invasive tumors from the biological and genetic standpoint; indeed they are invasive tumors that have not yet invaded muscle.

1. Aaboe,M., Birkenkamp-Demtroder,K., Wiuf,C., Sorensen,F.B., Thykjaer,T., Sauter,G., Jensen,K.M., Dyrskjot,L., and Orntoft,T. (2006). SOX4 expression in bladder carcinoma: clinical aspects and in vitro functional characterization. *Cancer Res.,* **66,** 3434-3442.

2. Abd,E.G., I, Moussa,H.S., Nasr,M.I., El Gemae,E.H., Masooud,A.M., Ibrahim,I.K., and El Hifnawy,N.M. (2005). Comparative Study of NMP-22, Telomerase, and BTA in the Detection of Bladder Cancer. *J. Egypt. Natl. Canc. Inst.,* **17,** 193-202.

3. Abdel-Fattah,R., Challen,C., Griffiths,T.R., Robinson,M.C., Neal,D.E., and Lunec,J. (1998). Alterations of TP53 in microdissected transitional cell carcinoma of the human urinary bladder: high frequency of TP53 accumulation in the absence of detected mutations is associated with poor prognosis. *Br. J. Cancer,* **77,** 2230-2238.

4. Aben,K.K., Macville,M.V., Smeets,D.F., Schoenberg,M.P., Witjes,J.A., and Kiemeney,L.A. (2001). Absence of karyotype abnormalities in patients with familial urothelial cell carcinoma. *Urology,* **57,** 266-269.

5. Aben,K.K., Witjes,J.A., Schoenberg,M.P., Hulsbergen-van de Kaa,C., Verbeek,A.L., and Kiemeney,L.A. (2002). Familial aggregation of urothelial cell carcinoma. *Int. J. Cancer,* **98,** 274-278.

6. Aboulkassim,T.O., Larue,H., Lemieux,P., Rousseau,F., and Fradet,Y. (2003). Alteration of the PATCHED locus in superficial bladder cancer. *Oncogene,* **22,** 2967-2971.

7. Adachi,H., Igawa,M., Shiina,H., Urakami,S., Shigeno,K., and Hino,O. (2003). Human bladder tumors with 2-hit mutations of tumor suppressor gene TSC1 and decreased expression of p27. *J. Urol.,* **170,** 601-604.

8. Adams,J., Cuthbert-Heavens,D., Bass,S., and Knowles,M.A. (2005). Infrequent mutation of TRAIL receptor 2 (TRAIL-R2/DR5) in transitional cell carcinoma of the bladder with 8p21 loss of heterozygosity. *Cancer Lett.,* **220,** 137-144.

9. Ambrosini,G., Adida,C., and Altieri,D.C. (1997). A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat. Med.,* **3,** 917-921.

10. Amendola,P., Audisio,R., Scaburri,A., Ariano,E., Cavuto,S., Imbriani,M., Massari,S., Scarselli,A., and Crosignani,P. (2005). [The active search for occupational cancer cases: bladder cancer in Lombardy Region]. *Epidemiol. Prev.,* **29,** 253-258.

11. Amira,N., Cancel-Tassin,G., Bernardini,S., Cochand-Priollet,B., Bittard,H., Mangin,P., Fournier,G., Latil,A., and Cussenot,O. (2004). Expression in bladder transitional cell carcinoma by real-time quantitative reverse transcription polymerase chain reaction array of 65 genes at the tumor suppressor locus 9q34.1-2: identification of 5 candidates tumor suppressor genes. *Int. J. Cancer,* **111,** 539-542.

12. Apodaca,G. (2004). The uroepithelium: not just a passive barrier. *Traffic.,* **5,** 117-128.

13. Appella,E. and Anderson,C.W. (2001). Post-translational modifications and activation of p53 by genotoxic stresses. *Eur. J. Biochem.,* **268,** 2764-2772.

14. Astrinidis,A., Senapedis,W., Coleman,T.R., and Henske,E.P. (2003). Cell cycle-regulated phosphorylation of hamartin, the product of the tuberous sclerosis complex 1 gene, by cyclin-dependent kinase 1/cyclin B. *J. Biol. Chem.,* **278,** 51372-51379.

15. Aveyard,J.S., Skilleter,A., Habuchi,T., and Knowles,M.A. (1999). Somatic mutation of PTEN in bladder carcinoma. *Br. J. Cancer,* **80,** 904-908.

16. Bader,A.G., Kang,S., and Vogt,P.K. (2006). Cancer-specific mutations in PIK3CA are oncogenic in vivo. *Proc. Natl. Acad. Sci. U. S. A,* **103,** 1475-1479.

17. Bader,A.G., Kang,S., Zhao,L., and Vogt,P.K. (2005). Oncogenic PI3K deregulates transcription and translation. *Nat. Rev. Cancer,* **5,** 921-929.

18. Baffa,R., Gomella,L.G., Vecchione,A., Bassi,P., Mimori,K., Sedor,J., Calviello,C.M., Gardiman,M., Minimo,C., Strup,S.E., McCue,P.A., Kovatich,A.J., Pagano,F., Huebner,K., and Croce,C.M. (2000). Loss of FHIT expression in transitional cell carcinoma of the urinary bladder. *Am. J. Pathol.,* **156,** 419-424.

19. Bakkar,A.A., Wallerand,H., Radvanyi,F., Lahaye,J.B., Pissard,S., Lecerf,L., Kouyoumdjian,J.C., Abbou,C.C., Pairon,J.C., Jaurand,M.C., Thiery,J.P., Chopin,D.K., and de Medina,S.G. (2003). FGFR3 and TP53 gene mutations define two distinct pathways in urothelial cell carcinoma of the bladder. *Cancer Res.,* **63,** 8108-8112.

20. Band,P.R., Le,N.D., MacArthur,A.C., Fang,R., and Gallagher,R.P. (2005). Identification of occupational cancer risks in British Columbia: a population-based case-control study of 1129 cases of bladder cancer. *J. Occup. Environ. Med.,* **47,** 854-858.

21. Barbacid,M. (1987). Ras Genes. *Annual Review of Biochemistry,* **56,** 779-827.

22. Bartkova,J., Horejsi,Z., Koed,K., Kramer,A., Tort,F., Zieger,K., Guldberg,P., Sehested,M., Nesland,J.M., Lukas,C., Orntoft,T., Lukas,J., and Bartek,J. (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature,* **434,** 864-870.

23. Bartoletti,R., Cai,T., Gacci,M., Giubilei,G., Viggiani,F., Santelli,G., Repetti,F., Nerozzi,S., Ghezzi,P., and Sisani,M. (2005a). Intravesical gemcitabine therapy for superficial transitional cell carcinoma: results of a Phase II prospective multicenter study. *Urology,* **66,** 726-731.

24. Bartoletti,R., Cai,T., Nesi,G., Sardi,I., and Rizzo,M. (2005b). Qualitative and quantitative analysis of angiogenetic factors in transitional cell bladder carcinoma: relationship with clinical course at 10 years follow-up. *Oncol. Rep.,* **14,** 251-255.

25. Baselga,J. (2001). The EGFR as a target for anticancer therapy--focus on cetuximab. *Eur. J. Cancer,* **37 Suppl 4,** S16-S22.

26. Benistant,C., Chapuis,H., and Roche,S. (2000). A specific function for phosphatidylinositol 3-kinase alpha (p85alpha-p110alpha) in cell survival and for phosphatidylinositol 3-kinase beta (p85alpha-p110beta) in de novo DNA synthesis of human colon carcinoma cells. *Oncogene,* **19,** 5083-5090.

27. Bernardini,S., Adessi,G.L., Chezy,E., Billerey,C., Carbillet,J.P., and Bittard,H. (2001). Influence of cigarette smoking on P53 gene mutations in bladder carcinomas. *Anticancer Res.,* **21,** 3001-3004.

28. Besson,A., Gurian-West,M., Schmidt,A., Hall,A., and Roberts,J.M. (2004). p27Kip1 modulates cell migration through the regulation of RhoA activation. *Genes Dev.,* **18,** 862-876.

29. Bianco,F.J., Jr., Justa,D., Grignon,D.J., Sakr,W.A., Pontes,J.E., and Wood,D.P., Jr. (2004). Management of clinical T1 bladder transitional cell carcinoma by radical cystectomy. *Urol. Oncol.,* **22,** 290-294.

30. Bignell,G.R., Huang,J., Greshock,J., Watt,S., Butler,A., West,S., Grigorova,M., Jones,K.W., Wei,W., Stratton,M.R., Futreal,P.A., Weber,B., Shapero,M.H., and Wooster,R. (2004). High-resolution analysis of DNA copy number using oligonucleotide microarrays. *Genome Res.,* **14,** 287-295.

31. Billerey,C., Chopin,D., Aubriot-Lorton,M.H., Ricol,D., Gil Diez,d.M., Van Rhijn,B., Bralet,M.P., Lefrere-Belda,M.A., Lahaye,J.B., Abbou,C.C., Bonaventure,J., Zafrani,E.S., van der,K.T., Thiery,J.P., and Radvanyi,F. (2001). Frequent FGFR3 mutations in papillary non-invasive bladder (pTa) tumors. *Am. J. Pathol.,* **158,** 1955-1959.

32. Birder,L.A. (2006). Urinary bladder urothelium: Molecular sensors of chemical/thermal/mechanical stimuli. *Vascul. Pharmacol.*.

33. Birkenhake,S., Martus,P., Kuhn,R., Schrott,K.M., and Sauer,R. (1998). Radiotherapy alone or radiochemotherapy with platin derivatives following transurethral resection of the bladder. Organ preservation and survival after treatment of bladder cancer. *Strahlenther. Onkol.,* **174,** 121-127.

34. Blanco,D.A., Ruibal,M.M., Suarez,P.G., Fernandez,R.E., Gomez,V.F., Alvarez,C.L., Chantada,A., V, and Gonzalez,M.M. (2003). [Staging of infiltrating bladder cancer. Role of C.T. scan]. *Arch. Esp. Urol.,* **56,** 23-29.

35. Blaveri,E., Brewer,J.L., Roydasgupta,R., Fridlyand,J., DeVries,S., Koppie,T., Pejavar,S., Mehta,K., Carroll,P., Simko,J.P., and Waldman,F.M. (2005). Bladder cancer stage and outcome by array-based comparative genomic hybridization. *Clin. Cancer Res.,* **11,** 7012-7022.

36. Blehm,K.N., Spiess,P.E., Bondaruk,J.E., Dujka,M.E., Villares,G.J., Zhao,Y.J., Bogler,O., Aldape,K.D., Grossman,H.B., Adam,L., McConkey,D.J., Czerniak,B.A., Dinney,C.P., and Bar-Eli,M. (2006). Mutations within the kinase domain and truncations of the epidermal growth factor receptor are rare events in bladder cancer: implications for therapy. *Clin. Cancer Res.,* **12,** 4671-4677.

37. Bochner,B.H., Cote,R.J., Weidner,N., Groshen,S., Chen,S.C., Skinner,D.G., and Nichols,P.W. (1995). Angiogenesis in bladder cancer: relationship between microvessel density and tumor prognosis. *J. Natl. Cancer Inst.,* **87,** 1603-1612.

38. Bohm,M., Kleine-Besten,R., and Wieland,I. (2000). Loss of heterozygosity analysis on chromosome 5p defines 5p13-12 as the critical region involved in tumor progression of bladder carcinomas. *Int. J. Cancer,* **89,** 194-197.

39. Bohm,M., Wieland,I., Schmidt,C., Rubben,H., and Allhoff,E.P. (2002). Loss of heterozygosity on chromosome 5p13-12 predicts adverse prognosis in advanced bladder cancer independent of tumor stage and grade. *J. Urol.,* **168,** 2655-2658.

40. Bond,G.L., Hu,W., and Levine,A. (2005a). A single nucleotide polymorphism in the MDM2 gene: from a molecular and cellular explanation to clinical effect. *Cancer Res.,* **65,** 5481-5484.

41. Bond,G.L., Hu,W., and Levine,A.J. (2005b). MDM2 is a central node in the p53 pathway: 12 years and counting. *Curr. Cancer Drug Targets.,* **5,** 3-8.

42. Borden,L.S., Jr., Clark,P.E., and Hall,M.C. (2003). Bladder cancer. *Curr. Opin. Oncol.,* **15,** 227-233.

43. Botteman,M.F., Pashos,C.L., Redaelli,A., Laskin,B., and Hauser,R. (2003). The health economics of bladder cancer: a comprehensive review of the published literature. *Pharmacoeconomics.,* **21,** 1315-1330.

44. Brennan,C., Zhang,Y., Leo,C., Feng,B., Cauwels,C., Aguirre,A.J., Kim,M., Protopopov,A., and Chin,L. (2004). High-resolution global profiling of genomic alterations with long oligonucleotide microarray. *Cancer Res.,* **64,** 4744-4748.

45. Brennan,P., Bogillot,O., Cordier,S., Greiser,E., Schill,W., Vineis,P., Lopez-Abente,G., Tzonou,A., Chang-Claude,J., Bolm-Audorff,U., Jockel,K.H., Donato,F., Serra,C., Wahrendorf,J., Hours,M., T'Mannetje,A., Kogevinas,M., and Boffetta,P. (2000). Cigarette smoking and bladder cancer in men: a pooled analysis of 11 case-control studies. *Int. J. Cancer,* **86,** 289-294.

46. Brockmoller,J., Kaiser,R., Kerb,R., Cascorbi,I., Jaeger,V., and Roots,I. (1996). Polymorphic enzymes of xenobiotic metabolism as modulators of acquired P53 mutations in bladder cancer. *Pharmacogenetics,* **6,** 535-545.

47. Broderick,D.K., Di,C., Parrett,T.J., Samuels,Y.R., Cummins,J.M., McLendon,R.E., Fults,D.W., Velculescu,V.E., Bigner,D.D., and Yan,H. (2004).

Mutations of PIK3CA in anaplastic oligodendrogliomas, high-grade astrocytomas, and medulloblastomas. *Cancer Res.,* **64,** 5048-5050.

48. Bruch,J., Schulz,W.A., Haussler,J., Melzner,I., Bruderlein,S., Moller,P., Kemmerling,R., Vogel,W., and Hameister,H. (2000). Delineation of the 6p22 amplification unit in urinary bladder carcinoma cell lines. *Cancer Res.,* **60,** 4526-4530.

49. Bubendorf,L., Grilli,B., Sauter,G., Mihatsch,M.J., Gasser,T.C., and Dalquen,P. (2001). Multiprobe FISH for enhanced detection of bladder cancer in voided urine specimens and bladder washings. *Am. J. Clin. Pathol.,* **116,** 79-86.

50. Bunz,F., Dutriaux,A., Lengauer,C., Waldman,T., Zhou,S., Brown,J.P., Sedivy,J.M., Kinzler,K.W., and Vogelstein,B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science,* **282,** 1497-1501.

51. Bunz,F., Fauth,C., Speicher,M.R., Dutriaux,A., Sedivy,J.M., Kinzler,K.W., Vogelstein,B., and Lengauer,C. (2002). Targeted inactivation of p53 in human cells does not result in aneuploidy. *Cancer Res.,* **62,** 1129-1133.

52. Burger,M., Burger,S.J., Denzinger,S., Wild,P.J., Wieland,W.F., Blaszyk,H., Obermann,E.C., Stoehr,R., and Hartmann,A. (2006). Elevated Microsatellite Instability at Selected Tetranucleotide Repeats does not Correlate with Clinicopathologic Features of Bladder Cancer. *Eur. Urol.*.

53. Buttitta,F., Felicioni,L., Barassi,F., Martella,C., Paolizzi,D., Fresu,G., Salvatore,S., Cuccurullo,F., Mezzetti,A., Campani,D., and Marchetti,A. (2006). PIK3CA mutation and histological type in breast carcinoma: high frequency of mutations in lobular carcinoma. *J. Pathol.,* **208,** 350-355.

54. Cahill,D.P., Lengauer,C., Yu,J., Riggins,G.J., Willson,J.K., Markowitz,S.D., Kinzler,K.W., and Vogelstein,B. (1998). Mutations of mitotic checkpoint genes in human cancers. *Nature,* **392,** 300-303.

55. Cairns,P., Evron,E., Okami,K., Halachmi,N., Esteller,M., Herman,J.G., Bose,S., Wang,S.I., Parsons,R., and Sidransky,D. (1998). Point mutation and homozygous deletion of PTEN/MMAC1 in primary bladder cancers. *Oncogene,* **16,** 3215-3218.

56. Calabro,F. and Sternberg,C.N. (2002). High-risk metastatic urothelial cancer: chances for cure? *Curr. Opin. Urol.,* **12,** 441-448.

57. Caliskan,M., Turkeri,L.N., Mansuroglu,B., Toktas,G., Aksoy,B., Unluer,E., and Akdas,A. (1997). Nuclear accumulation of mutant p53 protein: a possible predictor of failure of intravesical therapy in bladder cancer. *Br. J. Urol.,* **79,** 373-377.

58. Cappellen,D., De Oliveira,C., Ricol,D., de Medina,S., Bourdin,J., Sastre-Garau,X., Chopin,D., Thiery,J.P., and Radvanyi,F. (1999). Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas. *Nat. Genet.,* **23,** 18-20.

59. Cappellen,D., Gil Diez,d.M., Chopin,D., Thiery,J.P., and Radvanyi,F. (1997). Frequent loss of heterozygosity on chromosome 10q in muscle-invasive transitional cell carcinomas of the bladder. *Oncogene,* **14,** 3059-3066.

60. Carreon,T., LeMasters,G.K., Ruder,A.M., and Schulte,P.A. (2006). The genetic and environmental factors involved in benzidine metabolism and bladder carcinogenesis in exposed workers. *Front Biosci.,* **11,** 2889-2902.

61. Carter,S.L., Eklund,A.C., Kohane,I.S., Harris,L.N., and Szallasi,Z. (2006). A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. *Nat. Genet.,* **38,** 1043-1048.

62. CASE,R.A. and HOSKER,M.E. (1954). Tumour of the urinary bladder as an occupational disease in the rubber industry in England and Wales. *Br. J. Prev. Soc. Med.,* **8,** 39-50.

63. Cassidy,L.D., Youk,A.O., and Marsh,G.M. (2003). The Drake Health Registry Study: cause-specific mortality experience of workers potentially exposed to beta-naphthylamine. *Am. J. Ind. Med.,* **44,** 282-290.

64. Catto,J.W., Azzouzi,A.R., Amira,N., Rehman,I., Feeley,K.M., Cross,S.S., Fromont,G., Sibony,M., Hamdy,F.C., Cussenot,O., and Meuth,M. (2003). Distinct patterns of microsatellite instability are seen in tumours of the urinary tract. *Oncogene,* **22,** 8699-8706.

65. Catto,J.W., Azzouzi,A.R., Rehman,I., Feeley,K.M., Cross,S.S., Amira,N., Fromont,G., Sibony,M., Cussenot,O., Meuth,M., and Hamdy,F.C. (2005). Promoter hypermethylation is associated with tumor location, stage, and subsequent progression in transitional cell carcinoma. *J. Clin. Oncol.,* **23,** 2903-2910.

66. Catto,J.W., Meuth,M., and Hamdy,F.C. (2004). Genetic instability and transitional cell carcinoma of the bladder. *BJU. Int.,* **93,** 19-24.

67. Chang,F., Lee,J.T., Navolanic,P.M., Steelman,L.S., Shelton,J.G., Blalock,W.L., Franklin,R.A., and McCubrey,J.A. (2003). Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. *Leukemia,* **17,** 590-603.

68. Chang,S.S., Cookson,M.S., Baumgartner,R.G., Wells,N., and Smith,J.A., Jr. (2002). Analysis of early complications after radical cystectomy: results of a collaborative care pathway. *J. Urol.,* **167,** 2012-2016.

69. Chatterjee,S.J., Datar,R., Youssefzadeh,D., George,B., Goebell,P.J., Stein,J.P., Young,L., Shi,S.R., Gee,C., Groshen,S., Skinner,D.G., and Cote,R.J. (2004a). Combined effects of p53, p21, and pRb expression in the progression of bladder transitional cell carcinoma. *J. Clin. Oncol.,* **22,** 1007-1013.

70. Chatterjee,S.J., George,B., Goebell,P.J., Alavi-Tafreshi,M., Shi,S.R., Fung,Y.K., Jones,P.A., Cordon-Cardo,C., Datar,R.H., and Cote,R.J. (2004b).

Hyperphosphorylation of pRb: a mechanism for RB tumour suppressor pathway inactivation in bladder cancer. *J. Pathol.,* **203,** 762-770.

71. Chaudhary,R., Bromley,M., Clarke,N.W., Betts,C.D., Barnard,R.J., Ryder,W.D., and Kumar,S. (1999). Prognostic relevance of micro-vessel density in cancer of the urinary bladder. *Anticancer Res.,* **19,** 3479-3484.

72. Chen,D., Kon,N., Li,M., Zhang,W., Qin,J., and Gu,W. (2005). ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. *Cell,* **121,** 1071-1083.

73. Chen,J., Fujii,K., Zhang,L., Roberts,T., and Fu,H. (2001). Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 through a MEK-ERK independent mechanism. *Proc. Natl. Acad. Sci. U. S. A,* **98,** 7783-7788.

74. Chen,Y.C., Su,H.J., Guo,Y.L., Hsueh,Y.M., Smith,T.J., Ryan,L.M., Lee,M.S., and Christiani,D.C. (2003). Arsenic methylation and bladder cancer risk in Taiwan. *Cancer Causes Control,* **14,** 303-310.

75. Chene,P. (2003). Inhibiting the p53-MDM2 interaction: an important target for cancer therapy. *Nat. Rev. Cancer,* **3,** 102-109.

76. Cheng,L., Neumann,R.M., Scherer,B.G., Weaver,A.L., Leibovich,B.C., Nehra,A., Zincke,H., and Bostwick,D.G. (1999a). Tumor size predicts the survival of patients with pathologic stage T2 bladder carcinoma: a critical evaluation of the depth of muscle invasion. *Cancer,* **85,** 2638-2647.

77. Cheng,L., Neumann,R.M., Weaver,A.L., Spotts,B.E., and Bostwick,D.G. (1999b). Predicting cancer progression in patients with stage T1 bladder carcinoma. *J. Clin. Oncol.,* **17,** 3182-3187.

78. Chipuk,J.E., Kuwana,T., Bouchier-Hayes,L., Droin,N.M., Newmeyer,D.D., Schuler,M., and Green,D.R. (2004). Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science,* **303,** 1010-1014.

79. Cho,J.Y., Guo,C., Torello,M., Lunstrum,G.P., Iwata,T., Deng,C., and Horton,W.A. (2004). Defective lysosomal targeting of activated fibroblast growth factor receptor 3 in achondroplasia. *Proc. Natl. Acad. Sci. U. S. A,* **101,** 609-614.

80. Choi,C., Kim,M.H., Juhng,S.W., and Oh,B.R. (2000b). Loss of heterozygosity at chromosome segments 8p22 and 8p11.2-21.1 in transitional-cell carcinoma of the urinary bladder. *Int. J. Cancer,* **86,** 501-505.

81. Choi,C., Kim,M.H., Juhng,S.W., and Oh,B.R. (2000a). Loss of heterozygosity at chromosome segments 8p22 and 8p11.2-21.1 in transitional-cell carcinoma of the urinary bladder. *Int. J. Cancer,* **86,** 501-505.

82. Chopin,D.K. and Gattegno,B. (2002). Superficial bladder tumors. *Eur. Urol.,* **42,** 533-541.

83. Chow,N.H., Chan,S.H., Tzai,T.S., Ho,C.L., and Liu,H.S. (2001). Expression profiles of ErbB family receptors and prognosis in primary transitional cell carcinoma of the urinary bladder. *Clin. Cancer Res.,* **7,** 1957-1962.

84. Christensen,M., Jensen,M.A., Wolf,H., and Orntoft,T.F. (1998). Pronounced microsatellite instability in transitional cell carcinomas from young patients with bladder cancer. *Int. J. Cancer,* **79,** 396-401.

85. Christophorou,M.A., Ringshausen,I., Finch,A.J., Swigart,L.B., and Evan,G.I. (2006). The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. *Nature,* **443,** 214-217.

86. Ciardiello,F. and Tortora,G. (2002). Anti-epidermal growth factor receptor drugs in cancer therapy. *Expert. Opin. Investig. Drugs,* **11,** 755-768.

87. Citri,A. and Yarden,Y. (2006). EGF-ERBB signalling: towards the systems level. *Nat. Rev. Mol. Cell Biol.,* **7,** 505-516.

88. Clarke,N.S., Basu,S., Prescott,S., and Puri,R. (2006). Chemo-prevention in superficial bladder cancer using mitomycin C: a survey of the practice patterns of British urologists. *BJU. Int.,* **97,** 716-719.

89. Cohen,L.H., Pieterman,E., van Leeuwen,R.E., Overhand,M., Burm,B.E., van der Marel,G.A., and van Boom,J.H. (2000). Inhibitors of prenylation of Ras and other G-proteins and their application as therapeutics. *Biochem. Pharmacol.,* **60,** 1061-1068.

90. Cole,P. (1971). Coffee-drinking and cancer of the lower urinary tract. *Lancet,* **1,** 1335-1337.

91. Coleman,M.P., Gatta,G., Verdecchia,A., Esteve,J., Sant,M., Storm,H., Allemani,C., Ciccolallo,L., Santaquilani,M., and Berrino,F. (2003). EUROCARE-3 summary: cancer survival in Europe at the end of the 20th century. *Ann. Oncol.,* **14 Suppl 5,** v128-v149.

92. Connor,J.P., Olsson,C.A., Benson,M.C., Rapoport,F., and Sawczuk,I.S. (1989). Long-term follow-up in patients treated with methotrexate, vinblastine, doxorubicin, and cisplatin (M-VAC) for transitional cell carcinoma of urinary bladder: cause for concern. *Urology,* **34,** 353-356.

93. Cookson,M.S., Herr,H.W., Zhang,Z.F., Soloway,S., Sogani,P.C., and Fair,W.R. (1997). The treated natural history of high risk superficial bladder cancer: 15-year outcome. *J. Urol.,* **158,** 62-67.

94. Cordon-Cardo,C., Dalbagni,G., Saez,G.T., Oliva,M.R., Zhang,Z.F., Rosai,J., Reuter,V.E., and Pellicer,A. (1994). p53 mutations in human bladder cancer: genotypic versus phenotypic patterns. *Int. J. Cancer,* **56,** 347-353.

95. Corvera,S. and Czech,M.P. (1998). Direct targets of phosphoinositide 3-kinase products in membrane traffic and signal transduction. *Trends Cell Biol.,* **8,** 442-446.

96. Cote,R.J. and Datar,R.H. (2003). Therapeutic approaches to bladder cancer: identifying targets and mechanisms. *Crit Rev. Oncol. Hematol.,* **46 Suppl,** S67-S83.

97. Cote,R.J., Dunn,M.D., Chatterjee,S.J., Stein,J.P., Shi,S.R., Tran,Q.C., Hu,S.X., Xu,H.J., Groshen,S., Taylor,C.R., Skinner,D.G., and Benedict,W.F. (1998). Elevated and absent pRb expression is associated with bladder cancer progression and has cooperative effects with p53. *Cancer Res.,* **58,** 1090-1094.

98. Cote,R.J., Esrig,D., Groshen,S., Jones,P.A., and Skinner,D.G. (1997). p53 and treatment of bladder cancer. *Nature,* **385,** 123-125.

99. Crump,K.S. and Guess,H.A. (1982). Drinking water and cancer: review of recent epidemiological findings and assessment of risks. *Annu. Rev. Public Health,* **3,** 339-357.

100. Czerniak,B. and Herz F (1995). *Molecular Biology of Common Genito-Urinary Tumors in Cytology of Genito-Urinary Tumors. Ed. L.G. Koss. In Diagnostic Cytology of the Urinary Tract.* Lipincott-Raven, Philadelphia.

101. Czerniak,B., Li,L., Chaturvedi,V., Ro,J.Y., Johnston,D.A., Hodges,S., and Benedict,W.F. (2000). Genetic modeling of human urinary bladder carcinogenesis. *Genes Chromosomes. Cancer,* **27,** 392-402.

102. Dailey,L., Ambrosetti,D., Mansukhani,A., and Basilico,C. (2005). Mechanisms underlying differential responses to FGF signaling. *Cytokine Growth Factor Rev.,* **16,** 233-247.

103. Danaee,H., Nelson,H.H., Karagas,M.R., Schned,A.R., Ashok,T.D., Hirao,T., Perry,A.E., and Kelsey,K.T. (2002). Microsatellite instability at tetranucleotide repeats in skin and bladder cancer. *Oncogene,* **21,** 4894-4899.

104. Davies,H., Bignell,G.R., Cox,C., Stephens,P., Edkins,S., Clegg,S., Teague,J., Woffendin,H., Garnett,M.J., Bottomley,W., Davis,N., Dicks,E., Ewing,R., Floyd,Y., Gray,K., Hall,S., Hawes,R., Hughes,J., Kosmidou,V., Menzies,A., Mould,C., Parker,A., Stevens,C., Watt,S., Hooper,S., Wilson,R., Jayatilake,H., Gusterson,B.A., Cooper,C., Shipley,J., Hargrave,D., Pritchard-Jones,K., Maitland,N., Chenevix-Trench,G., Riggins,G.J., Bigner,D.D., Palmieri,G., Cossu,A., Flanagan,A., Nicholson,A., Ho,J.W., Leung,S.Y., Yuen,S.T., Weber,B.L., Seigler,H.F., Darrow,T.L., Paterson,H., Marais,R., Marshall,C.J., Wooster,R., Stratton,M.R., and Futreal,P.A. (2002). Mutations of the BRAF gene in human cancer. *Nature,* **417,** 949-954.

105. de Vries,A., Flores,E.R., Miranda,B., Hsieh,H.M., van Oostrom,C.T., Sage,J., and Jacks,T. (2002). Targeted point mutations of p53 lead to dominant-negative inhibition of wild-type p53 function. *Proc. Natl. Acad. Sci. U. S. A,* **99,** 2948-2953.

106. DeMatteo,R.P. (2002). The GIST of targeted cancer therapy: a tumor (gastrointestinal stromal tumor), a mutated gene (c-kit), and a molecular inhibitor (STI571). *Ann. Surg. Oncol.,* **9,** 831-839.

107. Dhawan,D., Hamdy,F.C., Rehman,I., Patterson,J., Cross,S.S., Feeley,K.M., Stephenson,Y., Meuth,M., and Catto,J.W. (2006). Evidence for the early onset of aberrant promoter methylation in urothelial carcinoma. *J. Pathol.,* **209,** 336-343.

108. Dickinson,A.J., Fox,S.B., Persad,R.A., Hollyer,J., Sibley,G.N., and Harris,A.L. (1994). Quantification of angiogenesis as an independent predictor of prognosis in invasive bladder carcinomas. *Br. J. Urol.,* **74,** 762-766.

109. Dominguez-Escrig,J.L., Kelly,J.D., Neal,D.E., King,S.M., and Davies,B.R. (2004). Evaluation of the therapeutic potential of the epidermal growth factor receptor tyrosine kinase inhibitor gefitinib in preclinical models of bladder cancer. *Clin. Cancer Res.,* **10,** 4874-4884.

110. Dornan,D., Bheddah,S., Newton,K., Ince,W., Frantz,G.D., Dowd,P., Koeppen,H., Dixit,V.M., and French,D.M. (2004). COP1, the negative regulator of p53, is overexpressed in breast and ovarian adenocarcinomas. *Cancer Res.,* **64,** 7226-7230.

111. Du,W. and Pogoriler,J. (2006). Retinoblastoma family genes. *Oncogene,* **25,** 5190-5200.

112. Duggan,B.J., Gray,S.B., McKnight,J.J., Watson,C.J., Johnston,S.R., and Williamson,K.E. (2004). Oligoclonality in bladder cancer: the implication for molecular therapies. *J. Urol.,* **171,** 419-425.

113. Dulaimi,E., Uzzo,R.G., Greenberg,R.E., Al Saleem,T., and Cairns,P. (2004). Detection of bladder cancer in urine by a tumor suppressor gene hypermethylation panel. *Clin. Cancer Res.,* **10,** 1887-1893.

114. Ebisuya,M., Kondoh,K., and Nishida,E. (2005). The duration, magnitude and compartmentalization of ERK MAP kinase activity: mechanisms for providing signaling specificity. *J. Cell Sci.,* **118,** 2997-3002.

115. Eble J, Sauter,G., Epstein,J.I., and Sesterhenn,I.A. (2004). *Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs.* IARC Press, Lyon, France.

116. Efeyan,A., Garcia-Cao,I., Herranz,D., Velasco-Miguel,S., and Serrano,M. (2006). Tumour biology: Policing of oncogene activity by p53. *Nature,* **443,** 159.

117. Eisenmann,K.M., VanBrocklin,M.W., Staffend,N.A., Kitchen,S.M., and Koo,H.M. (2003). Mitogen-activated protein kinase pathway-dependent tumor-specific survival signaling in melanoma cells through inactivation of the proapoptotic protein bad. *Cancer Res.,* **63,** 8330-8337.

118. Eissa,S., Ali,H.S., Al Tonsi,A.H., Zaglol,A., and El Ahmady,O. (2005). HER2/neu expression in bladder cancer: relationship to cell cycle kinetics. *Clin. Biochem.,* **38,** 142-148.

119. el Deiry,W.S., Tokino,T., Velculescu,V.E., Levy,D.B., Parsons,R., Trent,J.M., Lin,D., Mercer,W.E., Kinzler,K.W., and Vogelstein,B. (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell,* **75,** 817-825.

120. El Sebaie,M., Zaghloul,M.S., Howard,G., and Mokhtar,A. (2005). Squamous cell carcinoma of the bilharzial and non-bilharzial urinary bladder: a review of etiological features, natural history, and management. *Int. J. Clin. Oncol.,* **10,** 20-25.

121. Endo,K., Sasaki,H., Yano,M., Kobayashi,Y., Yukiue,H., Haneda,H., Suzuki,E., Kawano,O., and Fujii,Y. (2006). Evaluation of the epidermal growth factor receptor gene mutation and copy number in non-small cell lung cancer with gefitinib therapy. *Oncol. Rep.,* **16,** 533-541.

122. Epstein,J.I., Amin,M.B., Reuter,V.R., and Mostofi,F.K. (1998). The World Health Organization/International Society of Urological Pathology consensus classification of urothelial (transitional cell) neoplasms of the urinary bladder. Bladder Consensus Conference Committee. *Am. J. Surg. Pathol.,* **22,** 1435-1448.

123. Erill,N., Colomer,A., Verdu,M., Roman,R., Condom,E., Hannaoui,N., Banus,J.M., Cordon-Cardo,C., and Puig,X. (2004). Genetic and immunophenotype analyses of TP53 in bladder cancer: TP53 alterations are associated with tumor progression. *Diagn. Mol. Pathol.,* **13,** 217-223.

124. Esrig,D., Elmajian,D., Groshen,S., Freeman,J.A., Stein,J.P., Chen,S.C., Nichols,P.W., Skinner,D.G., Jones,P.A., and Cote,R.J. (1994). Accumulation of nuclear p53 and tumor progression in bladder cancer. *N. Engl. J. Med.,* **331,** 1259-1264.

125. Esser,C., Scheffner,M., and Hohfeld,J. (2005). The chaperone-associated ubiquitin ligase CHIP is able to target p53 for proteasomal degradation. *J. Biol. Chem.,* **280,** 27443-27448.

126. Eswarakumar,V.P., Lax,I., and Schlessinger,J. (2005). Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev.,* **16,** 139-149.

127. Evans,A.J., Gallie,B.L., Jewett,M.A., Pond,G.R., Vandezande,K., Underwood,J., Fradet,Y., Lim,G., Marrano,P., Zielenska,M., and Squire,J.A. (2004). Defining a 0.5-mb region of genomic gain on chromosome 6p22 in bladder cancer by quantitative-multiplex polymerase chain reaction. *Am. J. Pathol.,* **164,** 285-293.

128. Feber,A., Clark,J., Goodwin,G., Dodson,A.R., Smith,P.H., Fletcher,A., Edwards,S., Flohr,P., Falconer,A., Roe,T., Kovacs,G., Dennis,N., Fisher,C., Wooster,R., Huddart,R., Foster,C.S., and Cooper,C.S. (2004). Amplification and overexpression of E2F3 in human bladder cancer. *Oncogene,* **23,** 1627-1630.

129. Fei,P., Wang,W., Kim,S.H., Wang,S., Burns,T.F., Sax,J.K., Buzzai,M., Dicker,D.T., McKenna,W.G., Bernhard,E.J., and el Deiry,W.S. (2004). Bnip3L

is induced by p53 under hypoxia, and its knockdown promotes tumor growth. *Cancer Cell,* **6,** 597-609.

130. Felknor,S.A., Delclos,G.L., Lerner,S.P., Burau,K.D., Wood,S.M., Lusk,C.M., and Jalayer,A.D. (2003). Bladder cancer screening program for a petrochemical cohort with potential exposure to beta-napthylamine. *J. Occup. Environ. Med.,* **45,** 289-294.

131. Ferlay,J., Bray F, Pisani,P., and Parkin,D.M. (2004). *GLOBOCAN 2002: Cancer Incidence, Mortality and Prevalence Worldwide IARC CancerBase No. 5 version 2.0.* IARC Press, Lyon.

132. Folkman,J. (1992a). The role of angiogenesis in tumor growth. *Semin. Cancer Biol.,* **3,** 65-71.

133. Folkman,J. (1992b). The role of angiogenesis in tumor growth. *Semin. Cancer Biol.,* **3,** 65-71.

134. Fornari,D., Steven,K., Hansen,A.B., Jepsen,J.V., Poulsen,A.L., Vibits,H., and Horn,T. (2006). Transitional cell bladder tumor: predicting recurrence and progression by analysis of microsatellite loss of heterozygosity in urine sediment and tumor tissue. *Cancer Genet. Cytogenet.,* **167,** 15-19.

135. Frampton,J.E. and Plosker,G.L. (2006). Hexyl aminolevulinate: in the detection of bladder cancer. *Drugs,* **66,** 571-578.

136. Freeman,D.J., Li,A.G., Wei,G., Li,H.H., Kertesz,N., Lesche,R., Whale,A.D., Martinez-Diaz,H., Rozengurt,N., Cardiff,R.D., Liu,X., and Wu,H. (2003). PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms. *Cancer Cell,* **3,** 117-130.

137. Frere-Belda,M.A., Gil Diez,d.M., Daher,A., Martin,N., Albaud,B., Heudes,D., Abbou,C.C., Thiery,J.P., Zafrani,E.S., Radvanyi,F., and Chopin,D. (2004). Profiles of the 2 INK4a gene products, p16 and p14ARF, in human reference urothelium and bladder carcinomas, according to pRb and p53 protein status. *Hum. Pathol.,* **35,** 817-824.

138. Fujiwara,H., Matsunaga,K., Saito,M., Hagiya,S., Furukawa,K., Nakamura,H., and Ohizumi,Y. (2001). Halenaquinone, a novel phosphatidylinositol 3-kinase inhibitor from a marine sponge, induces apoptosis in PC12 cells. *Eur. J. Pharmacol.,* **413,** 37-45.

139. Gago-Dominguez,M., Castelao,J.E., Yuan,J.M., Yu,M.C., and Ross,R.K. (2001). Use of permanent hair dyes and bladder-cancer risk. *Int. J. Cancer,* **91,** 575-579.

140. Galmozzi,F., Rubagotti,A., Romagnoli,A., Carmignani,G., Perdelli,L., Gatteschi,B., and Boccardo,F. (2006). Prognostic value of cell cycle regulatory proteins in muscle-infiltrating bladder cancer. *J. Cancer Res. Clin. Oncol.*.

141. Ganguli,G. and Wasylyk,B. (2003). p53-independent functions of MDM2. *Mol. Cancer Res.,* **1,** 1027-1035.

142. Garcia,d.M., X, Condom,E., Vigues,F., Castellsague,X., Figueras,A., Munoz,J., Sola,J., Soler,T., Capella,G., and Germa,J.R. (2004). p53 and p21 Expression levels predict organ preservation and survival in invasive bladder carcinoma treated with a combined-modality approach. *Cancer,* **100,** 1859-1867.

143. Garcia,d.M., X, Torregrosa,A., Munoz,J., Castellsague,X., Condom,E., Vigues,F., Arance,A., Fabra,A., and Germa,J.R. (2000). Prognostic value of the expression of E-cadherin and beta-catenin in bladder cancer. *Eur. J. Cancer,* **36,** 357-362.

144. Garcia-Closas,M., Malats,N., Silverman,D., Dosemeci,M., Kogevinas,M., Hein,D.W., Tardon,A., Serra,C., Carrato,A., Garcia-Closas,R., Lloreta,J., Castano-Vinyals,G., Yeager,M., Welch,R., Chanock,S., Chatterjee,N., Wacholder,S., Samanic,C., Tora,M., Fernandez,F., Real,F.X., and Rothman,N. (2005). NAT2 slow acetylation, GSTM1 null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses. *Lancet,* **366,** 649-659.

145. Garcia-Rostan,G., Costa,A.M., Pereira-Castro,I., Salvatore,G., Hernandez,R., Hermsem,M.J., Herrero,A., Fusco,A., Cameselle-Teijeiro,J., and Santoro,M. (2005). Mutation of the PIK3CA gene in anaplastic thyroid cancer. *Cancer Res.,* **65,** 10199-10207.

146. Gardiner,R.A., Walsh,M.D., Allen,V., Rahman,S., Samaratunga,M.L., Seymour,G.J., and Lavin,M.F. (1994). Immunohistological expression of p53 in primary pT1 transitional cell bladder cancer in relation to tumour progression. *Br. J. Urol.,* **73,** 526-532.

147. Garnett,M.J. and Marais,R. (2004). Guilty as charged: B-RAF is a human oncogene. *Cancer Cell,* **6,** 313-319.

148. Gazzaniga,P., Gallucci,M., Gradilone,A., Gandini,O., Vincenzoni,A., Gianni,W., Naso,G., Frati,L., and Agliano,A.M. (1995). Detection of BCL-2 RNA in low grade tumours of the urinary bladder. *Eur. J. Cancer,* **31A,** 2119-2120.

149. Geng,L., Tan,J., Himmelfarb,E., Schueneman,A., Niermann,K., Brousal,J., Fu,A., Cuneo,K., Kesicki,E.A., Treiberg,J., Hayflick,J.S., and Hallahan,D.E. (2004). A specific antagonist of the p110delta catalytic component of phosphatidylinositol 3'-kinase, IC486068, enhances radiation-induced tumor vascular destruction. *Cancer Res.,* **64,** 4893-4899.

150. Giacinti,C. and Giordano,A. (2006). RB and cell cycle progression. *Oncogene,* **25,** 5220-5227.

151. Giglio,S., Mancini,F., Gentiletti,F., Sparaco,G., Felicioni,L., Barassi,F., Martella,C., Prodosmo,A., Iacovelli,S., Buttitta,F., Farsetti,A., Soddu,S., Marchetti,A., Sacchi,A., Pontecorvi,A., and Moretti,F. (2005). Identification of an aberrantly spliced form of HDMX in human tumors: a new mechanism for HDM2 stabilization. *Cancer Res.,* **65,** 9687-9694.

152. Glick,S.H., Howell,L.P., and White,R.W. (1996). Relationship of p53 and bcl-2 to prognosis in muscle-invasive transitional cell carcinoma of the bladder. *J. Urol.,* **155,** 1754-1757.

153. Gomez-Roman,J.J., Saenz,P., Molina,M., Cuevas,G.J., Escuredo,K., Santa,C.S., Junquera,C., Simon,L., Martinez,A., Gutierrez Banos,J.L., Lopez-Brea,M., Esparza,C., and Val-Bernal,J.F. (2005). Fibroblast growth factor receptor 3 is overexpressed in urinary tract carcinomas and modulates the neoplastic cell growth. *Clin. Cancer Res.,* **11,** 459-465.

154. Gonzalez,S. and Serrano,M. (2006). A new mechanism of inactivation of the INK4/ARF locus. *Cell Cycle,* **5,** 1382-1384.

155. Gonzalez-Zulueta,M., Ruppert,J.M., Tokino,K., Tsai,Y.C., Spruck,C.H., III, Miyao,N., Nichols,P.W., Hermann,G.G., Horn,T., Steven,K., and . (1993). Microsatellite instability in bladder cancer. *Cancer Res.,* **53,** 5620-5623.

156. Grand,E.K., Chase,A.J., Heath,C., Rahemtulla,A., and Cross,N.C. (2004). Targeting FGFR3 in multiple myeloma: inhibition of t(4;14)-positive cells by SU5402 and PD173074. *Leukemia,* **18,** 962-966.

157. Greshock,J., Naylor,T.L., Margolin,A., Diskin,S., Cleaver,S.H., Futreal,P.A., deJong,P.J., Zhao,S., Liebman,M., and Weber,B.L. (2004). 1-Mb resolution array-based comparative genomic hybridization using a BAC clone set optimized for cancer gene analysis. *Genome Res.,* **14,** 179-187.

158. Gu,J., Liang,D., Wang,Y., Lu,C., and Wu,X. (2005). Effects of N-acetyl transferase 1 and 2 polymorphisms on bladder cancer risk in Caucasians. *Mutat. Res.,* **581,** 97-104.

159. Gudkov,A.V. and Komarova,E.A. (2003). The role of p53 in determining sensitivity to radiotherapy. *Nat. Rev. Cancer,* **3,** 117-129.

160. Guler,G., Uner,A., Guler,N., Han,S.Y., Iliopoulos,D., Hauck,W.W., McCue,P., and Huebner,K. (2004). The fragile genes FHIT and WWOX are inactivated coordinately in invasive breast carcinoma. *Cancer,* **100,** 1605-1614.

161. Hafner,C., Knuechel,R., Stoehr,R., and Hartmann,A. (2002). Clonality of multifocal urothelial carcinomas: 10 years of molecular genetic studies. *Int. J. Cancer,* **101,** 1-6.

162. Hafner,C., van Oers,J.M., Hartmann,A., Landthaler,M., Stoehr,R., Blaszyk,H., Hofstaedter,F., Zwarthoff,E.C., and Vogt,T. (2006a). High Frequency of FGFR3 Mutations in Adenoid Seborrheic Keratoses. *J. Invest Dermatol.*.

163. Hafner,C., van Oers,J.M., Vogt,T., Landthaler,M., Stoehr,R., Blaszyk,H., Hofstaedter,F., Zwarthoff,E.C., and Hartmann,A. (2006b). Mosaicism of activating FGFR3 mutations in human skin causes epidermal nevi. *J. Clin. Invest,* **116,** 2201-2207.

164. Hainaut,P., Olivier,M., and Pfeifer,G.P. (2001). TP53 mutation spectrum in lung cancers and mutagenic signature of components of tobacco smoke: lessons from the IARC TP53 mutation database. *Mutagenesis,* **16,** 551-553.

165. Haliassos,A., Liloglou,T., Likourinas,M., Doumas,C., Ricci,N., and Spandidos,D.A. (1992). H-Ras Oncogene Mutations in the Urine of Patients with Bladder-Tumors - Description of A Noninvasive Method for the Detection of Neoplasia. *International Journal of Oncology,* **1,** 731-734.

166. Hall,P.A. and McCluggage,W.G. (2006). Assessing p53 in clinical contexts: unlearned lessons and new perspectives. *J. Pathol.,* **208,** 1-6.

167. Hamaguchi,M., Meth,J.L., von Klitzing,C., Wei,W., Esposito,D., Rodgers,L., Walsh,T., Welcsh,P., King,M.C., and Wigler,M.H. (2002). DBC2, a candidate for a tumor suppressor gene involved in breast cancer. *Proc. Natl. Acad. Sci. U. S. A,* **99,** 13647-13652.

168. Han,R.F. and Pan,J.G. (2006). Can intravesical bacillus Calmette-Guerin reduce recurrence in patients with superficial bladder cancer? A meta-analysis of randomized trials. *Urology,* **67,** 1216-1223.

169. Hanahan,D. and Weinberg,R.A. (2000). The hallmarks of cancer. *Cell,* **100,** 57-70.

170. Harris,S.L. and Levine,A.J. (2005). The p53 pathway: positive and negative feedback loops. *Oncogene,* **24,** 2899-2908.

171. Hartge,P., Hoover,R., Altman,R., Austin,D.F., Cantor,K.P., Child,M.A., Key,C.R., Mason,T.J., Marrett,L.D., Myers,M.H., Narayana,A.S., Silverman,D.T., Sullivan,J.W., Swanson,G.M., Thomas,D.B., and West,D.W. (1982). Use of hair dyes and risk of bladder cancer. *Cancer Res.,* **42,** 4784-4787.

172. Hartmann,A., Schlake,G., Zaak,D., Hungerhuber,E., Hofstetter,A., Hofstaedter,F., and Knuechel,R. (2002). Occurrence of chromosome 9 and p53 alterations in multifocal dysplasia and carcinoma in situ of human urinary bladder. *Cancer Res.,* **62,** 809-818.

173. Haupt,Y., Robles,A.I., Prives,C., and Rotter,V. (2002). Deconstruction of p53 functions and regulation. *Oncogene,* **21,** 8223-8231.

174. Hein,D.W., Doll,M.A., Fretland,A.J., Leff,M.A., Webb,S.J., Xiao,G.H., Devanaboyina,U.S., Nangju,N.A., and Feng,Y. (2000). Molecular genetics and epidemiology of the NAT1 and NAT2 acetylation polymorphisms. *Cancer Epidemiol. Biomarkers Prev.,* **9,** 29-42.

175. Hemal,A.K., Khaitan,A., Dinda,A.K., Gupta,N.P., Seth,A., Dogra,P.N., and Nabi,G. (2003). Prognostic significance of p53 nuclear overexpression in patients of muscle invasive urinary bladder carcinoma treated with cystectomy. *Urol. Int.,* **70,** 42-46.

176. Hennessy,B.T., Smith,D.L., Ram,P.T., Lu,Y., and Mills,G.B. (2005). Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat. Rev. Drug Discov.,* **4,** 988-1004.

177. Hermann,G.G., Horn,T., and Steven,K. (1998). The influence of the level of lamina propria invasion and the prevalence of p53 nuclear accumulation on survival in stage T1 transitional cell bladder cancer. *J. Urol.,* **159,** 91-94.

178. Hermeking,H., Lengauer,C., Polyak,K., He,T.C., Zhang,L., Thiagalingam,S., Kinzler,K.W., and Vogelstein,B. (1997). 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol. Cell,* **1,** 3-11.

179. Hernandez,S., Lopez-Knowles,E., Lloreta,J., Kogevinas,M., Amoros,A., Tardon,A., Carrato,A., Serra,C., Malats,N., and Real,F.X. (2006). Prospective study of FGFR3 mutations as a prognostic factor in nonmuscle invasive urothelial bladder carcinomas. *J. Clin. Oncol.,* **24,** 3664-3671.

180. Hernandez,S., Lopez-Knowles,E., Lloreta,J., Kogevinas,M., Jaramillo,R., Amoros,A., Tardon,A., Garcia-Closas,R., Serra,C., Carrato,A., Malats,N., and Real,F.X. (2005). FGFR3 and Tp53 mutations in T1G3 transitional bladder carcinomas: independent distribution and lack of association with prognosis. *Clin. Cancer Res.,* **11,** 5444-5450.

181. Herr,H.W. (2000). Tumor progression and survival of patients with high grade, noninvasive papillary (TaG3) bladder tumors: 15-year outcome. *J. Urol.,* **163,** 60-61.

182. Herr,H.W. and Donat,S.M. (2006). A re-staging transurethral resection predicts early progression of superficial bladder cancer. *BJU. Int.,* **97,** 1194-1198.

183. Herr,H.W. and Sogani,P.C. (2001). Does early cystectomy improve the survival of patients with high risk superficial bladder tumors? *J. Urol.,* **166,** 1296-1299.

184. Hinata,N., Shirakawa,T., Zhang,Z., Matsumoto,A., Fujisawa,M., Okada,H., Kamidono,S., and Gotoh,A. (2003). Radiation induces p53-dependent cell apoptosis in bladder cancer cells with wild-type- p53 but not in p53-mutated bladder cancer cells. *Urol. Res.,* **31,** 387-396.

185. Hirao,S., Hirao,T., Marsit,C.J., Hirao,Y., Schned,A., Devi-Ashok,T., Nelson,H.H., Andrew,A., Karagas,M.R., and Kelsey,K.T. (2005). Loss of heterozygosity on chromosome 9q and p53 alterations in human bladder cancer. *Cancer,* **104,** 1918-1923.

186. Hitchings,A.W., Kumar,M., Jordan,S., Nargund,V., Martin,J., and Berney,D.M. (2004). Prediction of progression in pTa and pT1 bladder carcinomas with p53, p16 and pRb. *Br. J. Cancer,* **91,** 552-557.

187. Ho,J.S., Ma,W., Mao,D.Y., and Benchimol,S. (2005). p53-Dependent transcriptional repression of c-myc is required for G1 cell cycle arrest. *Mol. Cell Biol.,* **25,** 7423-7431.

188. Holmang,S., Hedelin,H., Anderstrom,C., Holmberg,E., and Johansson,S.L. (1997). The importance of the depth of invasion in stage T1 bladder carcinoma: a prospective cohort study. *J. Urol.,* **157,** 800-803.

189. Hopenhayn-Rich,C., Biggs,M.L., and Smith,A.H. (1998). Lung and kidney cancer mortality associated with arsenic in drinking water in Cordoba, Argentina. *Int. J. Epidemiol.,* **27,** 561-569.

190. Hopman,A.H., Kamps,M.A., Speel,E.J., Schapers,R.F., Sauter,G., and Ramaekers,F.C. (2002). Identification of chromosome 9 alterations and p53 accumulation in isolated carcinoma in situ of the urinary bladder versus carcinoma in situ associated with carcinoma. *Am. J. Pathol.,* **161,** 1119-1125.

191. Huebner,K. and Croce,C.M. (2003). Cancer and the FRA3B/FHIT fragile locus: it's a HIT. *Br. J. Cancer,* **88,** 1501-1506.

192. Huncharek,M. and Kupelnick,B. (2005). Personal use of hair dyes and the risk of bladder cancer: results of a meta-analysis. *Public Health Rep.,* **120,** 31-38.

193. Hurst,C.D., Fiegler,H., Carr,P., Williams,S., Carter,N.P., and Knowles,M.A. (2004). High-resolution analysis of genomic copy number alterations in bladder cancer by microarray-based comparative genomic hybridization. *Oncogene,* **23,** 2250-2263.

194. Hussain,S.P. and Harris,C.C. (1999). p53 mutation spectrum and load: the generation of hypotheses linking the exposure of endogenous or exogenous carcinogens to human cancer. *Mutat. Res.,* **428,** 23-32.

195. Innocente,S.A., Abrahamson,J.L., Cogswell,J.P., and Lee,J.M. (1999). p53 regulates a G2 checkpoint through cyclin B1. *Proc. Natl. Acad. Sci. U. S. A,* **96,** 2147-2152.

196. Inoue,K., Slaton,J.W., Davis,D.W., Hicklin,D.J., McConkey,D.J., Karashima,T., Radinsky,R., and Dinney,C.P. (2000). Treatment of human metastatic transitional cell carcinoma of the bladder in a murine model with the anti-vascular endothelial growth factor receptor monoclonal antibody DC101 and paclitaxel. *Clin. Cancer Res.,* **6,** 2635-2643.

197. Ioachim,E., Charchanti,A., Stavropoulos,N.E., Skopelitou,A., Athanassiou,E.D., and Agnantis,N.J. (2000). Immunohistochemical expression of retinoblastoma gene product (Rb), p53 protein, MDM2, c-erbB-2, HLA-DR and proliferation indices in human urinary bladder carcinoma. *Histol. Histopathol.,* **15,** 721-727.

198. Ioachim,E., Michael,M.C., Salmas,M., Damala,K., Tsanou,E., Michael,M.M., Malamou-Mitsi,V., and Stavropoulos,N.E. (2006). Thrombospondin-1 expression in urothelial carcinoma: Prognostic significance and association with p53 alterations, tumour angiogenesis and extracellular matrix components. *BMC. Cancer,* **6,** 140.

199. Iovino,F., Lentini,L., Amato,A., and Di Leonardo,A. (2006). RB acute loss induces centrosome amplification and aneuploidy in murine primary fibroblasts. *Mol. Cancer,* **5,** 38.

200. Ishkanian,A.S., Malloff,C.A., Watson,S.K., DeLeeuw,R.J., Chi,B., Coe,B.P., Snijders,A., Albertson,D.G., Pinkel,D., Marra,M.A., Ling,V., MacAulay,C., and Lam,W.L. (2004). A tiling resolution DNA microarray with complete coverage of the human genome. *Nat. Genet.,* **36,** 299-303.

201. Iwakuma,T. and Lozano,G. (2003). MDM2, an introduction. *Mol. Cancer Res.,* **1,** 993-1000.

202. Izawa,J.I., Chin,J.L., and Winquist,E. (2006). Timing cystectomy and perioperative chemotherapy in the treatment of muscle invasive bladder cancer. *Can. J. Urol.,* **13,** 48-53.

203. Jaeger,T.M., Weidner,N., Chew,K., Moore,D.H., Kerschmann,R.L., Waldman,F.M., and Carroll,P.R. (1995). Tumor angiogenesis correlates with lymph node metastases in invasive bladder cancer. *J. Urol.,* **154,** 69-71.

204. Jain,R.K. (2002). Tumor angiogenesis and accessibility: role of vascular endothelial growth factor. *Semin. Oncol.,* **29,** 3-9.

205. Jankevicius,F., Goebell,P., Kushima,M., Schulz,W.A., Ackermann,R., and Schmitz-Drager,B.J. (2002). p21 and p53 Immunostaining and survival following systemic chemotherapy for urothelial cancer. *Urol. Int.,* **69,** 174-180.

206. Janmaat,M.L. and Giaccone,G. (2003). The epidermal growth factor receptor pathway and its inhibition as anticancer therapy. *Drugs Today (Barc.),* **39 Suppl C,** 61-80.

207. Jansen,B., Wacheck,V., Heere-Ress,E., Schlagbauer-Wadl,H., Hoeller,C., Lucas,T., Hoermann,M., Hollenstein,U., Wolff,K., and Pehamberger,H. (2000). Chemosensitisation of malignant melanoma by BCL2 antisense therapy. *Lancet,* **356,** 1728-1733.

208. Jebar,A.H., Hurst,C.D., Tomlinson,D.C., Johnston,C., Taylor,C.F., and Knowles,M.A. (2005). FGFR3 and Ras gene mutations are mutually exclusive genetic events in urothelial cell carcinoma. *Oncogene,* **24,** 5218-5225.

209. Jemal,A., Siegel,R., Ward,E., Murray,T., Xu,J., Smigal,C., and Thun,M.J. (2006). Cancer statistics, 2006. *CA Cancer J. Clin.,* **56,** 106-130.

210. Johansson,S.L. and Cohen,S.M. (1997). Epidemiology and etiology of bladder cancer. *Semin. Surg. Oncol.,* **13,** 291-298.

211. Jones,R.B., Gordus,A., Krall,J.A., and MacBeath,G. (2006). A quantitative protein interaction network for the ErbB receptors using protein microarrays. *Nature,* **439,** 168-174.

212. Jones,T.D., Wang,M., Eble,J.N., MacLennan,G.T., Lopez-Beltran,A., Zhang,S., Cocco,A., and Cheng,L. (2005). Molecular evidence supporting field effect in urothelial carcinogenesis. *Clin. Cancer Res.,* **11,** 6512-6519.

213. Jordan,A.M., Weingarten,J., and Murphy,W.M. (1987). Transitional cell neoplasms of the urinary bladder. Can biologic potential be predicted from histologic grading? *Cancer,* **60,** 2766-2774.

214. Jucker,M., Sudel,K., Horn,S., Sickel,M., Wegner,W., Fiedler,W., and Feldman,R.A. (2002). Expression of a mutated form of the p85alpha regulatory subunit of phosphatidylinositol 3-kinase in a Hodgkin's lymphoma-derived cell line (CO). *Leukemia,* **16,** 894-901.

215. Kahan,E., Ibrahim,A.S., El Najjar,K., Ron,E., Al Agha,H., Polliack,A., and El Bolkainy,M.N. (1997). Cancer patterns in the Middle East--special report from the Middle East Cancer Society. *Acta Oncol.,* **36,** 631-636.

216. Kamai,T., Takagi,K., Asami,H., Ito,Y., Oshima,H., and Yoshida,K.I. (2001). Decreasing of p27(Kip1)and cyclin E protein levels is associated with progression from superficial into invasive bladder cancer. *Br. J. Cancer,* **84,** 1242-1251.

217. Kannio,A., Ridanpaa,M., Koskinen,H., Partanen,T., Anttila,S., Collan,Y., Hietanen,E., Vainio,H., and Husgafvel-Pursiainen,K. (1996). A molecular and epidemiological study on bladder cancer: p53 mutations, tobacco smoking, and occupational exposure to asbestos. *Cancer Epidemiol. Biomarkers Prev.,* **5,** 33-39.

218. Kantor,A.F., Hartge,P., Hoover,R.N., and Fraumeni,J.F., Jr. (1985). Familial and environmental interactions in bladder cancer risk. *Int. J. Cancer,* **35,** 703-706.

219. Karakas,B., Bachman,K.E., and Park,B.H. (2006). Mutation of the PIK3CA oncogene in human cancers. *Br. J. Cancer,* **94,** 455-459.

220. Katayama,H., Sasai,K., Kawai,H., Yuan,Z.M., Bondaruk,J., Suzuki,F., Fujii,S., Arlinghaus,R.B., Czerniak,B.A., and Sen,S. (2004). Phosphorylation by aurora kinase A induces Mdm2-mediated destabilization and inhibition of p53. *Nat. Genet.,* **36,** 55-62.

221. Kawamoto,K., Enokida,H., Gotanda,T., Kubo,H., Nishiyama,K., Kawahara,M., and Nakagawa,M. (2006). p16INK4a and p14ARF methylation as a potential biomarker for human bladder cancer. *Biochem. Biophys. Res. Commun.,* **339,** 790-796.

222. Kawamura,K., Izumi,H., Ma,Z., Ikeda,R., Moriyama,M., Tanaka,T., Nojima,T., Levin,L.S., Fujikawa-Yamamoto,K., Suzuki,K., and Fukasawa,K. (2004). Induction of centrosome amplification and chromosome instability in human bladder cancer cells by p53 mutation and cyclin E overexpression. *Cancer Res.,* **64,** 4800-4809.

223. Kelsey,K.T., Hirao,T., Hirao,S., Devi-Ashok,T., Nelson,H.H., Andrew,A., Colt,J., Baris,D., Morris,J.S., Schned,A., and Karagas,M. (2005). TP53 alterations and patterns of carcinogen exposure in a U.S. population-based study of bladder cancer. *Int. J. Cancer,* **117,** 370-375.

224. Kelsey,K.T., Hirao,T., Schned,A., Hirao,S., Devi-Ashok,T., Nelson,H.H., Andrew,A., and Karagas,M.R. (2004). A population-based study of immunohistochemical detection of p53 alteration in bladder cancer. *Br. J. Cancer,* **90,** 1572-1576.

225. Kielb,S.J., Shah,N.L., Rubin,M.A., and Sanda,M.G. (2001). Functional p53 mutation as a molecular determinant of paclitaxel and gemcitabine susceptibility in human bladder cancer. *J. Urol.,* **166,** 482-487.

226. Kiemeney,L.A., Moret,N.C., Witjes,J.A., Schoenberg,M.P., and Tulinius,H. (1997). Familial transitional cell carcinoma among the population of Iceland. *J. Urol.,* **157,** 1649-1651.

227. Kiemeney,L.A. and Schoenberg,M. (1996). Familial transitional cell carcinoma. *J. Urol.,* **156,** 867-872.

228. Kim,J.H., Tuziak,T., Hu,L., Wang,Z., Bondaruk,J., Kim,M., Fuller,G., Dinney,C., Grossman,H.B., Baggerly,K., Zhang,W., and Czerniak,B. (2005). Alterations in transcription clusters underlie development of bladder cancer along papillary and nonpapillary pathways. *Lab Invest,* **85,** 532-549.

229. Kim,S.I., Kwon,S.M., Kim,Y.S., and Hong,S.J. (2002). Association of cyclooxygenase-2 expression with prognosis of stage T1 grade 3 bladder cancer. *Urology,* **60,** 816-821.

230. Kimura,T., Suzuki,H., Ohashi,T., Asano,K., Kiyota,H., and Eto,Y. (2001). The incidence of thanatophoric dysplasia mutations in FGFR3 gene is higher in low-grade or superficial bladder carcinomas. *Cancer,* **92,** 2555-2561.

231. Kirkali,Z., Chan,T., Manoharan,M., Algaba,F., Busch,C., Cheng,L., Kiemeney,L., Kriegmair,M., Montironi,R., Murphy,W.M., Sesterhenn,I.A., Tachibana,M., and Weider,J. (2005). Bladder cancer: epidemiology, staging and grading, and diagnosis. *Urology,* **66,** 4-34.

232. Knowles,M.A., Aveyard,J.S., Taylor,C.F., Harnden,P., and Bass,S. (2005). Mutation analysis of the 8p candidate tumour suppressor genes DBC2 (RHOBTB2) and LZTS1 in bladder cancer. *Cancer Lett.,* **225,** 121-130.

233. Knowles,M.A., Habuchi,T., Kennedy,W., and Cuthbert-Heavens,D. (2003). Mutation spectrum of the 9q34 tuberous sclerosis gene TSC1 in transitional cell carcinoma of the bladder. *Cancer Res.,* **63,** 7652-7656.

234. Koed,K., Wiuf,C., Christensen,L.L., Wikman,F.P., Zieger,K., Moller,K., von der,M.H., and Orntoft,T.F. (2005). High-density single nucleotide polymorphism array defines novel stage and location-dependent allelic imbalances in human bladder tumors. *Cancer Res.,* **65,** 34-45.

235. Kogevinas,M., Fernandez,F., Garcia-Closas,M., Tardon,A., Garcia-Closas,R., Serra,C., Carrato,A., Castano-Vinyals,G., Yeager,M., Chanock,S.J., Lloreta,J., Rothman,N., Real,F.X., Dosemeci,M., Malats,N., and Silverman,D. (2006). Hair dye use is not associated with risk for bladder cancer: Evidence from a case-control study in Spain. *Eur. J. Cancer,* **42,** 1448-1454.

236. Kogevinas,M., Sala,M., Boffetta,P., Kazerouni,N., Kromhout,H., and Hoar-Zahm,S. (1998). Cancer risk in the rubber industry: a review of the recent epidemiological evidence. *Occup. Environ. Med.,* **55,** 1-12.

237. Koksal,I.T., Yasar,D., Dirice,E., Usta,M.F., Karauzum,S., Luleci,G., Baykara,M., and Sanlioglu,S. (2005). Differential PTEN protein expression profiles in superficial versus invasive bladder cancers. *Urol. Int.,* **75,** 102-106.

238. Kondoh,K., Torii,S., and Nishida,E. (2005). Control of MAP kinase signaling to the nucleus. *Chromosoma,* **114,** 86-91.

239. Konety,B.R. (2006). Molecular markers in bladder cancer: a critical appraisal. *Urol. Oncol.,* **24,** 326-337.

240. Konety,B.R., Allareddy,V., and Herr,H. (2006). Complications after radical cystectomy: analysis of population-based data. *Urology*.

241. Koo,S.H., Kwon,K.C., Ihm,C.H., Jeon,Y.M., Park,J.W., and Sul,C.K. (1999). Detection of genetic alterations in bladder tumors by comparative genomic hybridization and cytogenetic analysis. *Cancer Genet. Cytogenet.,* **110,** 87-93.

242. Kramer,A.A., Graham,S., Burnett,W.S., and Nasca,P. (1991). Familial aggregation of bladder cancer stratified by smoking status. *Epidemiology,* **2,** 145-148.

243. Kruger,S., Mahnken,A., Kausch,I., and Feller,A.C. (2005). P16 immunoreactivity is an independent predictor of tumor progression in minimally invasive urothelial bladder carcinoma. *Eur. Urol.,* **47,** 463-467.

244. Kruger,S., Weitsch,G., Buttner,H., Matthiensen,A., Bohmer,T., Marquardt,T., Sayk,F., Feller,A.C., and Bohle,A. (2002). Overexpression of c-erbB-2 oncoprotein in muscle-invasive bladder carcinoma: relationship with gene amplification, clinicopathological parameters and prognostic outcome. *Int. J. Oncol.,* **21,** 981-987.

245. Ku,J.H., Kwak,C., Lee,H.S., Park,H.K., Lee,E., and Lee,S.E. (2004). Expression of survivin, a novel inhibitor of apoptosis, in superficial transitional cell carcinoma of the bladder. *J. Urol.,* **171,** 631-635.

246. Kunze,E., Chang-Claude,J., and Frentzel-Beyme,R. (1992). Life style and occupational risk factors for bladder cancer in Germany. A case-control study. *Cancer,* **69,** 1776-1790.

247. Kwiatkowski,D.J. and Manning,B.D. (2005). Tuberous sclerosis: a GAP at the crossroads of multiple signaling pathways. *Hum. Mol. Genet.,* **14 Spec No. 2,** R251-R258.

248. Lacombe,L., Dalbagni,G., Zhang,Z.F., Cordon-Cardo,C., Fair,W.R., Herr,H.W., and Reuter,V.E. (1996). Overexpression of p53 protein in a high-risk population of patients with superficial bladder cancer before and after bacillus Calmette-Guerin therapy: correlation to clinical outcome. *J. Clin. Oncol.,* **14,** 2646-2652.

249. Laird,A.D., Vajkoczy,P., Shawver,L.K., Thurnher,A., Liang,C., Mohammadi,M., Schlessinger,J., Ullrich,A., Hubbard,S.R., Blake,R.A., Fong,T.A., Strawn,L.M., Sun,L., Tang,C., Hawtin,R., Tang,F., Shenoy,N., Hirth,K.P., McMahon,G., and Cherrington (2000). SU6668 is a potent antiangiogenic and antitumor agent that induces regression of established tumors. *Cancer Res.,* **60,** 4152-4160.

250. Lang,G.A., Iwakuma,T., Suh,Y.A., Liu,G., Rao,V.A., Parant,J.M., Valentin-Vega,Y.A., Terzian,T., Caldwell,L.C., Strong,L.C., El Naggar,A.K., and Lozano,G. (2004). Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. *Cell,* **119,** 861-872.

251. Lebret,T., Bohin,D., Kassardjian,Z., Herve,J.M., Molinie,V., Barre,P., Lugagne,P.M., and Botto,H. (2000). Recurrence, progression and success in stage Ta grade 3 bladder tumors treated with low dose bacillus Calmette-Guerin instillations. *J. Urol.,* **163,** 63-67.

252. Lee,C.C., Yamamoto,S., Morimura,K., Wanibuchi,H., Nishisaka,N., Ikemoto,S., Nakatani,T., Wada,S., Kishimoto,T., and Fukushima,S. (1997a). Significance of cyclin D1 overexpression in transitional cell carcinomas of the urinary bladder and its correlation with histopathologic features. *Cancer,* **79,** 780-789.

253. Lee,C.C., Yamamoto,S., Wanibuchi,H., Wada,S., Sugimura,K., Kishimoto,T., and Fukushima,S. (1997b). Cyclin D1 overexpression in rat two-stage bladder carcinogenesis and its relationship with oncogenes, tumor suppressor genes, and cell proliferation. *Cancer Res.,* **57,** 4765-4776.

254. Lee,E., Park,I., and Lee,C. (1997c). Prognostic markers of intravesical bacillus Calmette-Guerin therapy for multiple, high-grade, stage T1 bladder cancers. *Int. J. Urol.,* **4,** 552-556.

255. Lee,J.W., Soung,Y.H., Kim,S.Y., Lee,H.W., Park,W.S., Nam,S.W., Kim,S.H., Lee,J.Y., Yoo,N.J., and Lee,S.H. (2005). PIK3CA gene is frequently mutated in breast carcinomas and hepatocellular carcinomas. *Oncogene,* **24,** 1477-1480.

256. Lee,S.H., Lee,J.W., Soung,Y.H., Kim,S.Y., Nam,S.W., Park,W.S., Kim,S.H., Yoo,N.J., and Lee,J.Y. (2004). Colorectal tumors frequently express phosphorylated mitogen-activated protein kinase. *APMIS,* **112,** 233-238.

257. Legeai-Mallet,L., Benoist-Lasselin,C., Munnich,A., and Bonaventure,J. (2004). Overexpression of FGFR3, Stat1, Stat5 and p21Cip1 correlates with

phenotypic severity and defective chondrocyte differentiation in FGFR3-related chondrodysplasias. *Bone,* **34,** 26-36.

258. Leng,R.P., Lin,Y., Ma,W., Wu,H., Lemmers,B., Chung,S., Parant,J.M., Lozano,G., Hakem,R., and Benchimol,S. (2003). Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. *Cell,* **112,** 779-791.

259. Lenz,D.L. and Harpster,L.E. (2003). Urothelial carcinoma in a man with hereditary nonpolyposis colon cancer. *Rev. Urol.,* **5,** 49-53.

260. Leonardo,C., Merola,R., Orlandi,G., Leonardo,F., Rondoni,M., and De Nunzio,C. (2005). C-erb-2 gene amplification and chromosomal anomalies in bladder cancer: preliminary results. *J. Exp. Clin. Cancer Res.,* **24,** 633-638.

261. Lerner,S.P. and Skinner,D.G. (2000). *Radical cystectomy for bladder cancer. In Vogelzang NJ, Scardino PT, Shipley WU, et al, Genitourinary Oncology, 2nd edition.* Lippincott Williams & Wilkins, Philadelphia.

262. Levrero,M., De,L., V, Costanzo,A., Gong,J., Wang,J.Y., and Melino,G. (2000). The p53/p63/p73 family of transcription factors: overlapping and distinct functions. *J. Cell Sci.,* **113 (Pt 10),** 1661-1670.

263. Lewis,S.A. (2000). Everything you wanted to know about the bladder epithelium but were afraid to ask. *Am. J. Physiol Renal Physiol,* **278,** F867-F874.

264. Li,A.G., Piluso,L.G., Cai,X., Wei,G., Sellers,W.R., and Liu,X. (2006a). Mechanistic insights into maintenance of high p53 acetylation by PTEN. *Mol. Cell,* **23,** 575-587.

265. Li,G., Yang,T., Li,L., Yan,J., Zeng,Y., Yu,J., and Zhang,Y. (2004). Cyclooxygenase-2 parallels invasive depth and increased MVD in transitional cell carcinoma. *Colloids Surf. B Biointerfaces.,* **37,** 15-19.

266. Li,S.Y., Rong,M., Grieu,F., and Iacopetta,B. (2006b). PIK3CA mutations in breast cancer are associated with poor outcome. *Breast Cancer Res. Treat.,* **96,** 91-95.

267. Liang,S.H. and Clarke,M.F. (2001). Regulation of p53 localization. *Eur. J. Biochem.,* **268,** 2779-2783.

268. Lichtenstein,P., Holm,N.V., Verkasalo,P.K., Iliadou,A., Kaprio,J., Koskenvuo,M., Pukkala,E., Skytthe,A., and Hemminki,K. (2000). Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. *N. Engl. J. Med.,* **343,** 78-85.

269. Lin,Y., Miyamoto,H., Fujinami,K., Uemura,H., Hosaka,M., Iwasaki,Y., and Kubota,Y. (1996). Telomerase activity in human bladder cancer. *Clin. Cancer Res.,* **2,** 929-932.

270. Liu,B., Wang,Z., Chen,B., Yu,J., Zhang,P., Ding,Q., and Zhang,Y. (2006). Randomized study of single instillation of epirubicin for superficial bladder carcinoma: long-term clinical outcomes. *Cancer Invest,* **24,** 160-163.

271. Liu,G. and Chen,X. (2006). Regulation of the p53 transcriptional activity. *J. Cell Biochem.,* **97,** 448-458.

272. Liu,J.L., Sheng,X., Hortobagyi,Z.K., Mao,Z., Gallick,G.E., and Yung,W.K. (2005). Nuclear PTEN-mediated growth suppression is independent of Akt down-regulation. *Mol. Cell Biol.,* **25,** 6211-6224.

273. Lockwood,W.W., Chari,R., Chi,B., and Lam,W.L. (2006). Recent advances in array comparative genomic hybridization technologies and their applications in human genetics. *Eur. J. Hum. Genet.,* **14,** 139-148.

274. Loeb,L.A. (1991). Mutator phenotype may be required for multistage carcinogenesis. *Cancer Res.,* **51,** 3075-3079.

275. Logie,A., Dunois-Larde,C., Rosty,C., Levrel,O., Blanche,M., Ribeiro,A., Gasc,J.M., Jorcano,J., Werner,S., Sastre-Garau,X., Thiery,J.P., and Radvanyi,F. (2005). Activating mutations of the tyrosine kinase receptor FGFR3 are associated with benign skin tumors in mice and humans. *Hum. Mol. Genet.,* **14,** 1153-1160.

276. Lokeshwar,V.B., Habuchi,T., Grossman,H.B., Murphy,W.M., Hautmann,S.H., Hemstreet,G.P., III, Bono,A.V., Getzenberg,R.H., Goebell,P., Schmitz-Drager,B.J., Schalken,J.A., Fradet,Y., Marberger,M., Messing,E., and Droller,M.J. (2005). Bladder tumor markers beyond cytology: International Consensus Panel on bladder tumor markers. *Urology,* **66,** 35-63.

277. Lopez-Abente,G., Aragones,N., Ramis,R., Hernandez-Barrera,V., Perez-Gomez,B., Escolar-Pujolar,A., and Pollan,M. (2006). Municipal distribution of bladder cancer mortality in Spain: possible role of mining and industry. *BMC. Public Health,* **6,** 17.

278. Lopez-Abente,G., Gonzalez,C.A., Errezola,M., Escolar,A., Izarzugaza,I., Nebot,M., and Riboli,E. (1991). Tobacco smoke inhalation pattern, tobacco type, and bladder cancer in Spain. *Am. J. Epidemiol.,* **134,** 830-839.

279. Lopez-Beltran,A. and Cheng,L. (2003). Stage pT1 bladder carcinoma: diagnostic criteria, pitfalls and prognostic significance. *Pathology,* **35,** 484-491.

280. Lopez-Beltran,A., Luque,R.J., Alvarez-Kindelan,J., Quintero,A., Merlo,F., Carrasco,J.C., Requena,M.J., and Montironi,R. (2004). Prognostic factors in stage T1 grade 3 bladder cancer survival: the role of G1-S modulators (p53, p21Waf1, p27kip1, Cyclin D1, and Cyclin D3) and proliferation index (ki67-MIB1). *Eur. Urol.,* **45,** 606-612.

281. Lopez-Beltran,A., Requena,M.J., Luque,R.J., Alvarez-Kindelan,J., Quintero,A., Blanca,A.M., Rodriguez,M.E., Siendones,E., and Montironi,R. (2006). Cyclin D3 expression in primary Ta/T1 bladder cancer. *J. Pathol.,* **209,** 106-113.

282. Lorenzo-Romero,J.G., Salinas-Sanchez,A.S., Gimenez-Bachs,J.M., Sanchez-Sanchez,F., Escribano-Martinez,J., Segura-Martin,M., Hernandez-Millan,I.R., and Virseda-Rodriguez,J.A. (2003). Prognostic implications of p53 gene mutations in bladder tumors. *J. Urol.,* **169,** 492-499.

283. Lotan,Y. and Roehrborn,C.G. (2002). Cost-effectiveness of a modified care protocol substituting bladder tumor markers for cystoscopy for the followup of patients with transitional cell carcinoma of the bladder: a decision analytical approach. *J. Urol.,* **167,** 75-79.

284. Lotan,Y., Svatek,R.S., and Sagalowsky,A.I. (2006). Should we screen for bladder cancer in a high-risk population?: a cost per life-year saved analysis. *Cancer*.

285. Luo,J., Manning,B.D., and Cantley,L.C. (2003). Targeting the PI3K-Akt pathway in human cancer: rationale and promise. *Cancer Cell,* **4,** 257-262.

286. Lynch,C.J. and Milner,J. (2006). Loss of one p53 allele results in four-fold reduction of p53 mRNA and protein: a basis for p53 haplo-insufficiency. *Oncogene,* **25,** 3463-3470.

287. Maas,S., Warskulat,U., Steinhoff,C., Mueller,W., Grimm,M.O., Schulz,W.A., and Seifert,H.H. (2004). Decreased Fas expression in advanced-stage bladder cancer is not related to p53 status. *Urology,* **63,** 392-397.

288. Malats,N., Bustos,A., Nascimento,C.M., Fernandez,F., Rivas,M., Puente,D., Kogevinas,M., and Real,F.X. (2005). P53 as a prognostic marker for bladder cancer: a meta-analysis and review. *Lancet Oncol.,* **6,** 678-686.

289. Malavaud,B. (2004). T1G3 bladder tumours: the case for radical cystectomy. *Eur. Urol.,* **45,** 406-410.

290. Mannervik,B. (1985). The isoenzymes of glutathione transferase. *Adv. Enzymol. Relat Areas Mol. Biol.,* **57,** 357-417.

291. Mantripragada,K.K., Buckley,P.G., de Stahl,T.D., and Dumanski,J.P. (2004). Genomic microarrays in the spotlight. *Trends Genet.,* **20,** 87-94.

292. Mao,L., Lee,D.J., Tockman,M.S., Erozan,Y.S., Askin,F., and Sidransky,D. (1994). Microsatellite alterations as clonal markers for the detection of human cancer. *Proc. Natl. Acad. Sci. U. S. A,* **91,** 9871-9875.

293. Marine,J.C. and Jochemsen,A.G. (2005). Mdmx as an essential regulator of p53 activity. *Biochem. Biophys. Res. Commun.,* **331,** 750-760.

294. Marsit,C.J., Karagas,M.R., Andrew,A., Liu,M., Danaee,H., Schned,A.R., Nelson,H.H., and Kelsey,K.T. (2005). Epigenetic inactivation of SFRP genes and TP53 alteration act jointly as markers of invasive bladder cancer. *Cancer Res.,* **65,** 7081-7085.

295. Martone,T., Vineis,P., Malaveille,C., and Terracini,B. (2000). Impact of polymorphisms in xeno(endo)biotic metabolism on pattern and frequency of p53 mutations in bladder cancer. *Mutat. Res.,* **462,** 303-309.

296. Maruyama,R., Toyooka,S., Toyooka,K.O., Harada,K., Virmani,A.K., Zochbauer-Muller,S., Farinas,A.J., Vakar-Lopez,F., Minna,J.D., Sagalowsky,A., Czerniak,B., and Gazdar,A.F. (2001). Aberrant promoter methylation profile of bladder cancer and its relationship to clinicopathological features. *Cancer Res.,* **61,** 8659-8663.

297. Masters,J.R., Vani,U.D., Grigor,K.M., Griffiths,G.O., Crook,A., Parmar,M.K., and Knowles,M.A. (2003). Can p53 staining be used to identify patients with aggressive superficial bladder cancer? *J. Pathol.,* **200,** 74-81.

298. McCubrey,J.A., Steelman,L.S., Abrams,S.L., Lee,J.T., Chang,F., Bertrand,F.E., Navolanic,P.M., Terrian,D.M., Franklin,R.A., D'Assoro,A.B., Salisbury,J.L., Mazzarino,M.C., Stivala,F., and Libra,M. (2006). Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Adv. Enzyme Regul.*.

299. McNeish,I.A., Bell,S.J., and Lemoine,N.R. (2004). Gene therapy progress and prospects: cancer gene therapy using tumour suppressor genes. *Gene Ther.,* **11,** 497-503.

300. McPhillips,F., Mullen,P., Monia,B.P., Ritchie,A.A., Dorr,F.A., Smyth,J.F., and Langdon,S.P. (2001). Association of c-Raf expression with survival and its targeting with antisense oligonucleotides in ovarian cancer. *Br. J. Cancer,* **85,** 1753-1758.

301. Menendez,D., Inga,A., and Resnick,M.A. (2006). The biological impact of the human master regulator p53 can be altered by mutations that change the spectrum and expression of its target genes. *Mol. Cell Biol.,* **26,** 2297-2308.

302. Mhawech,P., Greloz,V., Oppikofer,C., Szalay-Quinodoz,I., and Herrmann,F. (2004). Expression of cell cycle proteins in T1a and T1b urothelial bladder carcinoma and their value in predicting tumor progression. *Cancer,* **100,** 2367-2375.

303. Mhawech-Fauceglia,P., Cheney,R.T., Fischer,G., Beck,A., and Herrmann,F.R. (2006a). FGFR3 and p53 protein expressions in patients with pTa and pT1 urothelial bladder cancer. *Eur. J. Surg. Oncol.,* **32,** 231-237.

304. Mhawech-Fauceglia,P., Cheney,R.T., and Schwaller,J. (2006b). Genetic alterations in urothelial bladder carcinoma: an updated review. *Cancer,* **106,** 1205-1216.

305. Mhawech-Fauceglia,P., Fischer,G., Beck,A., Cheney,R.T., and Herrmann,F.R. (2006c). Raf1, Aurora-A/STK15 and E-cadherin biomarkers expression in patients with pTa/pT1 urothelial bladder carcinoma; a retrospective TMA study of 246 patients with long-term follow-up. *Eur. J. Surg. Oncol.,* **32,** 439-444.

306. Michael,D. and Oren,M. (2003). The p53-Mdm2 module and the ubiquitin system. *Semin. Cancer Biol.,* **13,** 49-58.

307. Miyamoto,H., Shuin,T., Torigoe,S., Iwasaki,Y., and Kubota,Y. (1995). Retinoblastoma gene mutations in primary human bladder cancer. *Br. J. Cancer,* **71,** 831-835.

308. Mizutani,Y., Okada,Y., and Yoshida,O. (1997). Expression of platelet-derived endothelial cell growth factor in bladder carcinoma. *Cancer,* **79,** 1190-1194.

309. Mohammadi,M., McMahon,G., Sun,L., Tang,C., Hirth,P., Yeh,B.K., Hubbard,S.R., and Schlessinger,J. (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science,* **276,** 955-960.

310. Momand,J., Zambetti,G.P., Olson,D.C., George,D., and Levine,A.J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell,* **69,** 1237-1245.

311. Moore,L.E., Smith,A.H., Eng,C., DeVries,S., Kalman,D., Bhargava,V., Chew,K., Ferreccio,C., Rey,O.A., Hopenhayn,C., Biggs,M.L., Bates,M.N., and Waldman,F.M. (2003). P53 alterations in bladder tumors from arsenic and tobacco exposed patients. *Carcinogenesis,* **24,** 1785-1791.

312. Moore,L.E., Smith,A.H., Eng,C., Kalman,D., DeVries,S., Bhargava,V., Chew,K., Moore,D., Ferreccio,C., Rey,O.A., and Waldman,F.M. (2002). Arsenic-related chromosomal alterations in bladder cancer. *J. Natl. Cancer Inst.,* **94,** 1688-1696.

313. Mostofi,F.K., Sobin,L.H., and Torloni H (1973). *Histological Typing of Urinary Bladder Tumors.* World Health Organization, Geneva.

314. Munro,N.P. and Knowles,M.A. (2003). Fibroblast growth factors and their receptors in transitional cell carcinoma. *J. Urol.,* **169,** 675-682.

315. Naito,S., Tanaka,K., Koga,H., Kotoh,S., Hirohata,T., and Kumazawa,J. (1995). Cancer occurrence among dyestuff workers exposed to aromatic amines. A long term follow-up study. *Cancer,* **76,** 1445-1452.

316. Najem,G.R., Louria,D.B., Seebode,J.J., Thind,I.S., Prusakowski,J.M., Ambrose,R.B., and Fernicola,A.R. (1982). Life time occupation, smoking, caffeine, saccharine, hair dyes and bladder carcinogenesis. *Int. J. Epidemiol.,* **11,** 212-217.

317. Nakopoulou,L., Vourlakou,C., Zervas,A., Tzonou,A., Gakiopoulou,H., and Dimopoulos,M.A. (1998). The prevalence of bcl-2, p53, and Ki-67 immunoreactivity in transitional cell bladder carcinomas and their clinicopathologic correlates. *Hum. Pathol.,* **29,** 146-154.

318. Naski,M.C., Wang,Q., Xu,J., and Ornitz,D.M. (1996). Graded activation of fibroblast growth factor receptor 3 by mutations causing achondroplasia and thanatophoric dysplasia. *Nat. Genet.,* **13,** 233-237.

319. Natale,R.B. and Zaretsky,S.L. (2002). ZD1839 (Iressa): what's in it for the patient? *Oncologist.,* **7 Suppl 4,** 25-30.

320. Neal,D.E., Sharples,L., Smith,K., Fennelly,J., Hall,R.R., and Harris,A.L. (1990). The epidermal growth factor receptor and the prognosis of bladder cancer. *Cancer,* **65,** 1619-1625.

321. Nicholson,D.W. (2000). From bench to clinic with apoptosis-based therapeutic agents. *Nature,* **407,** 810-816.

322. Nicolaas Bouwes (EPA WAM) (2006). *The cost of illness handbook. Office of Pollution Prevention and Toxics (EETD, EPAB).* Abt Associates, Cambridge, Massachusetts.

323. Nikolaev,A.Y., Li,M., Puskas,N., Qin,J., and Gu,W. (2003). Parc: a cytoplasmic anchor for p53. *Cell,* **112,** 29-40.

324. Nishizaki,M., Sasaki,J., Fang,B., Atkinson,E.N., Minna,J.D., Roth,J.A., and Ji,L. (2004). Synergistic tumor suppression by coexpression of FHIT and p53 coincides with FHIT-mediated MDM2 inactivation and p53 stabilization in human non-small cell lung cancer cells. *Cancer Res.,* **64,** 5745-5752.

325. Normanno,N., Maiello,M.R., and De Luca,A. (2003). Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs): simple drugs with a complex mechanism of action? *J. Cell Physiol,* **194,** 13-19.

326. Nutt,J.E., Mellon,J.K., Qureshi,K., and Lunec,J. (1998). Matrix metalloproteinase-1 is induced by epidermal growth factor in human bladder tumour cell lines and is detectable in urine of patients with bladder tumours. *Br. J. Cancer,* **78,** 215-220.

327. Nyormoi,O., Wang,Z., Doan,D., Ruiz,M., McConkey,D., and Bar-Eli,M. (2001). Transcription factor AP-2alpha is preferentially cleaved by caspase 6 and degraded by proteasome during tumor necrosis factor alpha-induced apoptosis in breast cancer cells. *Mol. Cell Biol.,* **21,** 4856-4867.

328. Obermann,E.C., Junker,K., Stoehr,R., Dietmaier,W., Zaak,D., Schubert,J., Hofstaedter,F., Knuechel,R., and Hartmann,A. (2003). Frequent genetic alterations in flat urothelial hyperplasias and concomitant papillary bladder cancer as detected by CGH, LOH, and FISH analyses. *J. Pathol.,* **199,** 50-57.

329. Oeggerli,M., Schraml,P., Ruiz,C., Bloch,M., Novotny,H., Mirlacher,M., Sauter,G., and Simon,R. (2006). E2F3 is the main target gene of the 6p22 amplicon with high specificity for human bladder cancer. *Oncogene*.

330. Oeggerli,M., Tomovska,S., Schraml,P., Calvano-Forte,D., Schafroth,S., Simon,R., Gasser,T., Mihatsch,M.J., and Sauter,G. (2004). E2F3 amplification and overexpression is associated with invasive tumor growth and rapid tumor cell proliferation in urinary bladder cancer. *Oncogene,* **23,** 5616-5623.

331. Ohkubo,S., Tanaka,T., Taya,Y., Kitazato,K., and Prives,C. (2006). Excess HDM2 impacts cell cycle and apoptosis and has a selective effect on p53-dependent transcription. *J. Biol. Chem.,* **281,** 16943-16950.

332. Ohta,J.I., Miyoshi,Y., Uemura,H., Fujinami,K., Mikata,K., Hosaka,M., Tokita,Y., and Kubota,Y. (2001). Fluorescence in situ hybridization evaluation of c-erbB-2 gene amplification and chromosomal anomalies in bladder cancer. *Clin. Cancer Res.,* **7,** 2463-2467.

333. Okamura,T., Akita,H., Kawai,N., Tozawa,K., Yamada,Y., and Kohri,K. (1998). Immunohistochemical evaluation of p53, proliferating cell nuclear antigen (PCNA) and bcl-2 expression during bacillus Calmette-Guerin (BCG) intravesical instillation therapy for superficial bladder cancers. *Urol. Res.,* **26,** 161-164.

334. Okumura,A., Mizuno,I., Nagakawa,O., and Fuse,H. (2004). Telomerase activity is correlated with lower grade and lower stage bladder carcinomas. *Int. J. Urol.,* **11,** 1082-1086.

335. Olive,K.P., Tuveson,D.A., Ruhe,Z.C., Yin,B., Willis,N.A., Bronson,R.T., Crowley,D., and Jacks,T. (2004). Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell,* **119,** 847-860.

336. Olivier,M., Eeles,R., Hollstein,M., Khan,M.A., Harris,C.C., and Hainaut,P. (2002). The IARC TP53 database: new online mutation analysis and recommendations to users. *Hum. Mutat.,* **19,** 607-614.

337. Ong,F., Moonen,L.M., Gallee,M.P., ten Bosch,C., Zerp,S.F., Hart,A.A., Bartelink,H., and Verheij,M. (2001). Prognostic factors in transitional cell cancer of the bladder: an emerging role for Bcl-2 and p53. *Radiother. Oncol.,* **61,** 169-175.

338. Oosterlinck,W., Kurth,K.H., Schroder,F., Bultinck,J., Hammond,B., and Sylvester,R. (1993). A prospective European Organization for Research and Treatment of Cancer Genitourinary Group randomized trial comparing transurethral resection followed by a single intravesical instillation of epirubicin or water in single stage Ta, T1 papillary carcinoma of the bladder. *J. Urol.,* **149,** 749-752.

339. Ornitz,D.M., Xu,J., Colvin,J.S., McEwen,D.G., MacArthur,C.A., Coulier,F., Gao,G., and Goldfarb,M. (1996). Receptor specificity of the fibroblast growth factor family. *J. Biol. Chem.,* **271,** 15292-15297.

340. Owen-Schaub,L.B. (2002a). Fas function and tumor progression: use it and lose it. *Cancer Cell,* **2,** 95-96.

341. Owen-Schaub,L.B. (2002b). Fas function and tumor progression: use it and lose it. *Cancer Cell,* **2,** 95-96.

342. Ozer,E., Mungan,M.U., Tuna,B., Kazimoglu,H., Yorukoglu,K., and Kirkali,Z. (1999). Prognostic significance of angiogenesis and immunoreactivity of

cathepsin D and type IV collagen in high-grade stage T1 primary bladder cancer. *Urology,* **54,** 50-55.

343. Pagliaro,L.C. (2000). Gene therapy for bladder cancer. *World J. Urol.,* **18,** 148-151.

344. Paik,M.L., Scolieri,M.J., Brown,S.L., Spirnak,J.P., and Resnick,M.I. (2000). Limitations of computerized tomography in staging invasive bladder cancer before radical cystectomy. *J. Urol.,* **163,** 1693-1696.

345. Pendyala,L., Velagapudi,S., Toth,K., Zdanowicz,J., Glaves,D., Slocum,H., Perez,R., Huben,R., Creaven,P.J., and Raghavan,D. (1997). Translational studies of glutathione in bladder cancer cell lines and human specimens. *Clin. Cancer Res.,* **3,** 793-798.

346. Petrylak,D.P., Scher,H.I., Reuter,V., O'Brien,J.P., and Cordon-Cardo,C. (1994). P-glycoprotein expression in primary and metastatic transitional cell carcinoma of the bladder. *Ann. Oncol.,* **5,** 835-840.

347. Peyromaure,M., Weibing,S., Sebe,P., Verpillat,P., Toublanc,M., Dauge,M.C., Boccon-Gibod,L., and Ravery,V. (2002). Prognostic value of p53 overexpression in T1G3 bladder tumors treated with bacillus Calmette-Guerin therapy. *Urology,* **59,** 409-413.

348. Peyromaure,M. and Zerbib,M. (2004). T1G3 transitional cell carcinoma of the bladder: recurrence, progression and survival. *BJU. Int.,* **93,** 60-63.

349. Pfeifer,G.P., Denissenko,M.F., Olivier,M., Tretyakova,N., Hecht,S.S., and Hainaut,P. (2002). Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. *Oncogene,* **21,** 7435-7451.

350. Phillips,D.H. (2002). Smoking-related DNA and protein adducts in human tissues. *Carcinogenesis,* **23,** 1979-2004.

351. Picchio,M., Treiber,U., Beer,A.J., Metz,S., Bossner,P., van Randenborgh,H., Paul,R., Weirich,G., Souvatzoglou,M., Hartung,R., Schwaiger,M., and Piert,M. (2006). Value of 11C-choline PET and contrast-enhanced CT for staging of bladder cancer: correlation with histopathologic findings. *J. Nucl. Med.,* **47,** 938-944.

352. Pinkel,D., Segraves,R., Sudar,D., Clark,S., Poole,I., Kowbel,D., Collins,C., Kuo,W.L., Chen,C., Zhai,Y., Dairkee,S.H., Ljung,B.M., Gray,J.W., and Albertson,D.G. (1998). High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. *Nat. Genet.,* **20,** 207-211.

353. Piolatto,G., Negri,E., La Vecchia,C., Pira,E., Decarli,A., and Peto,J. (1991). Bladder cancer mortality of workers exposed to aromatic amines: an updated analysis. *Br. J. Cancer,* **63,** 457-459.

354. Pisani,P., Parkin,D.M., Munoz,N., and Ferlay,J. (1997). Cancer and infection: estimates of the attributable fraction in 1990. *Cancer Epidemiol. Biomarkers Prev.,* **6,** 387-400.

355. Planz,B., Jochims,E., Deix,T., Caspers,H.P., Jakse,G., and Boecking,A. (2005). The role of urinary cytology for detection of bladder cancer. *Eur. J. Surg. Oncol.,* **31,** 304-308.

356. Pollack,J.R., Sorlie,T., Perou,C.M., Rees,C.A., Jeffrey,S.S., Lonning,P.E., Tibshirani,R., Botstein,D., Borresen-Dale,A.L., and Brown,P.O. (2002). Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors. *Proc. Natl. Acad. Sci. U. S. A,* **99,** 12963-12968.

357. Polyak,K., Lee,M.H., Erdjument-Bromage,H., Koff,A., Roberts,J.M., Tempst,P., and Massague,J. (1994). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell,* **78,** 59-66.

358. Popov,Z., Gil-Diez,d.M., Lefrere-Belda,M.A., Hoznek,A., Bastuji-Garin,S., Abbou,C.C., Thiery,J.P., Radvanyi,F., and Chopin,D.K. (2000). Low E-cadherin expression in bladder cancer at the transcriptional and protein level provides prognostic information. *Br. J. Cancer,* **83,** 209-214.

359. Potter,C.J., Pedraza,L.G., Huang,H., and Xu,T. (2003). The tuberous sclerosis complex (TSC) pathway and mechanism of size control. *Biochem. Soc. Trans.,* **31,** 584-586.

360. Powers,C.J., McLeskey,S.W., and Wellstein,A. (2000). Fibroblast growth factors, their receptors and signaling. *Endocr. Relat Cancer,* **7,** 165-197.

361. Powis,G., Ihle,N., and Kirkpatrick,D.L. (2006). Practicalities of drugging the phosphatidylinositol-3-kinase/Akt cell survival signaling pathway. *Clin. Cancer Res.,* **12,** 2964-2966.

362. Poyurovsky,M.V. and Prives,C. (2006). Unleashing the power of p53: lessons from mice and men. *Genes Dev.,* **20,** 125-131.

363. Prat,E., Bernues,M., Caballin,M.R., Egozcue,J., Gelabert,A., and Miro,R. (2001). Detection of chromosomal imbalances in papillary bladder tumors by comparative genomic hybridization. *Urology,* **57,** 986-992.

364. Primdahl,H., Wikman,F.P., von der,M.H., Zhou,X.G., Wolf,H., and Orntoft,T.F. (2002). Allelic imbalances in human bladder cancer: genome-wide detection with high-density single-nucleotide polymorphism arrays. *J. Natl. Cancer Inst.,* **94,** 216-223.

365. Priore,R., Dailey,L., and Basilico,C. (2006). Downregulation of Akt activity contributes to the growth arrest induced by FGF in chondrocytes. *J. Cell Physiol,* **207,** 800-808.

366. Prout,G.R., Jr., Wesley,M.N., McCarron,P.G., Chen,V.W., Greenberg,R.S., Mayberry,R.M., and Edwards,B.K. (2004). Survival experience of black patients and white patients with bladder carcinoma. *Cancer,* **100,** 621-630.

367. Pryor,W.A. and Stone,K. (1993). Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. *Ann. N. Y. Acad. Sci.,* **686,** 12-27.

368. Przybojewska,B., Jagiello,A., and Jalmuzna,P. (2000). H-RAS, K-RAS, and N-RAS gene activation in human bladder cancers. *Cancer Genet. Cytogenet.,* **121,** 73-77.

369. Pugh,R.C. (1973). Proceedings: The pathology of cancer of the bladder. *Cancer,* **32,** 1267-1274.

370. Puig,P., Capodieci,P., Drobnjak,M., Verbel,D., Prives,C., Cordon-Cardo,C., and Di Como,C.J. (2003). p73 Expression in human normal and tumor tissues: loss of p73alpha expression is associated with tumor progression in bladder cancer. *Clin. Cancer Res.,* **9,** 5642-5651.

371. Qu,L., Huang,S., Baltzis,D., Rivas-Estilla,A.M., Pluquet,O., Hatzoglou,M., Koumenis,C., Taya,Y., Yoshimura,A., and Koromilas,A.E. (2004). Endoplasmic reticulum stress induces p53 cytoplasmic localization and prevents p53-dependent apoptosis by a pathway involving glycogen synthase kinase-3beta. *Genes Dev.,* **18,** 261-277.

372. Queipo Zaragoza,J.A., Ruiz Cerda,J.L., Palmero,M.L., Rubio Martinez,L.A., Vera,S.F., and Jimenez Cruz,J.F. (2005). [Prognostic value for progression of the regulating proteins of the cellular cycle in PT1G3 bladder tumours]. *Actas Urol. Esp.,* **29,** 261-268.

373. Quek,M.L., Stein,J.P., Clark,P.E., Daneshmand,S., Miranda,G., Cai,J., Groshen,S., Cote,R.J., Lieskovsky,G., Quinn,D.I., and Skinner,D.G. (2004). Microscopic and gross extravesical extension in pathological staging of bladder cancer. *J. Urol.,* **171,** 640-645.

374. Raghavan,D. (2003). Molecular targeting and pharmacogenomics in the management of advanced bladder cancer. *Cancer,* **97,** 2083-2089.

375. Resnick-Silverman,L. and Manfredi,J.J. (2006). Gene-specific mechanisms of p53 transcriptional control and prospects for cancer therapy. *J. Cell Biochem.,* **99,** 679-689.

376. Reuter,V.E. (2006). The pathology of bladder cancer. *Urology,* **67,** 11-17.

377. Reuter,V.E., Epstein,J.I., Amin,M.B., and Mostofi,F.K. (1999). The "WHO/ISUP Consensus Classification of Urothelial (Transitional Cell) Neoplasms": continued discussion. *Hum. Pathol.,* **30,** 879-880.

378. Ribeiro-Filho,L.A., Franks,J., Sasaki,M., Shiina,H., Li,L.C., Nojima,D., Arap,S., Carroll,P., Enokida,H., Nakagawa,M., Yonezawa,S., and Dahiya,R. (2002).

CpG hypermethylation of promoter region and inactivation of E-cadherin gene in human bladder cancer. *Mol. Carcinog.,* **34,** 187-198.

379. Richter,J., Beffa,L., Wagner,U., Schraml,P., Gasser,T.C., Moch,H., Mihatsch,M.J., and Sauter,G. (1998). Patterns of chromosomal imbalances in advanced urinary bladder cancer detected by comparative genomic hybridization. *Am. J. Pathol.,* **153,** 1615-1621.

380. Richter,J., Jiang,F., Gorog,J.P., Sartorius,G., Egenter,C., Gasser,T.C., Moch,H., Mihatsch,M.J., and Sauter,G. (1997). Marked genetic differences between stage pTa and stage pT1 papillary bladder cancer detected by comparative genomic hybridization. *Cancer Res.,* **57,** 2860-2864.

381. Richter,J., Wagner,U., Schraml,P., Maurer,R., Alund,G., Knonagel,H., Moch,H., Mihatsch,M.J., Gasser,T.C., and Sauter,G. (1999). Chromosomal imbalances are associated with a high risk of progression in early invasive (pT1) urinary bladder cancer. *Cancer Res.,* **59,** 5687-5691.

382. Riechmann,V., van,C., I, and Sablitzky,F. (1994b). The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2 and Id3. *Nucleic Acids Res.,* **22,** 749-755.

383. Riechmann,V., van,C., I, and Sablitzky,F. (1994a). The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2 and Id3. *Nucleic Acids Res.,* **22,** 749-755.

384. Rieger-Christ,K.M., Cain,J.W., Braasch,J.W., Dugan,J.M., Silverman,M.L., Bouyounes,B., Libertino,J.A., and Summerhayes,I.C. (2001). Expression of classic cadherins type I in urothelial neoplastic progression. *Hum. Pathol.,* **32,** 18-23.

385. Romih,R., Korosec,P., de,M.W., Jr., and Jezernik,K. (2005). Differentiation of epithelial cells in the urinary tract. *Cell Tissue Res.,* **320,** 259-268.

386. Roos,W.P. and Kaina,B. (2006). DNA damage-induced cell death by apoptosis. *Trends Mol. Med.*.

387. Rosenman,K.D. and Reilly,M.J. (2004). Cancer mortality and incidence among a cohort of benzidine and dichlorobenzidine dye manufacturing workers. *Am. J. Ind. Med.,* **46,** 505-512.

388. Rosner,M., Freilinger,A., and Hengstschlager,M. (2006). The tuberous sclerosis genes and regulation of the cyclin-dependent kinase inhibitor p27. *Mutat. Res.*.

389. Rotterud,R., Berner,A., Holm,R., Skovlund,E., and Fossa,S.D. (2001). p53, p21 and mdm2 expression vs the response to radiotherapy in transitional cell carcinoma of the bladder. *BJU. Int.,* **88,** 202-208.

390. Rotterud,R., Nesland,J.M., Berner,A., and Fossa,S.D. (2005). Expression of the epidermal growth factor receptor family in normal and malignant urothelium. *BJU. Int.,* **95,** 1344-1350.

391. Roz,L., Andriani,F., Ferreira,C.G., Giaccone,G., and Sozzi,G. (2004). The apoptotic pathway triggered by the Fhit protein in lung cancer cell lines is not affected by Bcl-2 or Bcl-x(L) overexpression. *Oncogene,* **23,** 9102-9110.

392. Rubio-Moscardo,F., Blesa,D., Mestre,C., Siebert,R., Balasas,T., Benito,A., Rosenwald,A., Climent,J., Martinez,J.I., Schilhabel,M., Karran,E.L., Gesk,S., Esteller,M., deLeeuw,R., Staudt,L.M., Fernandez-Luna,J.L., Pinkel,D., Dyer,M.J., and Martinez-Climent,J.A. (2005). Characterization of 8p21.3 chromosomal deletions in B-cell lymphoma: TRAIL-R1 and TRAIL-R2 as candidate dosage-dependent tumor suppressor genes. *Blood,* **106,** 3214-3222.

393. Ryk,C., Berggren,P., Kumar,R., Hemminki,K., Larsson,P., Steineck,G., Lambert,B., and Hou,S.M. (2005). Influence of GSTM1, GSTT1, GSTP1 and NAT2 genotypes on the p53 mutational spectrum in bladder tumours. *Int. J. Cancer,* **113,** 761-768.

394. Saint,F., Frere Belda,M.A., Quintela,R., Hoznek,A., Patard,J.J., Bellot,J., Popov,Z., Zafrani,E.S., Abbou,C.C., Chopin,D.K., and de Medina,S.G. (2004). Pretreatment p53 nuclear overexpression as a prognostic marker in superficial bladder cancer treated with Bacillus Calmette-Guerin (BCG). *Eur. Urol.,* **45,** 475-482.

395. Saito,S. (2001). Transurethral en bloc resection of bladder tumors. *J. Urol.,* **166,** 2148-2150.

396. Samanic,C., Kogevinas,M., Dosemeci,M., Malats,N., Real,F.X., Garcia-Closas,M., Serra,C., Carrato,A., Garcia-Closas,R., Sala,M., Lloreta,J., Tardon,A., Rothman,N., and Silverman,D.T. (2006). Smoking and bladder cancer in Spain: effects of tobacco type, timing, environmental tobacco smoke, and gender. *Cancer Epidemiol. Biomarkers Prev.,* **15,** 1348-1354.

397. Samaratunga,H., Makarov,D.V., and Epstein,J.I. (2002). Comparison of WHO/ISUP and WHO classification of noninvasive papillary urothelial neoplasms for risk of progression. *Urology,* **60,** 315-319.

398. Samuels,Y. and Velculescu,V.E. (2004). Oncogenic mutations of PIK3CA in human cancers. *Cell Cycle,* **3,** 1221-1224.

399. Sanchini,M.A., Gunelli,R., Nanni,O., Bravaccini,S., Fabbri,C., Sermasi,A., Bercovich,E., Ravaioli,A., Amadori,D., and Calistri,D. (2005). Relevance of urine telomerase in the diagnosis of bladder cancer. *JAMA,* **294,** 2052-2056.

400. Santos,L.L., Amaro,T., Pereira,S.A., Lameiras,C.R., Lopes,P., Bento,M.J., Oliveira,J., Criado,B., and Lopes,C.S. (2003). Expression of cell-cycle regulatory proteins and their prognostic value in superficial low-grade urothelial cell carcinoma of the bladder. *Eur. J. Surg. Oncol.,* **29,** 74-80.

401. Sarkis,A.S., Bajorin,D.F., Reuter,V.E., Herr,H.W., Netto,G., Zhang,Z.F., Schultz,P.K., Cordon-Cardo,C., and Scher,H.I. (1995). Prognostic value of

p53 nuclear overexpression in patients with invasive bladder cancer treated with neoadjuvant MVAC. *J. Clin. Oncol.,* **13,** 1384-1390.

402. Sarkis,A.S., Dalbagni,G., Cordon-Cardo,C., Melamed,J., Zhang,Z.F., Sheinfeld,J., Fair,W.R., Herr,H.W., and Reuter,V.E. (1994). Association of P53 nuclear overexpression and tumor progression in carcinoma in situ of the bladder. *J. Urol.,* **152,** 388-392.

403. Sarkis,A.S., Dalbagni,G., Cordon-Cardo,C., Zhang,Z.F., Sheinfeld,J., Fair,W.R., Herr,H.W., and Reuter,V.E. (1993). Nuclear overexpression of p53 protein in transitional cell bladder carcinoma: a marker for disease progression. *J. Natl. Cancer Inst.,* **85,** 53-59.

404. Sarosdy,M.F., Kahn,P.R., Ziffer,M.D., Love,W.R., Barkin,J., Abara,E.O., Jansz,K., Bridge,J.A., Johansson,S.L., Persons,D.L., and Gibson,J.S. (2006). Use of a multitarget fluorescence in situ hybridization assay to diagnose bladder cancer in patients with hematuria. *J. Urol.,* **176,** 44-47.

405. Saunders,W.S., Shuster,M., Huang,X., Gharaibeh,B., Enyenihi,A.H., Petersen,I., and Gollin,S.M. (2000). Chromosomal instability and cytoskeletal defects in oral cancer cells. *Proc. Natl. Acad. Sci. U. S. A,* **97,** 303-308.

406. Schlessinger,J. (2000). Cell signaling by receptor tyrosine kinases. *Cell,* **103,** 211-225.

407. Schlott,T., Quentin,T., Korabiowska,M., Budd,B., and Kunze,E. (2004). Alteration of the MDM2-p73-P14ARF pathway related to tumour progression during urinary bladder carcinogenesis. *Int. J. Mol. Med.,* **14,** 825-836.

408. Schmidt,C.A., Oettle,H., Ludwig,W.D., Serke,S., Pawlaczyk-Peter,B., Wilborn,F., Binder,L.T., Huhn,D., and Siegert,W. (1994). Overexpression of the Raf-1 proto-oncogene in human myeloid leukemia. *Leuk. Res.,* **18,** 409-413.

409. Schmitz-Drager,B.J., Goebell,P.J., Ebert,T., and Fradet,Y. (2000). p53 immunohistochemistry as a prognostic marker in bladder cancer. Playground for urology scientists? *Eur. Urol.,* **38,** 691-699.

410. Schrier,B.P., Vriesema,J.L., Witjes,J.A., Kiemeney,L.A., and Schalken,J.A. (2006). The Predictive Value of p53, p27(Kip1), and alpha-Catenin for Progression in Superficial Bladder Carcinoma. *Eur. Urol.,* **50,** 76-82.

411. Schroeder,J.C., Conway,K., Li,Y., Mistry,K., Bell,D.A., and Taylor,J.A. (2003). p53 mutations in bladder cancer: evidence for exogenous versus endogenous risk factors. *Cancer Res.,* **63,** 7530-7538.

412. Schultz,I.J., Witjes,J.A., Swinkels,D.W., and de Kok,J.B. (2006). Bladder cancer diagnosis and recurrence prognosis: comparison of markers with emphasis on survivin. *Clin. Chim. Acta,* **368,** 20-32.

413. Sears,R.C. and Nevins,J.R. (2002). Signaling networks that link cell proliferation and cell fate. *J. Biol. Chem.,* **277,** 11617-11620.

414. Sebolt-Leopold,J.S. and Herrera,R. (2004). Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nat. Rev. Cancer,* **4,** 937-947.

415. Sen,S., Zhou,H., Zhang,R.D., Yoon,D.S., Vakar-Lopez,F., Ito,S., Jiang,F., Johnston,D., Grossman,H.B., Ruifrok,A.C., Katz,R.L., Brinkley,W., and Czerniak,B. (2002). Amplification/overexpression of a mitotic kinase gene in human bladder cancer. *J. Natl. Cancer Inst.,* **94,** 1320-1329.

416. Sengupta,S., Vonesch,J.L., Waltzinger,C., Zheng,H., and Wasylyk,B. (2000). Negative cross-talk between p53 and the glucocorticoid receptor and its role in neuroblastoma cells. *EMBO J.,* **19,** 6051-6064.

417. Serdar,A., Turhan,C., Soner,G., Cem,S.N., Bayram,K., Damla,B.E., and Erbil,E. (2005). The prognostic importance of e-cadherin and p53 gene expression in transitional bladder carcinoma patients. *Int. Urol. Nephrol.,* **37,** 485-492.

418. Serra,C., Bonfill,X., Sunyer,J., Urrutia,G., Turuguet,D., Bastus,R., Roque,M., 't,M.A., and Kogevinas,M. (2000). Bladder cancer in the textile industry. *Scand. J. Work Environ. Health,* **26,** 476-481.

419. Serth,J., Kuczyk,M.A., Bokemeyer,C., Hervatin,C., Nafe,R., Tan,H.K., and Jonas,U. (1995). p53 immunohistochemistry as an independent prognostic factor for superficial transitional cell carcinoma of the bladder. *Br. J. Cancer,* **71,** 201-205.

420. Shahin,O., Thalmann,G.N., Rentsch,C., Mazzucchelli,L., and Studer,U.E. (2003). A retrospective analysis of 153 patients treated with or without intravesical bacillus Calmette-Guerin for primary stage T1 grade 3 bladder cancer: recurrence, progression and survival. *J. Urol.,* **169,** 96-100.

421. Shariat,S.F., Casella,R., Khoddami,S.M., Hernandez,G., Sulser,T., Gasser,T.C., and Lerner,S.P. (2004a). Urine detection of survivin is a sensitive marker for the noninvasive diagnosis of bladder cancer. *J. Urol.,* **171,** 626-630.

422. Shariat,S.F., Kim,J., Raptidis,G., Ayala,G.E., and Lerner,S.P. (2003). Association of p53 and p21 expression with clinical outcome in patients with carcinoma in situ of the urinary bladder. *Urology,* **61,** 1140-1145.

423. Shariat,S.F., Pahlavan,S., Baseman,A.G., Brown,R.M., Green,A.E., Wheeler,T.M., and Lerner,S.P. (2001). E-cadherin expression predicts clinical outcome in carcinoma in situ of the urinary bladder. *Urology,* **57,** 60-65.

424. Shariat,S.F., Tokunaga,H., Zhou,J., Kim,J., Ayala,G.E., Benedict,W.F., and Lerner,S.P. (2004b). p53, p21, pRB, and p16 expression predict clinical outcome in cystectomy with bladder cancer. *J. Clin. Oncol.,* **22,** 1014-1024.

425. Sharpless,N.E. (2005). INK4a/ARF: a multifunctional tumor suppressor locus. *Mutat. Res.,* **576,** 22-38.

426. Shepherd,F.A. (2001). Angiogenesis inhibitors in the treatment of lung cancer. *Lung Cancer,* **34 Suppl 3,** S81-S89.

427. Shigemasa,K., Tian,X., Gu,L., Shiroyama,Y., Nagai,N., and Ohama,K. (2003). Expression of cyclooxygenase-2 and its relationship to p53 accumulation in ovarian adenocarcinomas. *Int. J. Oncol.,* **22,** 99-105.

428. Shiina,H., Igawa,M., Urakami,S., Honda,S., Shirakawa,H., and Ishibe,T. (1996). Immunohistochemical analysis of bcl-2 expression in transitional cell carcinoma of the bladder. *J. Clin. Pathol.,* **49,** 395-399.

429. Shiraishi,K., Eguchi,S., Mohri,J., and Kamiryo,Y. (2003b). P53 mutation predicts intravesical adriamycin instillation failure in superficial transitional cell carcinoma of bladder. *Anticancer Res.,* **23,** 3475-3478.

430. Shiraishi,K., Eguchi,S., Mohri,J., and Kamiryo,Y. (2003a). P53 mutation predicts intravesical adriamycin instillation failure in superficial transitional cell carcinoma of bladder. *Anticancer Res.,* **23,** 3475-3478.

431. Siemiatycki,J., Dewar,R., Nadon,L., and Gerin,M. (1994). Occupational risk factors for bladder cancer: results from a case-control study in Montreal, Quebec, Canada. *Am. J. Epidemiol.,* **140,** 1061-1080.

432. Silverman,D.T., Levin,L.I., Hoover,R.N., and Hartge,P. (1989). Occupational risks of bladder cancer in the United States: I. White men. *J. Natl. Cancer Inst.,* **81,** 1472-1480.

433. Simon,R., Burger,H., Brinkschmidt,C., Bocker,W., Hertle,L., and Terpe,H.J. (1998). Chromosomal aberrations associated with invasion in papillary superficial bladder cancer. *J. Pathol.,* **185,** 345-351.

434. Simon,R., Burger,H., Semjonow,A., Hertle,L., Terpe,H.J., and Bocker,W. (2000). Patterns of chromosomal imbalances in muscle invasive bladder cancer. *Int. J. Oncol.,* **17,** 1025-1029.

435. Simon,R., Richter,J., Wagner,U., Fijan,A., Bruderer,J., Schmid,U., Ackermann,D., Maurer,R., Alund,G., Knonagel,H., Rist,M., Wilber,K., Anabitarte,M., Hering,F., Hardmeier,T., Schonenberger,A., Flury,R., Jager,P., Fehr,J.L., Schraml,P., Moch,H., Mihatsch,M.J., Gasser,T., and Sauter,G. (2001). High-throughput tissue microarray analysis of 3p25 (RAF1) and 8p12 (FGFR1) copy number alterations in urinary bladder cancer. *Cancer Res.,* **61,** 4514-4519.

436. Simoneau,M., Larue,H., Aboulkassim,T.O., Meyer,F., Moore,L., and Fradet,Y. (2000). Chromosome 9 deletions and recurrence of superficial bladder cancer: identification of four regions of prognostic interest. *Oncogene,* **19,** 6317-6323.

437. Siu,L.L., Banerjee,D., Khurana,R.J., Pan,X., Pflueger,R., Tannock,I.F., and Moore,M.J. (1998). The prognostic role of p53, metallothionein, P-glycoprotein, and MIB-1 in muscle-invasive urothelial transitional cell carcinoma. *Clin. Cancer Res.,* **4,** 559-565.

438. Skopelitou,A.S., Gloustianou,G., Bai,M., and Huebner,K. (2001). FHIT gene expression in human urinary bladder transitional cell carcinomas. *In Vivo,* **15,** 169-173.

439. Slaton,J.W., Benedict,W.F., and Dinney,C.P. (2001). P53 in bladder cancer: mechanism of action, prognostic value, and target for therapy. *Urology,* **57,** 852-859.

440. Smith,N.D., Rubenstein,J.N., Eggener,S.E., and Kozlowski,J.M. (2003). The p53 tumor suppressor gene and nuclear protein: basic science review and relevance in the management of bladder cancer. *J. Urol.,* **169,** 1219-1228.

441. Smith,S.D., Wheeler,M.A., Plescia,J., Colberg,J.W., Weiss,R.M., and Altieri,D.C. (2001). Urine detection of survivin and diagnosis of bladder cancer. *JAMA,* **285,** 324-328.

442. Sobin,L.H. and Wittekind CH (2002). *TNM Classification of Malignant Tumors, 6th Edition.* John Wiley and Sons, New York.

443. Solsona,E., Iborra,I., Ricos,J.V., Monros,J.L., Casanova,J., and Dumont,R. (1999). Effectiveness of a single immediate mitomycin C instillation in patients with low risk superficial bladder cancer: short and long-term followup. *J. Urol.,* **161,** 1120-1123.

444. Sordella,R., Bell,D.W., Haber,D.A., and Settleman,J. (2004). Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science,* **305,** 1163-1167.

445. Soucek,T., Yeung,R.S., and Hengstschlager,M. (1998). Inactivation of the cyclin-dependent kinase inhibitor p27 upon loss of the tuberous sclerosis complex gene-2. *Proc. Natl. Acad. Sci. U. S. A,* **95,** 15653-15658.

446. Soussi,T. and Beroud,C. (2001). Assessing TP53 status in human tumours to evaluate clinical outcome. *Nat. Rev. Cancer,* **1,** 233-240.

447. Soussi,T., Kato,S., Levy,P.P., and Ishioka,C. (2005). Reassessment of the TP53 mutation database in human disease by data mining with a library of TP53 missense mutations. *Hum. Mutat.,* **25,** 6-17.

448. Sozzi,G., Pastorino,U., Moiraghi,L., Tagliabue,E., Pezzella,F., Ghirelli,C., Tornielli,S., Sard,L., Huebner,K., Pierotti,M.A., Croce,C.M., and Pilotti,S. (1998). Loss of FHIT function in lung cancer and preinvasive bronchial lesions. *Cancer Res.,* **58,** 5032-5037.

449. Spiess,P.E. and Czerniak,B. (2006). Dual-track pathway of bladder carcinogenesis: practical implications. *Arch. Pathol. Lab Med.,* **130,** 844-852.

450. SPITZ,S., MAGUIGAN,W.H., and DOBRINER,K. (1950). The carcinogenic action of benzidine. *Cancer,* **3,** 789-804.

451. Spruck,C.H., III, Ohneseit,P.F., Gonzalez-Zulueta,M., Esrig,D., Miyao,N., Tsai,Y.C., Lerner,S.P., Schmutte,C., Yang,A.S., Cote,R., and . (1994). Two molecular pathways to transitional cell carcinoma of the bladder. *Cancer Res.,* **54,** 784-788.

452. Spruck,C.H., III, Rideout,W.M., III, Olumi,A.F., Ohneseit,P.F., Yang,A.S., Tsai,Y.C., Nichols,P.W., Horn,T., Hermann,G.G., Steven,K., and . (1993). Distinct pattern of p53 mutations in bladder cancer: relationship to tobacco usage. *Cancer Res.,* **53,** 1162-1166.

453. Steiner,G., Bierhoff,E., Schmidt,D., Leissner,J., Wolf,H.K., and Albers,P. (2000). p53 immunoreactivity in biopsy specimens of T1G3 transitional cell carcinoma of the bladder--a helpful parameter in guiding the decision for or against cystectomy? *Eur. J. Cancer,* **36,** 610-614.

454. Steinmaus,C.M., Nunez,S., and Smith,A.H. (2000). Diet and bladder cancer: a meta-analysis of six dietary variables. *Am. J. Epidemiol.,* **151,** 693-702.

455. Sternberg,C.N. (2002). Neo-adjuvant and adjuvant chemotherapy of bladder cancer: Is there a role? *Ann. Oncol.,* **13 Suppl 4,** 273-279.

456. Sternberg,C.N., Yagoda,A., Scher,H.I., Watson,R.C., Geller,N., Herr,H.W., Morse,M.J., Sogani,P.C., Vaughan,E.D., Bander,N., and . (1989). Methotrexate, vinblastine, doxorubicin, and cisplatin for advanced transitional cell carcinoma of the urothelium. Efficacy and patterns of response and relapse. *Cancer,* **64,** 2448-2458.

457. Stevaux,O. and Dyson,N.J. (2002). A revised picture of the E2F transcriptional network and RB function. *Curr. Opin. Cell Biol.,* **14,** 684-691.

458. Stoehr,R., Brinkmann,A., Filbeck,T., Gamper,C., Wild,P., Blaszyk,H., Hofstaedter,F., Knuechel,R., and Hartmann,A. (2004). No evidence for mutation of B-RAF in urothelial carcinomas of the bladder and upper urinary tract. *Oncol. Rep.,* **11,** 137-141.

459. Streeter,E.H. and Crew,J.P. (2001). Angiogenesis, angiogenic factor expression and prognosis of bladder cancer. *Anticancer Res.,* **21,** 4355-4363.

460. Swana,H.S., Grossman,D., Anthony,J.N., Weiss,R.M., and Altieri,D.C. (1999). Tumor content of the antiapoptosis molecule survivin and recurrence of bladder cancer. *N. Engl. J. Med.,* **341,** 452-453.

461. Swiatkowski,S., Seifert,H.H., Steinhoff,C., Prior,A., Thievessen,I., Schliess,F., and Schulz,W.A. (2003). Activities of MAP-kinase pathways in normal uroepithelial cells and urothelial carcinoma cell lines. *Exp. Cell Res.,* **282,** 48-57.

462. Sylvester,R.J., van der Meijden,A.P., Witjes,J.A., and Kurth,K. (2005). Bacillus calmette-guerin versus chemotherapy for the intravesical treatment of patients with carcinoma in situ of the bladder: a meta-analysis of the published results of randomized clinical trials. *J. Urol.,* **174,** 86-91.

463. Takihana,Y., Tsuchida,T., Fukasawa,M., Araki,I., Tanabe,N., and Takeda,M. (2006). Real-time quantitative analysis for human telomerase reverse transcriptase mRNA and human telomerase RNA component mRNA expressions as markers for clinicopathologic parameters in urinary bladder cancer. *Int. J. Urol.,* **13,** 401-408.

464. Takkouche,B., Etminan,M., and Montes-Martinez,A. (2005). Personal use of hair dyes and risk of cancer: a meta-analysis. *JAMA,* **293,** 2516-2525.

465. Tanaka,M., Koul,D., Davies,M.A., Liebert,M., Steck,P.A., and Grossman,H.B. (2000). MMAC1/PTEN inhibits cell growth and induces chemosensitivity to doxorubicin in human bladder cancer cells. *Oncogene,* **19,** 5406-5412.

466. Tarkkanen,A. and Karjalainen,K. (1984). Excess of cancer deaths in close relatives of patients with bilateral retinoblastoma. *Ophthalmologica,* **189,** 143-146.

467. Tetu,B., Fradet,Y., Allard,P., Veilleux,C., Roberge,N., and Bernard,P. (1996). Prevalence and clinical significance of HER/2neu, p53 and Rb expression in primary superficial bladder cancer. *J. Urol.,* **155,** 1784-1788.

468. Tiguert,R., Bianco,F.J., Jr., Oskanian,P., Li,Y., Grignon,D.J., Wood,D.P., Jr., Pontes,J.E., and Sarkar,F.H. (2001). Structural alteration of p53 protein in patients with muscle invasive bladder transitional cell carcinoma. *J. Urol.,* **166,** 2155-2160.

469. Tolley,D.A., Parmar,M.K., Grigor,K.M., Lallemand,G., Benyon,L.L., Fellows,J., Freedman,L.S., Grigor,K.M., Hall,R.R., Hargreave,T.B., Munson,K., Newling,D.W., Richards,B., Robinson,M.R., Rose,M.B., Smith,P.H., Williams,J.L., and Whelan,P. (1996). The effect of intravesical mitomycin C on recurrence of newly diagnosed superficial bladder cancer: a further report with 7 years of follow up. *J. Urol.,* **155,** 1233-1238.

470. Toncheva,D.I. and Zaharieva,B.M. (2003). High-throughput tissue microarray analysis of erbB-2 gene amplification in urinary bladder cancer. A study of Bulgarian patients. *Urol. Int.,* **71,** 408-411.

471. Tonini,T., Hillson,C., and Claudio,P.P. (2002). Interview with the retinoblastoma family members: do they help each other? *J. Cell Physiol,* **192,** 138-150.

472. Torii,S., Yamamoto,T., Tsuchiya,Y., and Nishida,E. (2006). ERK MAP kinase in G cell cycle progression and cancer. *Cancer Sci.,* **97,** 697-702.

473. Tremblay,C., Armstrong,B., Theriault,G., and Brodeur,J. (1995). Estimation of risk of developing bladder cancer among workers exposed to coal tar pitch volatiles in the primary aluminum industry. *Am. J. Ind. Med.,* **27,** 335-348.

474. Trudel,S., Li,Z.H., Wei,E., Wiesmann,M., Chang,H., Chen,C., Reece,D., Heise,C., and Stewart,A.K. (2005). CHIR-258, a novel, multitargeted tyrosine kinase inhibitor for the potential treatment of t(4;14) multiple myeloma. *Blood,* **105,** 2941-2948.

475. Uchida,C., Miwa,S., Kitagawa,K., Hattori,T., Isobe,T., Otani,S., Oda,T., Sugimura,H., Kamijo,T., Ookawa,K., Yasuda,H., and Kitagawa,M. (2005). Enhanced Mdm2 activity inhibits pRB function via ubiquitin-dependent degradation. *EMBO J.,* **24,** 160-169.

476. Uchida,T., Wang,C., Wada,C., Iwamura,M., Egawa,S., and Koshiba,K. (1996). Microsatellite instability in transitional cell carcinoma of the urinary tract and its relationship to clinicopathological variables and smoking. *Int. J. Cancer,* **69,** 142-145.

477. Uchikoba,T., Horiuchi,K., Oka,F., Sato,M., Tsuboi,N., Ohaki,Y., and Nishimura,T. (2005). Diagnosing the location of carcinoma in situ (CIS) of the urinary bladder using pirarubicin hydrochloride. *Urol. Int.,* **74,** 235-239.

478. Udeani,G.O., Gerhauser,C., Thomas,C.F., Moon,R.C., Kosmeder,J.W., Kinghorn,A.D., Moriarty,R.M., and Pezzuto,J.M. (1997). Cancer chemopreventive activity mediated by deguelin, a naturally occurring rotenoid. *Cancer Res.,* **57,** 3424-3428.

479. Ulukan,H. and Swaan,P.W. (2002). Camptothecins: a review of their chemotherapeutic potential. *Drugs,* **62,** 2039-2057.

480. Urist,M.J., Di Como,C.J., Lu,M.L., Charytonowicz,E., Verbel,D., Crum,C.P., Ince,T.A., McKeon,F.D., and Cordon-Cardo,C. (2002). Loss of p63 expression is associated with tumor progression in bladder cancer. *Am. J. Pathol.,* **161,** 1199-1206.

481. van der Aa,M.N., van Leenders,G.J., Steyerberg,E.W., van Rhijn,B.W., Jobsis,A.C., Zwarthoff,E.C., and van der Kwast,T.H. (2005). A new system for substaging pT1 papillary bladder cancer: a prognostic evaluation. *Hum. Pathol.,* **36,** 981-986.

482. van Dyke,T. (2005). Cancer biology: sense out of missense. *Nature,* **434,** 287-288.

483. van Oers,J.M., Lurkin,I., van Exsel,A.J., Nijsen,Y., van Rhijn,B.W., van der Aa,M.N., and Zwarthoff,E.C. (2005). A simple and fast method for the simultaneous detection of nine fibroblast growth factor receptor 3 mutations in bladder cancer and voided urine. *Clin. Cancer Res.,* **11,** 7743-7748.

484. van Rhijn,B.W., Lurkin,I., Radvanyi,F., Kirkels,W.J., van der Kwast,T.H., and Zwarthoff,E.C. (2001). The fibroblast growth factor receptor 3 (FGFR3) mutation is a strong indicator of superficial bladder cancer with low recurrence rate. *Cancer Res.,* **61,** 1265-1268.

485. van Rhijn,B.W., van der Kwast,T.H., Vis,A.N., Kirkels,W.J., Boeve,E.R., Jobsis,A.C., and Zwarthoff,E.C. (2004). FGFR3 and P53 characterize alternative genetic pathways in the pathogenesis of urothelial cell carcinoma. *Cancer Res.,* **64,** 1911-1914.

486. van Rhijn,B.W., van Tilborg,A.A., Lurkin,I., Bonaventure,J., de Vries,A., Thiery,J.P., van der Kwast,T.H., Zwarthoff,E.C., and Radvanyi,F. (2002). Novel

fibroblast growth factor receptor 3 (FGFR3) mutations in bladder cancer previously identified in non-lethal skeletal disorders. *Eur. J. Hum. Genet.,* **10,** 819-824.

487. van Rhijn,B.W., Vis,A.N., van der Kwast,T.H., Kirkels,W.J., Radvanyi,F., Ooms,E.C., Chopin,D.K., Boeve,E.R., Jobsis,A.C., and Zwarthoff,E.C. (2003). Molecular grading of urothelial cell carcinoma with fibroblast growth factor receptor 3 and MIB-1 is superior to pathologic grade for the prediction of clinical outcome. *J. Clin. Oncol.,* **21,** 1912-1921.

488. van Tilborg,A.A., de Vries,A., de Bont,M., Groenfeld,L.E., van der Kwast,T.H., and Zwarthoff,E.C. (2000). Molecular evolution of multiple recurrent cancers of the bladder. *Hum. Mol. Genet.,* **9,** 2973-2980.

489. Vecchione,A., Sevignani,C., Giarnieri,E., Zanesi,N., Ishii,H., Cesari,R., Fong,L.Y., Gomella,L.G., Croce,C.M., and Baffa,R. (2004). Inactivation of the FHIT gene favors bladder cancer development. *Clin. Cancer Res.,* **10,** 7607-7612.

490. Veltman,J.A., Fridlyand,J., Pejavar,S., Olshen,A.B., Korkola,J.E., DeVries,S., Carroll,P., Kuo,W.L., Pinkel,D., Albertson,D., Cordon-Cardo,C., Jain,A.N., and Waldman,F.M. (2003a). Array-based comparative genomic hybridization for genome-wide screening of DNA copy number in bladder tumors. *Cancer Res.,* **63,** 2872-2880.

491. Veltman,J.A., Fridlyand,J., Pejavar,S., Olshen,A.B., Korkola,J.E., DeVries,S., Carroll,P., Kuo,W.L., Pinkel,D., Albertson,D., Cordon-Cardo,C., Jain,A.N., and Waldman,F.M. (2003b). Array-based comparative genomic hybridization for genome-wide screening of DNA copy number in bladder tumors. *Cancer Res.,* **63,** 2872-2880.

492. Vermeulen,K., Van Bockstaele,D.R., and Berneman,Z.N. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif.,* **36,** 131-149.

493. Veys,C.A. (2004). Bladder tumours in rubber workers: a factory study 1946-1995. *Occup. Med. (Lond),* **54,** 322-329.

494. Vicent,S., Lopez-Picazo,J.M., Toledo,G., Lozano,M.D., Torre,W., Garcia-Corchon,C., Quero,C., Soria,J.C., Martin-Algarra,S., Manzano,R.G., and Montuenga,L.M. (2004). ERK1/2 is activated in non-small-cell lung cancer and associated with advanced tumours. *Br. J. Cancer,* **90,** 1047-1052.

495. Villanueva,C.M., Cantor,K.P., Cordier,S., Jaakkola,J.J., King,W.D., Lynch,C.F., Porru,S., and Kogevinas,M. (2004). Disinfection byproducts and bladder cancer: a pooled analysis. *Epidemiology,* **15,** 357-367.

496. Villanueva,C.M., Cantor,K.P., King,W.D., Jaakkola,J.J., Cordier,S., Lynch,C.F., Porru,S., and Kogevinas,M. (2006). Total and specific fluid consumption as determinants of bladder cancer risk. *Int. J. Cancer,* **118,** 2040-2047.

497. Vleminckx,K. and Kemler,R. (1999). Cadherins and tissue formation: integrating adhesion and signaling. *Bioessays,* **21,** 211-220.

498. Vogelstein,B., Lane,D., and Levine,A.J. (2000). Surfing the p53 network. *Nature,* **408,** 307-310.

499. von der,M.H., Hansen,S.W., Roberts,J.T., Dogliotti,L., Oliver,T., Moore,M.J., Bodrogi,I., Albers,P., Knuth,A., Lippert,C.M., Kerbrat,P., Sanchez,R.P., Wersall,P., Cleall,S.P., Roychowdhury,D.F., Tomlin,I., Visseren-Grul,C.M., and Conte,P.F. (2000). Gemcitabine and cisplatin versus methotrexate, vinblastine, doxorubicin, and cisplatin in advanced or metastatic bladder cancer: results of a large, randomized, multinational, multicenter, phase III study. *J. Clin. Oncol.,* **18,** 3068-3077.

500. Voorter,C., Joos,S., Bringuier,P.P., Vallinga,M., Poddighe,P., Schalken,J., du,M.S., Ramaekers,F., Lichter,P., and Hopman,A. (1995). Detection of chromosomal imbalances in transitional cell carcinoma of the bladder by comparative genomic hybridization. *Am J. Pathol.,* **146,** 1341-1354.

501. Vousden,K.H. (2002). Activation of the p53 tumor suppressor protein. *Biochim. Biophys. Acta,* **1602,** 47-59.

502. Vousden,K.H. and Prives,C. (2005). P53 and prognosis: new insights and further complexity. *Cell,* **120,** 7-10.

503. Wagner,H.E., Joyce,A.D., Beatrice,K., and Summerhayes,I.C. (1990). ras induced lesions in a heterotopic mouse bladder. *Oncogene,* **5,** 557-563.

504. Wallerand,H., Bakkar,A.A., de Medina,S.G., Pairon,J.C., Yang,Y.C., Vordos,D., Bittard,H., Fauconnet,S., Kouyoumdjian,J.C., Jaurand,M.C., Zhang,Z.F., Radvanyi,F., Thiery,J.P., and Chopin,D.K. (2005). Mutations in TP53, but not FGFR3, in urothelial cell carcinoma of the bladder are influenced by smoking: contribution of exogenous versus endogenous carcinogens. *Carcinogenesis,* **26,** 177-184.

505. Wang,D.S., Rieger-Christ,K., Latini,J.M., Moinzadeh,A., Stoffel,J., Pezza,J.A., Saini,K., Libertino,J.A., and Summerhayes,I.C. (2000). Molecular analysis of PTEN and MXI1 in primary bladder carcinoma. *Int. J. Cancer,* **88,** 620-625.

506. Wang,M., Wang,Z., Ran,L., Han,H., Wang,Y., and Yang,D. (2003). [Study on food contaminants monitoring in China during 2000-2001]. *Wei Sheng Yan. Jiu.,* **32,** 322-326.

507. Wang,W., Kim,S.H., and el Deiry,W.S. (2006). Small-molecule modulators of p53 family signaling and antitumor effects in p53-deficient human colon tumor xenografts. *Proc. Natl. Acad. Sci. U. S. A,* **103,** 11003-11008.

508. Wang,X.W., Zhan,Q., Coursen,J.D., Khan,M.A., Kontny,H.U., Yu,L., Hollander,M.C., O'Connor,P.M., Fornace,A.J., Jr., and Harris,C.C. (1999). GADD45 induction of a G2/M cell cycle checkpoint. *Proc. Natl. Acad. Sci. U. S. A,* **96,** 3706-3711.

509. Ward,E., Halperin,W., Thun,M., Grossman,H.B., Fink,B., Koss,L., Osorio,A.M., and Schulte,P. (1990). Screening workers exposed to 4,4'-methylenebis(2-chloroaniline) for bladder cancer by cystoscopy. *J. Occup. Med.,* **32,** 865-868.

510. Waters,S.B., Holt,K.H., Ross,S.E., Syu,L.J., Guan,K.L., Saltiel,A.R., Koretzky,G.A., and Pessin,J.E. (1995). Desensitization of Ras activation by a feedback disassociation of the SOS-Grb2 complex. *J. Biol. Chem.,* **270,** 20883-20886.

511. Webster,M.K., D'Avis,P.Y., Robertson,S.C., and Donoghue,D.J. (1996). Profound ligand-independent kinase activation of fibroblast growth factor receptor 3 by the activation loop mutation responsible for a lethal skeletal dysplasia, thanatophoric dysplasia type II. *Mol. Cell Biol.,* **16,** 4081-4087.

512. Webster,M.K. and Donoghue,D.J. (1997). FGFR activation in skeletal disorders: too much of a good thing. *Trends Genet.,* **13,** 178-182.

513. Weikert,S., Krause,H., Wolff,I., Christoph,F., Schrader,M., Emrich,T., Miller,K., and Muller,M. (2005). Quantitative evaluation of telomerase subunits in urine as biomarkers for noninvasive detection of bladder cancer. *Int. J. Cancer,* **117,** 274-280.

514. Werner,H., Karnieli,E., Rauscher,F.J., and LeRoith,D. (1996). Wild-type and mutant p53 differentially regulate transcription of the insulin-like growth factor I receptor gene. *Proc. Natl. Acad. Sci. U. S. A,* **93,** 8318-8323.

515. Wiley,H.S. (2003). Trafficking of the ErbB receptors and its influence on signaling. *Exp. Cell Res.,* **284,** 78-88.

516. Williamson,M.P., Elder,P.A., Shaw,M.E., Devlin,J., and Knowles,M.A. (1995). p16 (CDKN2) is a major deletion target at 9p21 in bladder cancer. *Hum. Mol. Genet.,* **4,** 1569-1577.

517. Witjes,J.A. (2004). Bladder carcinoma in situ in 2003: state of the art. *Eur. Urol.,* **45,** 142-146.

518. World Health Organization (1994). *World health organization evaluation of carcinogenic risk to humans. Schistosome, liver flukes and Helicobacter pylori.* IARC monograms.

519. Wright,K.O., Messing,E.M., and Reeder,J.E. (2004). DBCCR1 mediates death in cultured bladder tumor cells. *Oncogene,* **23,** 82-90.

520. Wu,C.S., Pollack,A., Czerniak,B., Chyle,V., Zagars,G.K., Dinney,C.P., Hu,S.X., and Benedict,W.F. (1996). Prognostic value of p53 in muscle-invasive bladder cancer treated with preoperative radiotherapy. *Urology,* **47,** 305-310.

521. Wu,Q., Hoffmann,M.J., Hartmann,F.H., and Schulz,W.A. (2005). Amplification and overexpression of the ID4 gene at 6p22.3 in bladder cancer. *Mol. Cancer,* **4,** 16.

522. Wu,X., Obata,T., Khan,Q., Highshaw,R.A., De Vere,W.R., and Sweeney,C. (2004). The phosphatidylinositol-3 kinase pathway regulates bladder cancer cell invasion. *BJU. Int.,* **93,** 143-150.

523. Wu,X.R. (2005). Urothelial tumorigenesis: a tale of divergent pathways. *Nat. Rev. Cancer,* **5,** 713-725.

524. Wu,X.R., Manabe,M., Yu,J., and Sun,T.T. (1990). Large scale purification and immunolocalization of bovine uroplakins I, II, and III. Molecular markers of urothelial differentiation. *J. Biol. Chem.,* **265,** 19170-19179.

525. Wulfing,C., von Struensee,D., Bierer,S., Bogemann,M., Hertle,L., and Eltze,E. (2005). [Expression of Her2/neu in locally advanced bladder cancer: implication for a molecular targeted therapy]. *Aktuelle Urol.,* **36,** 423-429.

526. Xia,F. and Altieri,D.C. (2006). Mitosis-independent survivin gene expression in vivo and regulation by p53. *Cancer Res.,* **66,** 3392-3395.

527. Xia,G., Kumar,S.R., Hawes,D., Cai,J., Hassanieh,L., Groshen,S., Zhu,S., Masood,R., Quinn,D.I., Broek,D., Stein,J.P., and Gill,P.S. (2006). Expression and significance of vascular endothelial growth factor receptor 2 in bladder cancer. *J. Urol.,* **175,** 1245-1252.

528. Xu,H.J., Cairns,P., Hu,S.X., Knowles,M.A., and Benedict,W.F. (1993). Loss of RB protein expression in primary bladder cancer correlates with loss of heterozygosity at the RB locus and tumor progression. *Int. J. Cancer,* **53,** 781-784.

529. Xu,X., Sun,T.T., Gupta,P.K., Zhang,P., and Nasuti,J.F. (2001). Uroplakin as a marker for typing metastatic transitional cell carcinoma on fine-needle aspiration specimens. *Cancer,* **93,** 216-221.

530. Yamamoto,Y., Matsuyama,H., Furuya,T., Oga,A., Yoshihiro,S., Okuda,M., Kawauchi,S., Sasaki,K., and Naito,K. (2004). Centrosome hyperamplification predicts progression and tumor recurrence in bladder cancer. *Clin. Cancer Res.,* **10,** 6449-6455.

531. Yamana,K., Bilim,V., Hara,N., Kasahara,T., Itoi,T., Maruyama,R., Nishiyama,T., Takahashi,K., and Tomita,Y. (2005). Prognostic impact of FAS/CD95/APO-1 in urothelial cancers: decreased expression of Fas is associated with disease progression. *Br. J. Cancer,* **93,** 544-551.

532. Yamasaki,L. (2003). Role of the RB tumor suppressor in cancer. *Cancer Treat. Res.,* **115,** 209-239.

533. Yan,J., Roy,S., Apolloni,A., Lane,A., and Hancock,J.F. (1998). Ras isoforms vary in their ability to activate Raf-1 and phosphoinositide 3-kinase. *J. Biol. Chem.,* **273,** 24052-24056.

534. Yang,Y., Li,C.C., and Weissman,A.M. (2004). Regulating the p53 system through ubiquitination. *Oncogene,* **23,** 2096-2106.

535. Yarden,Y. and Sliwkowski,M.X. (2001). Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell Biol.,* **2,** 127-137.

536. Yasunaga,Y., Nakanishi,H., Naka,N., Miki,T., Tsujimura,T., Itatani,H., Okuyama,A., and Aozasa,K. (1997). Alterations of the p53 gene in occupational bladder cancer in workers exposed to aromatic amines. *Lab Invest,* **77,** 677-684.

537. Yossepowitch,O., Dalbagni,G., Golijanin,D., Donat,S.M., Bochner,B.H., Herr,H.W., Fair,W.R., and Russo,P. (2003). Orthotopic urinary diversion after cystectomy for bladder cancer: implications for cancer control and patterns of disease recurrence. *J. Urol.,* **169,** 177-181.

538. Yu,D.S., Hsieh,D.S., and Chang,S.Y. (2001). Detection of chromosomal alterations in bladder cancer by comparative genomic hybridization. *BJU. Int.,* **87,** 889-893.

539. Yu,J., Manabe,M., and Sun,T.T. (1992). Identification of an 85-100 kDa glycoprotein as a cell surface marker for an advanced stage of urothelial differentiation: association with the inter-plaque ('hinge') area. *Epithelial Cell Biol.,* **1,** 4-12.

540. Yu,X., Sharma,K.D., Takahashi,T., Iwamoto,R., and Mekada,E. (2002). Ligand-independent dimer formation of epidermal growth factor receptor (EGFR) is a step separable from ligand-induced EGFR signaling. *Mol. Biol. Cell,* **13,** 2547-2557.

541. Zacharatos,P., Kotsinas,A., Evangelou,K., Karakaidos,P., Vassiliou,L.V., Rezaei,N., Kyroudi,A., Kittas,C., Patsouris,E., Papavassiliou,A.G., and Gorgoulis,V.G. (2004). Distinct expression patterns of the transcription factor E2F-1 in relation to tumour growth parameters in common human carcinomas. *J. Pathol.,* **203,** 744-753.

542. Zeegers,M.P., Tan,F.E., Dorant,E., and van Den Brandt,P.A. (2000). The impact of characteristics of cigarette smoking on urinary tract cancer risk: a meta-analysis of epidemiologic studies. *Cancer,* **89,** 630-639.

543. Zellweger,T., Benz,G., Cathomas,G., Mihatsch,M.J., Sulser,T., Gasser,T.C., and Bubendorf,L. (2006). Multi-target fluorescence in situ hybridization in bladder washings for prediction of recurrent bladder cancer. *Int. J. Cancer*.

544. Zeng,Y.X. and el Deiry,W.S. (1996). Regulation of p21WAF1/CIP1 expression by p53-independent pathways. *Oncogene,* **12,** 1557-1564.

545. Zhang,Z.T., Pak,J., Huang,H.Y., Shapiro,E., Sun,T.T., Pellicer,A., and Wu,X.R. (2001). Role of Ha-ras activation in superficial papillary pathway of urothelial tumor formation. *Oncogene,* **20,** 1973-1980.

546. Zhang,Z.T., Pak,J., Shapiro,E., Sun,T.T., and Wu,X.R. (1999). Urothelium-specific expression of an oncogene in transgenic mice induced the formation of carcinoma in situ and invasive transitional cell carcinoma. *Cancer Res.,* **59,** 3512-3517.

547. Zhao,J., Richter,J., Wagner,U., Roth,B., Schraml,P., Zellweger,T., Ackermann,D., Schmid,U., Moch,H., Mihatsch,M.J., Gasser,T.C., and Sauter,G. (1999). Chromosomal imbalances in noninvasive papillary bladder neoplasms (pTa). *Cancer Res.,* **59,** 4658-4661.

548. Zheng,M., Simon,R., Mirlacher,M., Maurer,R., Gasser,T., Forster,T., Diener,P.A., Mihatsch,M.J., Sauter,G., and Schraml,P. (2004). TRIO amplification and abundant mRNA expression is associated with invasive tumor growth and rapid tumor cell proliferation in urinary bladder cancer. *Am. J. Pathol.,* **165,** 63-69.

549. Zhu,Q., Yao,J., Wani,G., Wani,M.A., and Wani,A.A. (2001). Mdm2 mutant defective in binding p300 promotes ubiquitination but not degradation of p53: evidence for the role of p300 in integrating ubiquitination and proteolysis. *J. Biol. Chem.,* **276,** 29695-29701.

550. Zieger,K., Dyrskjot,L., Wiuf,C., Jensen,J.L., Andersen,C.L., Jensen,K.M., and Orntoft,T.F. (2005). Role of activating fibroblast growth factor receptor 3 mutations in the development of bladder tumors. *Clin. Cancer Res.,* **11,** 7709-7719.

1. Patients and cell lines

2. DNA extraction
    a. Phenol Chloroform
    b. Qiagen kit
    c. PK protocol

3. DNA quantitation

4. PCR
    a. TP53
    b. PIK3CA
    c. FGFR3

5. PCR product purification

6. Sequencing reaction

7. Sequencing precipitation

8. Taqman quantitative PCR
    a. CDK5
    b. MDM2
    c. 10q 6 genes

9. Immunohistochemistry
    a. P53, MDM2, PTEN, Annexin, FGFR3, COX2, IGFR1, 14-3-3, Survivin

10. Array CGH analysis

1. Patients and tumor samples.

Cases were drawn from the EPICURO study which comprises 1,356 consecutive incident bladder cancer cases recruited prospectively, between 1997-2001, in 18 general hospitals in Spain.

Sociodemographic and clinical information was retrieved from hospital records.

Tumors were staged and graded according to the TNM classification and the World Health Organization-International Society of Urological Pathology as described in detail elsewhere. All blocks from the initial tumor were sent to the coordinating center where expert pathologists reviewed a section from each block and classified the tumor following strict criteria. If various blocks were received from the same patient, the tissue that best represents the disease was chosen. Three and 10 μm thick sections were cut from each block for processing. Three μm sections were used for immunohistochemistry and 10 μm sections were used for DNA extraction.

Cases were prospectively followed-up both through hospital records and by telephone interviews to the patients or a next-of-kin when the former was not reachable or was deceased. The median follow-up period for patients with nonmuscle invasive tumors who were alive and free of disease was 62 months. Written informed consent was obtained from all patients. The study was approved by the Ethics Committees of all participating institutions.

Bladder cancer cell lines.

A panel of 14 bladder cancer cell lines were obtained from the Ludwig Institute for Cancer Research New York Branch (Sloan-Kettering Institute) or from Yves Fradet (Laval University, Québec, Canada): MGHU4, RT4, MGHU3, J82, **T24,** VMCub3, VMCub1, SW1710, JON, 5637, LGW01, SW800, 253J and 575 A.

2. DNA extraction.

H-E-stained sections were used to select areas containing ≥50% tumor cells for manual microdissection.

Methods used for DNA extraction:

- Phenol chloroform extraction: first manual microdissections.

- Kit Qiagen: DNA used for mutational analysis, quantitative PCR. DNA extracted from microdissected paraffin sections and cell lines.

- PK protocol: DNA used for array CGH, obtains more quantity of material.
    - Purification by Microcon columns for array CGH

** Do not start many microdissections at once, as leaving the sample in xylene for long periods is not beneficial for the DNA.

a) DNA extraction by phenol-chloroform

Day 1- Deparaffinization

- Incubate 60ºC 30 min OVEN 60ºC
- Wash in xylene 3x 5 min
- Dry at room temperature (RT)

Day 1- Microdissection

- Scrape with a tip/blade and introduce in eppendorf with 400µl xylene and mix
- Spin 5 min at 13000 rpm and discard supernatant
- Add 400µl ethanol and mix
- Spin 5 min at 13000 rpm and discard supernatant
- Dry at room temperature (RT)
- Add 6µl proteinase K, 4 µl buffer, 30µl ddwater and incubate 55ºC ON **OVEN 55ºC**

Day 2- Purification

- Spin 1 min at 13000 rpm
- Incubate 95ºC 10 min PLATE 95ºC
- Spin 1 min at 13000 rpm
- Add 60µl ddwater
- Add 100µl Phenol/Chloroform and mix
- Spin 3 min at 14000 rpm, transfer supernatant to new eppendorf
- Add 100µl Cloroformo and mix
- Spin 3 min at 14000 rpm, transfer supernatant to new eppendorf

Day 2- Precipitation

- Add 10µl Sodium Acetate 3M (Final concentration 0,3M)
- Add 40µl ethanol 100% at –20ºC Keep ethanol at -20ºC
- Leave at –20ºC Overnight (ON)

Day 3- Precipitation

- Spin 20 min at 14000 rpm and discard supernatant
- Add 250µl etanol 70% at -20ºC Keep ethanol at -20ºC
- Spin 5 min at 14000 rpm and discard supernatant
- Dry at room temperature (RT)
- Resuspend in 40µl ddwater and keep at -20ºC

b) DNA extraction by Qiagen Kit (Qiamp DNA mini kit)

Day 1- Deparaffinization

- Incubate 60ºC 30 min OVEN 60ºC
- Wash in xylene 3x 5 min
- Dry at room temperature (RT)

Day 1- Microdissection

- Scrape with a tip/blade and introduce in eppendorf with 1200µl xylene and mix
- Spin 5 min at 13000 rpm and discard supernatant
- Add 1200µl ethanol and mix
- Spin 5 min at 13000 rpm and discard supernatant
- Add 1200µl ethanol and mix
- Spin 5 min at 13000 rpm and discard supernatant
- Incubate 37ºC 10-15 min with open eppendorf PLATE 37ºC
- Resuspend in 180µl buffer ATL
- Add 20µl proteinasa K and vortex
- Cover eppendorfs with parafilm
- Incubate 55ºC ON OVEN 55ºC

Day 2- Purification and precipitation

- Add 200µl Buffer AL, vortex and incubate 70ºC 10 min PLATE 70ºC
- Add 200µl Ethanol 100%, vortex transfer into column
- Spin 1min at 8000 rpm and discard flow through
- Add 500µl Buffer AW1
- Spin 1 min at 8000 rpm and discard flow through
- Add 500µl Buffer AW2
- Spin 3 min at 14000 rpm and discard flow through
- Put column onto new eppendorf.

- Add 40µl ddwater at 70ºC Ddwater 70ºC
- Leave at RT 1 min
- Spin 1 min at 8000 rpm and keep at -20ºC

c) DNA extraction by PK protocol

Day 1- Deparaffinization

- Incubate 60ºC 30 min OVEN 60ºC
- Wash in xylene 3x 2 min
- Wash in alcohol 100% 2 min
- Wash in alcohol 95% 2 min
- Wash in alcohol 70% 2 min
- Wash in alcohol 50% 2 min
- Wash in Ddwater 2 min
- Dry at room temperature (RT)

Day 1- Microdissection

- Scrape with a tip/blade and introduce in eppendorf with 15µl Digestion buffer* and mix
- Add 20µl mineral oil
- Cover eppendorf with parafilm
- Incubate 55ºC ON OVEN 55ºC

Day 2: Proteinase K treatment

Add 0,3 µl of PK for every 15µl of buffer through the oil

- Incubate 55º C ON

Day 3: Proteinase K treatment

Add 0,3 µl of PK for every 15µl of buffer through the oil

- Incubate 55º C ON

Day 4: Extraction

- Inactive PK at 95ºC for 10-15 min.
- Remove the aqueous phase (DNA), trying not to remove any oil

* Digestion Buffer (100 µl)

PCR Buffer 10X	10µl	(1x)
Tween 20	0,5µl	(0,5%)
PK 20 mg/ml	2µl	(4 mg/ml)
Distilled water	87,5µl	

Add 15 µl for anything less than 1 mm, and proportionally more if larger

For DNA extracted for array CGH analysis. After DNA is extracted by PK protocol, it has to be purified using Amicon Microcon YM-30 columns (Millipore)

- Add 450 µl ddwater to column, spin 8 min at 12000 g at RT
- Discard flow through
- Add ddwater to the DNA to be concentrated up to a volume of 450 µl
- Apply to column, spin 8 mins. at 12000 g at RT
- Discard flow through
- Add 450 µl ddwater to column, spin 8 min at 12000 g at RT
- Discard flow through
- Add 450 µl ddwater to column, spin 8 min at 12000 g at RT
- Dump flow through. Check for amount of ddwater remaining on top of the column. If it looks like more than 15 µl, then spin column again for 2 min
- Flip the column into a new 1.5 ml tube. Spin 1 min at 13000 g

3. DNA quantitation

DNA for general use was quantitated by spectrophotometer. Dilution 1:100 in ddwater, and read at 260/280.

DNA for array CGH analysis was quantitated by Taqman real time polymerase chain reaction amplification of sequences containing CA repeats at: D1S2868 (1p22), D2S385 (2q31), D4S1605 (4p16), D5S643 (5q32), D10S586 (10p12), and D11S1315 (11p15).

4. Polymerase Chain Reaction

50 µl PCR reactions were done using:

10-50 ng of DNA	
0.4 µmol/L of each primer	(2µl 10µM aliquot)
500 nmol/L deoxynucleotide triphosphates	(5µl dNTP aliquot- madre 25µM)
3.5 mmol/L $MgCl_2$	(7µl magnesium)
1xPCR II buffer	(5µl buffer)
1.5 units of Amplitaq Gold DNA Polymerase	(0.3µl Taq)

PCR conditions were as follows:

94 ºC	(10 min) for 1 cycle	
94 ºC	(40 sec)	nº cycles- 35 if cell line, 40 if DNA extracted from tissue
Annealing temp ºC	(40 sec)	
72 ºC	(40 sec)	
72 ºC	(10 min)	

Primer sequences for Tp53: exons 4-9.

Exon		Primer sequence	Fragment (bp)	Annealing temperature
4	F	CACCCATCTACAGTCCCCCTTG	307	63
	R	CTTGCACGGTCAGTTGCCCTGAG		
5	F	TTTCAACTCTGTCTCCTTCCT	257	63
	R	GACAGGGCTGGTTGCCCA		
6	F	ACGACAGGGCTGGTTGCCCA	200	67
	R	GCAACTGGGGTCTCTGGGAG		
7	F	CCTCATCTTGGGCCTGTGTT	209	63
	R	CTTGCCGCTGACCCCTGG		
8	F	CTGCCTCTTGCTTCTCTTTT	204	63

	R	ACAAGAAGCGGTGGAGGAGA		
9	F	TTATGCCTCAGATTCACTTTTAT	212	63
	R	TGAGCTGTTTTACCTGCAATTG		

FGFR3: exons 7, 10 and 15

Exon		Primer sequence	Fragment (bp)	Annealing temperature
7	F	AGTGGCGGTGGTGGTGAGGGAG	161	63
	R	CTGCAAGGTGTACAGTGACGCACA		
10	F	CAACGCCCATGTCTTTGCAG	199	63
	R	CAAGATCTCCCGCTTCCCG		
15	F	GAGAGGTGGAGAGGCTTCAG	228	63
	R	TCATGCCAGTAGGACGCCT		

PIK3CA: exons 9 and 20.

Exon		Primer sequence	Fragment (bp)	Annealing temperature
9	F	TGAAAATGTATTTGCTTTTTCTGT	340	60
	R	TGTAAATTCTGCTTTATTTATTCC		
20.1	F	TTTGCTCCAAACTGACCAA	300	60
	R	GCATGCTGTTTAATTGTGTGG		
20.2	F	ACTGAGCAAGAGGCTTTGGA	372	60
	R	TTTGGACTTAAGGCATAACATGAA		

All mutations were confirmed by analyzing the products of a second, independent, PCR. When a previously undescribed sequence variant was found in tumor DNA, it was confirmed using independent PCR reaction products. Germline DNA was used to determine the somatic nature of previously undescribed variants.

Samples without DNA template were included in all assays as negative controls.

PCR products were separated by electrophoresis in 1% agarose gels and visualized with ethidium bromide.

5. PCR product purification

PCR products are purified by two different kits according to the number of samples. Small number of samples are purified by the GFX kit, and high number of samples are purified by plate with the Montage PCR Cleanup Kit.

GFX Kit (Amersham biosciences)

- Add 500 µl of CAPTURE BUFFER to PCR product and tranfer onto column
- Spin 30 sec at 14000 rpm and discard flow through
- Add 500 µl of WASH BUFFER
- Spin 30 sec at 14000 rpm and discard flow through
- Transfer column onto new eppendorf
- Add 40 µl of TE BUFFER
- Leave 1 min at RT
- Spin 1 min at 14000 rpm and store at -20ºC

Montage PCR Cleanup Kit (Millipore)

- Add 50µl of TE buffer (0.01M) to PCR product and tranfer onto purple plate
- Filter with Millipore Vaccum Manifold for 7 min and discard flow through
- Add 25µl of Ddwater and cover with parafilm
- Shake for 10 min
- Transfer to clean plate and store at -20ºC

6. Sequencing reaction

10 µl Sequencing reactions were done using:

5µl PCR product	
0.32 µmol/L of each primer	(1µM aliquot)
5x Sequencing buffer	(1µl buffer)
1 µl units Sequencing mix	(0.3µl Taq)

Sequencing conditions were as follows:

96 ºC	(10 min) for 1 cycle	
96 ºC	(10 sec)	25 cycles
55 ºC	(5 sec)	
60 ºC	(4min)	

7. Sequencing precipitation

Sequencing products are purified by two different protocols according to the number of samples. Small number of samples are precipitated by Sodium Acetate protocol, and high number of samples are precipitated by plate with the Montage Seq Cleanup Kit.

Sodium Acetate protocol

- Mix and vortex
    - 50 µl 100% Ethanol (from 4ºC)
    - 10 µl Ddwater
    - 2 µl 3M Sodium Acetate
- Add 62 µl of mix en to each sequencing reaction product
- Mix and transfer to 1.5ml eppendorf
- Leave at RT 15min
- Spin at 4ºC for 30 min at 13000rpm and discard supernatant
- Add 70 µl of 70% Ethanol (at -20ºC)
- Spin at 4ºC for 1 min at 13000 rpm and discard supernatant
- Incubate 5 min at 37ºC and store -20ºC

Montage Seq Cleanup Kit (Millipore)

- Add 20µl of injection buffer to sequencing reaction product and tranfer onto blue plate
- Filter with Millipore Vaccum Manifold for 4 min and discard flow through
- Add 25µl of injection buffer
- Filter with Millipore Vaccum Manifold for 4 min and discard flow through
- Add 25µl of injection buffer and cover with parafilm
- Shake for 10 min
- Transfer to clean plate and store at -20ºC

8. Taqman Quantitative PCR analysis.

Primers and probes for the 6 genes at 10q were designed by the Custom Assay Gene expression facility of Applied Biosystems or Primer Express software (Applied Biosystems) and synthesized by The Oligo Factory (Applied Biosystems). Primers and probes for CDK5 were designed by …. Dr. Perez Jurado and for MDM2 were taken from …

Gene		Primer sequence	Probe 6'FAM
CDK5	F	GGAACTGTGTTCAAGGCCAAA	ACCGGGAGACTCATG
	R	CCCGTTTCAGAGCCACGAT	
MDM2	F	CGGGAGTTCAGGGTAAAGGT	ATCATCCGGACCTCCC
	R	GCGCAGCGTTCACACTAG	
MXI1	F	TCAGAAGAGAATGGAGGGTGTGT	CTGCATCAAGAACAAAAC
	R	CCCCTAGTCCTTAAAGACCTATAATTGAAC	
FAS	F	TGGGCATCCATAGCAAGTTGAT	TTGTCCTACGGCTTCTGC
	R	CGGCAAGACTATGGCAGGAT	
DMBT1	F	AGCCCCTGGTTCCCCTAA	ACTGCCAGGTAAAATG
	R	AGACGCCATTGCTGAAGCT	
FGFR2	F	TCTTCTGCGTTTGGAGTTGCT	ACAACCCCGGGCTCG
	R	GGGTCGGGATGGAGAAAGC	
BUB3	F	GGACTTACGGAACATGGGTTACG	CAGGCTGGACTCCCTG
	R	CGTATGCAGCGAGTCTGGTATTT	
DNTT	F	GAAAAGCTCAGGTTGCCTAGCA	TTGGATCATTTTCAAAAGT
	R	CCACTCTTTGACGAGGCAATTT	

20 µl reactions were done using:

CDK5

10ng DNA	
900 nM of each primer	(100µM aliquot - 0.18µl)
10nM probe	(5µM aliquot- 0.4µl)
1x Taqman PCR master mix	(10µl mix)

MDM2 and 6x 10q genes

10ng DNA

1 µl primer/probe mix

1x Taqman PCR master mix (10µl mix)

Taqman PCR parameters

50 ºC (2 min) for 1 cycle

95 ºC (10 min) for 1 cycle

95 ºC (10 sec) ⎫

60 ºC (1 min) ⎭ 40 cycles

Assays were performed using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). All assays were performed in duplicate.

TaqMan software was used to calculate a *Ct* value for each reaction, where the *Ct* value is the point in the extension phase of the PCR reaction at which the product is distinguishable from the background. To quantify gene copy number in each sample and calculate fold changes, a calibrator was used. This consisted of a pool of DNA isolated from paraffin-embedded normal tonsil (n=9), liver (n=4), kidney (n=4), and spleen (n=1).

Taqman results were classified as gain, when the gene dose was >1.3 compared to DNA from normal tissue, >5 (amplification), and <0.4 (loss).

9. Immunohistochemistry.

All immunohistochemical assays were performed using formalin-fixed, paraffin-embedded tissue.

Deparaffinization: as done for DNA extraction by PK protocol except you leave the slides in the ddwater in the last step.

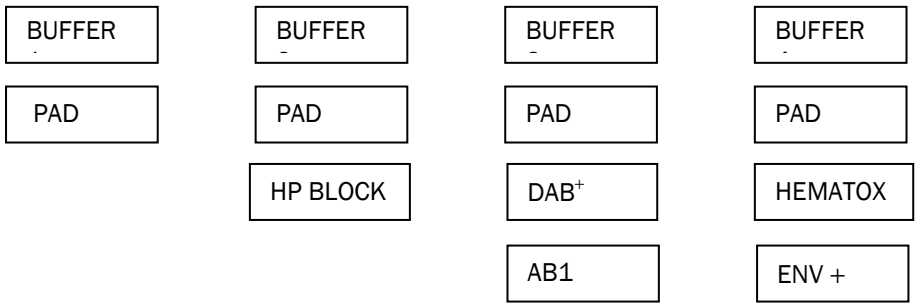
Antigenic recuperation: the recuperation depends on the Antibody used. The following table summarizes the Antibodies, dilution, species and recuperation.

Gene	Ab	Dilution	Species	Antigenic recuperation
P53	DO7	1:50	mouse	10 mM citrate pH 7.3 at 120 ºC for 1 min in an autoclave
HDM2	2A10	1:500	mouse	10 mM citrate pH 7.3 for 15 min in a microwave
Survivin	RB-9245	1:100	rabbit	10 mM citrate pH 6.0 and pressure cooker for 20 min
IGF1-R	24-31	1:100	mouse	50 mM EDTA pH 8.0 and pressure cooker for 20 min
14-3-3 σ	1433S01	1:100	mouse	10 mM citrate pH 6.0 and pressure cooker for 20 min
COX-2	RB-9072	1:100	rabbit	10 mM citrate pH 6.0 and pressure cooker for 20 min
PTEN	17A	1:2	mouse	No retrieval (O.N. 1ºAb)
FGFR3		1:50	mouse	10 mM citrate pH 6.0 and pressure cooker for 20 min
Annexin		1:1000	rabbit	10 mM citrate pH 7.3 at 120 ºC for 1 min in an autoclave

A Tech-Mate 500 instrument (Ventana Medical System, Tucson, AZ) was used for immunostaining. As secondary antibody, the Envision+ anti-mouse or anti rabbit reagent was applied (DAKO, Copenhagen, Denmark). Reactions were developed using diaminobenzidine. Sections were counterstained, dehydrated, and mounted. All tissue areas were examined for staining; both intensity (1+ to 3+) and proportion of reactive cells were assessed.

Tech-Mate 500 instrument:

- Program: Envisión+
- Fill wells as appropriate:
  - AB1 is the primary antibody
  - Pad: 1-2 are new pads, once used they are recyled onto locations 3-4, these are thrown after each IHC in a special container.
  - DAB^+ : 1 drop of chromogen for 1ml of substrate
  - ENV+ is the secondary antibody. Put 9 drops per well
  - Buffers: 400µl per well



- Place tissue sections in the correct arm of the instrument. If there are less than 10 slides use row B, if there are more, compensate them between row A and C.
- Click on the RUN button***

Once the Tech-Mate machine has finished slides should be placed in ddwater.

Dehydration:

- Wash in alcohol 50% 2 min
- Wash in alcohol 70% 2 min
- Wash in alcohol 95% 2 min
- Wash in alcohol 100% 2 min
- Wash in xylene 3x 2 min

Mount the slides and they are ready for scoring.

***Envision+ program

- 4 washes with buffer 1 of 10'' each
- 1º Ab 30 min (except PTEN 12h)
- 3 washes with buffer 1 of 10'' each
- Peroxidase blocking with H_20_2 blocking solution 7min and 30''
- 3 washes with buffer 2 of 10'' each
- 2º Ab 30 min
- 2 washes with buffer 2 of 10'' each
- 3 washes with buffer 3 of 10'' each
- DAB+ 7 min
- 5 washes with buffer 3 of 10'' each
- Hematoxilin for 1min
- 2 washes with buffer 2 of 10'' each
- 2 washes with buffer 3 of 10'' each
- 3 washes with H_20 of 10'' each

10. Array CGH.

1) Random Prime Amplification

RP DNA labeling system (Cat. No: 18094-11) contains the 2.5x Random Prime solution [125mM Tris-HCL (pH 6.8), 12.5 mM MgCl2, 25mM 2-mercaptoethanol, 750 micrograms/ml oligodeozyribonucleotide primers (randome octamers)] and the Klenow Fragment (large fragment of DNA polymerase I) [40 U/microl Klenow Fragment in 50mM Potassium Phosphate (pH 7.0), 100 mM KCl, 1mM DTT, 50% Glycerol]. The random primers are annealed to the denatured DNA template and extended by klenow fragment. Considerable DNA synthesis occurs resulting in an 10-40 amplification of the probe.

Test:	***Reference:***
50-100 ng paraffin tumor DNA	50 ng fresh genomic DNA
10 µl 2.5x RP mix (from Invitrogen BioPrime Kit)	10 µl 2.5x RP mix
xx µl ddH_2O	xx µl ddH_2O
22 µl total	22 µl total

- Vortex, quickspin. Heat to 100°C 5-10 min. Use thin-walled PCR tubes only. Place on ice immediately for 10 minutes.
- Add 2.5 µl RPA dNTPs and 0.5 µl Klenow enzyme, vortex gently, quickspin.
- Incubate for 2 hours at 37°C. Do not incubate for more than 2 hours.

RPA dNTPS: 200 µl total: 4 µl 100 mM dATP, 4 µl 100 mM dCTP, 4 µl 100 mM dGTP, 4 µl 100 mM dTTP, 2 µl 1 M Tris pH 8, 0.4 µl 0.5 M EDTA pH 8 & 181.6 µl dH_2O

Product could be stored on ice for up to half an hour or for a couple of hours in the freezer before the columns.

2) Qiaquick purification of amplified DNA product:

After amplification the product is cleaned up to remove unincorporated nucleotides and for buffer exchange into ddwater for the next step of random prime labeling with the Qiagen PCR purification Kit (Cat. No 28106).

- Add 125 µl of PB buffer to RPA reaction (5x volume)
- Place Qiaquick column in 2 ml tube, pipet reaction onto column
- Spin at 12400 x g for 60 sec, 25°C, dump liquid
- Add 750 µl PE buffer to the column
- Spin at 12400 x g for 60 sec, 25°C, dump liquid
- Spin again at 13000 x g for 60 sec, 25°C
- Place column in a clean collecting tube
- Add 34 µl EB, leave 4 min at room temperature
- Spin at 12400 x g for 60 sec, 25°C. The eluent should be around 32-33 ul.
- Product can be stored at -20°C if needed.

3) Random Prime Labeling

In PCR room; always make sure about lot number

The RP mix and Klenow come from the RP DNA labelling kit as in the amplification protocol. The dNTPs should have been made yesterday with the RPA dNTPs, they last for about 2 weeks. For each Test sample labeled with Cy3 a corresponding Reference (gender matched) is labeled with Cy5. Avoid exposure to light as much as possible for the cy dyes, specially when washing as higher temperatures accelerate photodestruction of the fluorophore. Cy dyes are from Amersham Biosciences (Product code PA 53022 Cy3-dUTP and PA 55022 Cy5-dUTP) and are at 25nmoles in 10mM phosphate buffer, pH 7.0. They exhibit low non-specific binding, high photo stability, high ddwater solubility and pH insensitivity. Cy3-dUTP has an absorbance max of 550nm and emission max of 570nm; Cy5-dUTP has an absorbance max of 649nm and emission max of 670nm. Cy3 is more stable than Cy5. Minimize freeze-thaw cycles.

- The following is prepared in separate tubes 0.5ml

1) Test:	2) Reference
32.4 µl of the cleaned RPA	32.4 µl of cleaned reference RPA DNA normal
32 µl 2.5x RP mix	32 µl 2.5x RP mix
64.4 µl	64.4 µl

- Vortex, quickspin. Heat to 100°C for 10 min. Use thin-walled PCR tubes. Place on ice immediately for 10 minutes
    - Thaw dNTPs for labeling, Cy5 and Cy3 (25 µl per tube), klenow enzyme
- Remove from ice, quick spin then add

    8 µl RPL dNTPs

    1.6 µl Klenow enzyme (from Invitrogen BioPrime Kit)

    6 µl **cy3-dUTP** (test) or **cy5-dUTP** (reference)

    15.6 µl total volume
- Vortex gently, quickspin. Incubate for 2 hours at 37°C. Do not incubate the amplified DNA for more than the 2 hours. In biosafety room

RPL dNTPS: 200 µl total: 4 µl 100 mM dATP, 4 µl 100 mM dCTP, 4 µl 100 mM dGTP, 2 µl 100 mM dTTP, 2 µl 1 M Tris pH 8, 0.4 µl 0.5 M EDTA pH 8 & 183.6 µl dH_2O

SLIDE PREPARATION..... step 6

4) Sephadex column purification of labeled probe:

Labelled products are purified in Amersham MicroSpin G-50 Columns (Product number: 27-5330-02). The columns are designed for removal of unincorporated nucleotides. The columns contain sephadex G-50 DNA. Columns are supplied pre-equilibrated in TE

buffer (10mM Tris-HCl pH 8.0 and 1mM EDTA) containing 0.05% Kathon CG/ICP Biocide as a preservative.

- Prepare two columns per sample as it takes a maximum volume of 40 µl.
- Mix column by vortexing, snap off bottom, loosen cap, place column in 1.5 ml screw cap tall tube, spin 1 min at 750 x g.
- Place column in new tube, add 40 µl of sample into each column and spin 2 mins at 750 x g. Join the two tubes of each sample into one. You should be able to see a pinkish tint for the test and a bluish tint for the reference. If it is difficult to see the color when holding the tube over a white piece of paper then the samples probably did not label efficiently. Samples may be stored at -20°C protected from light.
    - Turn on a centrifuge at 4 degrees- fast cool
    - Thaw Cot-1
    - Turn on slide warmer to 37ºC
    - Put 100% ethanol at –20ºC
    - Take out 3M NaAc to RT
    - Label 1.5 ml eppendorf tubes with your test numbers

Alternative……Use of **New bioprime kit** (Cat. No 18095-011). Amplification protocol is the same but when purifying by qiagen columns resuspend in 22 µl of EB. In the random prime labelling mix 21 µl of RPA with 20 µl of RP mix and use the dUTPs from the new kit (not own made).

5 µl RPL dNTPs

1 µl Klenow enzyme (from Invitrogen BioPrime Kit)

3 µl **cy3-dUTP** (test) or **cy5-dUTP** (reference)

9 µl total volume

When purifying by sephadex columns use 1 column per sample as max volume of reaction is 50 µl. The rest of the protocol is the same.

5) Reprecipitate DNA's From here onwards always outside PCR room and no filter tips

Avoid exposure to light as much as possible for the cy dyes. Measure the DNA and combine test with reference. Then add in order:

- 100 µl Human Cot-1 DNA (500 µl Cot-1 DNA per stock and 1mg/ml). Human Cot-1 DNA (Cat. No. 15279-011) from Invitrogen is obtained from human placental DNA and can be used to suppress cross-hybridization to human repetitive DNA.
- Add X µl room temperature 3M Na Acetate, mixing with pipet (1/10th of Cot-1 + sephadex purified RPL)
- Add Y µl 100% EtOH at -20, vortex gently (2.5X Cot-1 + sephadex purified RPL)
- Spin 30 mins at 14,000 rpm (20K x g), 4 °C.
- *SLIDE PREPARATION.....step 6*
    - Prepare slide incubation chamber. We use a flat rectangular plastic slide holder with the opening on one end. Add 300ul of HWB wash buffer in the bottom of the chamber and place chamber on the 37 °C slide warmer or place gasquettes and glass slides on warmer.
    - Put array slides onto 37 °C slide warmer
    - Turn on ddwater bath to 70 °C
- Decant supernatant; kimwipe ethanol, being careful to avoid DNA pellet. Allow pellet to dry for about 15 minutes at RT.
- Carefully add 4 µl dH20, 8 µl 20% SDS, 6µl yeast tRNA (Invitrogen Cat. No. 15401-029, 100 µg/ml). Do not resuspend with pipet, all for 10 min, quick spinning down every couple of min. It is critical that the pellet is resuspended well before the next step.
- Add 42 µl of room temperature MasterMix 1.0, do not resuspend with pipet, vortex vigorously for 10 min, quick spinning down every couple of min. It is ok to leave it longer to ensure full resupension of the probe.

- Quickly spin (1 sec) to bring the probe mix to bottom of tube.

6) Slide Preparation:

- Select slides with arrays that have no marks on the chromium or missing chromium. Array spots should all be nice and round and not touching.
- Etch the information on the slide on the label side with a diamond pencil so it is clear which is the top of the slide and remove any pen marks from the slide with a cotton rod and ethanol. Mark the array carefully on the top edges with a minimal mark using a diamond pencil (about 2 mm away from outer edges of array). You do not want to etch too deeply or into where the rubber cement border is going to be. By blowing you will be able to see the array and mark the slide clearly.
- UV cross link the slide at 1200 mjoules in a stratalinker. Situated between Pinkel's and Albertson's lab. Make sure slides are face up, press autocross link and start.
- Using a 21 Ga Needle and a 3 cc syringe carefully make a rubber cement dam around the two arrays. Check all the rubber cements in the lab and use the most liquidy. Fill the syringe of rubber cement without the needle, put the needle on and make sure to leave at least 2 mm from the edge of the array and the rubber cement border. Apply rubber cement around 10 times cause it shrinks. Allow rubber cement to dry at RT for about 10 minutes or more. Protect arrays from dust with the top of a tip box. If using gasquette system allow to dry for 4 hours.
- Place slide on 37 °C slide warmer for a minimum of 30 minutes before adding the probe mix.

7) Hybridization:

- Denature the resuspended probe mix at 70 °C for 10-15 minutes.

- Transfer immediately to a 37 °C water bath and incubate for 30 minutes. (probes can be at 37 °C a little longer if necessary due to handling multiple samples at once. It is best to time the denaturation and 37 °C incubation for the probes in sets of 4 (for two slides).
- Quick spin down probe mix, resuspend gently with pipet, then apply warm probe immediately onto warmed slide on the hot plate without gloves!!!!. Spread out the probe mix while adding, and avoid bubbles as much as possible. Repeat for second array, then immediately rock the slide back and forth to spread out the probe mix over the whole array, making sure it reaches all edges of the rubber cement. Sometimes it needs a little help and you can use a pipet tip to gently coach it to the edge.
- Apply probes to second slide, and repeat the spreading out technique, leaving first one on the 37°C warmer. Bubbles from the SDS should disperse.
- It is critical that the probe mix is well dispersed, has minimal bubbles before placing it into the slide chamber. Place slides into chamber taking care to keep the slides level, and to avoid disrupting the rubber cement. Seal the chamber with a layer of rubber cement, then a layer of parafilm. Keep the bottom of chamber as flat as possible, do not fold the parafilm on the bottom. If using gasquette system just put gasquette on top of slide and sandwich it with the glass slide. Then clip with metal clips and put onto plastic support in the incubator.
- Incubate for 2 days at 37 °C in a humid chamber on a slowly rocking platform. Move the chamber to the other end of the rocker 2 times per day to endure equal mixing.

DAY 2: WASHES AND STAINING

- Remove slide from chamber keeping it level, and carefully remove the rubber cement on a warm surface, make sure the array is remaining, moist. If it is

starting to dry out then stop and place it into the washes, you can remove the remainder while in the PN step.

- Rinse the slide once with PN, then place into pre-warmed HWB (50% formamide/2XSSC) at 45 °C, Swish slide and then incubate for 15 minutes.
- Wash 1X for 20-30 minutes at 45°C in 2X SSC/ 0.1% SDS. (the longer time is for when there are more than 2 slides). Do not wash more than 4-5 slides at a time.
- Wash 1X for 10 mins at RT in PN, remove any remaining rubber cement during this step, taking care to keep slide fully wet.
- Wash 1X for 10 mins at RT in PN.
- Remove slide from PN, blot back and one side, and leave slide wet, then add 100 µl of RT 0.3µg/ml DAPI in PBS buffered glycerol, and carefully place a 22x50 mm coverslip over arrays, avoiding bubbles. Blot out excess DAPI and PN from the edges onto kimwipes and carefully seal the edges with clear nail enamel.

DAY 3: IMAGING

- Slides are usually left flat, overnight at RT in dark container and imaged the following day.
- Chromium slides must be imaged using a CCD camera set up, typical exposure times on our current in house system, are: DAPI 3000ms; Cy3 45-60,000 ms; cy5 180,000 ms. Clear slides can be imaged on the axon scanner, with typical pmts being for cy3 540, and for cy5 680

SOLUTIONS

20X SSC: 3.0M NaCl: (87.66g /500ml) pH 7.00; 0.3M Na Citrate (citric acid) (44.115g/500ml) pH 7; autoclave, keep sterile.

Master Mix 1: 5.0 ml formamide (final=50%) (50% form, 2X SSC); 1.0 ml 20X SSC (final=2X); 1.0 g dextran sulfate (final=10%). Must heat at 70°C 1-2 hours to dissolve the dextran sulfate. Bring to pH 7.0 with HCl using pH paper; bring volume to 7.0 ml with ddH_20

Hybridization Wash Buf: 50% formamide in 2X SSC (0.50 volume formamide + 0.1 volume 20X SSC) pH 7.0 with HCl; bring to volume with ddH_20.

0.33M Dibasic PN Stock: 353.76g $Na_2HPO_47H_20$ (mwt 268), 13.3 mls NP40, make up to 4 Liters

0.33M Monobasic PN Stock: 45.5g $NaH_2PO_4.H_20$ (mwt 138), 3.3 mls NP40, make up to 1 Liter

0.1M $NaH_2PO_4.H_20$/0.1%NP40: METHOD A: Take 300 mls of 0.33M Monobasic stock and 700 mls dH20. METHOD B: 13.799g sodium phosphate monobasic hydrate +1.0ml NP40 per liter ddH20; autoclave.

0.1M $Na_2HPO_47H_20$/0.1%NP40: METHOD A: Take 1.0 Liter 0.33M Dibasic Stock, add 2.3 Liters dH20 METHOD B: 26.807g sodium phosphate dibasic eptahydrate+1.0ml NP40 perliter ddH20; autoclave.

PN Buffer: METHOD A and METHOD B: Add 0.1M $NaH_2PO_4.H_20$/0.1%NP40 to the desired volume of 0.1M $Na_2HPO_47H_20$/0.1%NP40 to obtain pH 8. About .05X volume of MONOBASIC PN must be added to DIBASIC PN; autoclave

PNM Buffer: 25 g (5% final) carnation instant milk protein int 500 ml autoclaved PN buffer, and shake, do not stir!! Add 0.5g (0.1%) Na azide, incubate at 37°C X 1-2 hr, leave at RT ON. . Spin before use to remove particulates. Store at 4°C.

2X SSC: Dilute 20X SSC 1:10 , pH to 7.0 (100 mls 20X, up to 990mls with dH20 and pH to 7.0, and bring to final volume of 1000 ml; autoclave

0.5 M EDTA, pH 8.0 186.1g disodium EDTA.2H20 to 800 ml dH20. Mix with magnetic stirrer, and adjust pH to 8.0 with conc NaOH solution (5N-10N). The EDTA does not dissolve fully until it is around pH 8.0.

3M Sodium Acetate,pH 5.2: 408.1g of sodium acetate.3H20 in 800 ml dH20, adjust to pH 5.2 with glacial acetic acid, adjust to 1 Liter volume.

Human 2.0 arrays were obtained from the University of California, San Francisco Cancer Center array core. The array consisted of 2464 BAC clones spotted in triplicate and distributed approximately uniformly across the genome with an average genome-wide resolution of 1.4 Mb.

The acquired microarray images were analyzed using Spot and Sproc software.

http://jainlab.ucsf.edu/Downloads.html

Hernández S, López-Knowles E, Lloreta J, Kogevinas M, Jaramillo R, Amorós A, Tardón A, García-Closas R, Serra C, Carrato A, Malats N, Real FX.
FGFR3 and Tp53 mutations in T1G3 transitional bladder carcinomas: independent distribution and lack of association with prognosis.
Clin Cancer Res. 2005 Aug 1;11(15):5444-50.

Hernández S, López-Knowles E, Lloreta J, Kogevinas M, Amorós A, Tardón A, Carrato A, Serra C, Malats N, Real FX.
Prospective study of FGFR3 mutations as a prognostic factor in nonmuscle invasive urothelial bladder carcinomas.
J Clin Oncol. 2006 Aug 1;24(22):3664-71.