3.4 Anàlisi d'expressió de gens apoptòtics (V)

Casas S, Ollila J, Aventin A, Vihinen M, Sierra J, Knuutila S. Changes in apoptosis-related pathways in acute myeloid leukemia. Cancer Genet and Cytogenet 2003;acceptat, pendent de publicació.

3.4.1 cADN array

Es va analitzar l'expressió de 205 gens relacionats amb el cicle cel.lular i l'apoptosi en cèl.lules mononucleades de 10 mostres de MO de pacients de LMA mitjançant cADN array. La mostra de referència provenia de cèl.lules mononucleades aïllades de 5 mostres de MO d'individus sans.

En relació a la mostra de referència, l'anàlisi va identificar 34 gens amb expressió anòmala, dels quals 22 eren sotaexpressats i 12 sobreexpressats (Taula 3 i Fig. 2). El perfil d'expressió gènica es representa a la figura 1, on gens i pacients s'han agrupat segons semblança d'expressió mitjançant una anàlisis no dirigida, jeràrquica i aglomerativa. Pel què als 9 gens *housekeeping* inclosos a l'array, la seva expressió va ser semblant entre mostres amb una variància del 0,09.

El resultats indicaven alteracions en diverses vies de senyalització cel.lular relacionades amb l'apoptosi. Entre aquestes destacar, canvis en l'expressió de gens relacionats amb la via de senyalització de TNF, ja que *ADAM17* i *RIPK1* estaven sobreexpresats, i *TNFSF5*, *TNFA*, *NSMAF* i *LTB* eren sotaexpressats (Fig. 3). Així mateix, es van observar alterats nivells d'expressió de diversos gens de la via de senyalització de IGF, com sobreexpressió de *IGF10* i sotaexpressió de *IGF1R*, *IGF2*, *IGFBP3* i *IGFBP5* (Fig. 4). Alhora, els gens relacionats amb la via de senyalització de GSH, *GPX1*, *GSR*, *GSTM1*, *GSTP1* i *MGST1*, es van detectar tots sotaexpressats (Fig.5). A més, en relació a la reacció de les caspases, es va identificar sobreexpressió de *CASP8*, i desregulació de l'expressió de varis gens codificants per proteïnes de tipus BCL2 com, sobreexpressió de *BAG-1* i *BCL2A1*, i sotaexpressió de *NIP3* i *DAD1* (Fig. 6). També es van observar

nivells anòmals d'expressió en gens de resposta a lesions en l'ADN, com sotaexpressió dels supressors de tumors *P53* i *CSE1L*, i sobreexpressió de *GADD45A* (Fig. 7A). Tanmateix es van detectar alteracions en l'expressió de diversos gens relacionats amb el cicle cel.lular (Fig. 7B). S'observava sobreexpressió de *RBBP4*, alteració en el punt de control de cicle cel.lular G1/M per sobreexpressió de *CDC37* i sotaexpressió de *CDKN2A*, així com en G2/M per sobreexpressió de *NEDD5*. Finalment, es va detectar sotaexpressió del gen codificant per la proteïna de senyalització *MAPK7*, mentre que els factors de transcripció *JUN* i *E2F5* estaven sobreexpressats i *NF-ATC1* sotaexpressat.

3.4.2 Validació dels resultats

Per tal de validar els resultats obtinguts per cADN array i per augmentar l'anàlisi d'expressió en una sèrie més extensa de pacients, es va quantificar l'expressió de 4 gens relacionats amb l'apoptosi (*MCL1*, *MYC*, *BAX* i *DAPK1*) emprant QRT-PCR a temps real. La mitja dels valors d'expressió relativa en relació a la mostra de referència dels 15 pacients de LMA analitzats, en escala $\log_2$, eren 6,4 ± 7,7 per *MCL1*, 3,8 ± 5,2 per *MYC*, 3 ± 3,4 per *BAX* i 5,5 ± 7,3 per *DAPK1*. Aquests valors es correlacionaven significativament amb els obtinguts mitjançant cADN array (Fig. 8).

Changes in apoptosis-related pathways in acute myeloid leukemia

Sílvia Casas[a,b], Juha Ollila[c], Anna Aventín[b], Mauno Vihinen[c,e], Jorge Sierra[b], Sakari Knuutila[a,]*

[a]*Departments of Pathology and Medical Genetics, Haartman Institute and Helsinki University Central Hospital, University of Helsinki, Helsinki, Finland;* [b]*Department of Haematology, Hospital Sant Pau - Autonomous University of Barcelona, Barcelona, Spain;* [c]*Division of Biochemistry, Department of Biosciences, University of Helsinki, Helsinki, Finland;* [e]*Institute of Medical Technology, University of Tampere, Tampere, Finland.*

Running title: Changes in apoptosis-related pathways in AML

Key words: cDNA array, gene expression, apoptosis-related pathways, acute myeloid leukemia

*Correspondence: Sakari Knuutila, PhD,
Department of Medical Genetics,
POB 21 (Haartmaninkatu 3, 4th flr)
FIN-00014 University of Helsinki, Helsinki, Finland.
Phone: +358-9-191 26527
Fax +358-9-191 26788
E-mail: Sakari.Knuutila@helsinki.fi

Abstract

Expression analysis of apoptotic genes was performed for 15 patients with acute myeloid leukemia (AML) at the time of diagnosis to identify genes and signaling pathways involved in the regulation of cell survival and apoptosis during leukemogenesis. cDNA array analysis revealed 34 genes whose expression was significantly different as compared to the reference. Tumor suppressor genes *P53* and *CDKN2A* were down-regulated, whereas proto-oncogenes *JUN* and *GRB10* were up-regulated. Furthermore, several cellular signaling pathways acting either in cell cycle egulation or in apoptosis were altered. Deregulation was found in pathways which contribute to genomic stability (by down-regulation of either *P53* or *CSE1L,* and by up-regulation of *GADD45*) and regulate cell cycle progression (by down-regulation of *CDKN2A* and up-regulation of *RBBP4, CDC37* and *NEDD5*). Alterations at the transcriptional level were identified, namely up-regulation of *JUN* and *E2F5*. Abnormalities were observed in the regulation of the caspases through up-regulation of *CASP8* and by altered expression of BCL2-related pathway. Extrinsic apoptotic signals mediated by IGFs were deregulated, whereas the glutathione detoxification pathway was down-regulated. These findings provide novel insight into the regulation of balance between apoptosis and cell proliferation signals, and suggest that these genes and pathways may have an important role in the pathogenesis of AML.

1. Introduction

Acute myeloid leukemia (AML) is a hematologic disease with heterogeneous clinical and biological features. The genesis of AML is induced by alteration of the normal structure and role of genes that control both proliferation and differentiation and prevent the normal apoptosis of hematopoietic precursor cells. The multipotential leukemic stem cell pool could therefore differentiate into committed progenitor cells, which retain the capability of maturing into a lineage spectrum of different morphological AML subtypes [1]. AML is characterized by the presence of specific balanced chromosome abnormalities which lead to specific gene fusions. The novel hybrid protein directly interferes with the control of myeloid differentiation and regulation of cell survival and apoptosis [2, 3].

An alteration due to a defect in the normal regulation of either apoptosis or cell proliferation signaling induces the cell population to survive and accumulate, promoting further genetic aberrations and tumorigenesis. The importance of apoptosis in the pathogenesis of AML has been well established. Several fusion proteins interact with mediators of apoptosis, sending anti-apoptotic signals, such as PML/RARα or CBFβ/SMMHC through the P53 pathway, or AML1/ETO through the BCL2-related pathway [4-6]. Furthermore, it has been suggested that MLL fusion proteins could modify the GADD34 function and inhibit apoptosis [7, 8]. Recently, higher levels of *BCL2A1* were found to be associated with normal karyotype in AML patients [9]. However, neither the specific disrupted genes nor the altered pathway of apoptosis have been well described. Conventional anti-cancer drugs in AML are designed to promote apoptosis in target cells, but a leukemic cell population with defects in normal apoptosis signaling could be refractory to conventional treatment [10, 11]. Studies concerning apoptosis regulation in AML are therefore needed to identify therapeutic targets that can defeat the resistance to apoptosis of leukemic cells.

Cellular mechanisms and proteins have been described to function at different points in the programmed cell death process illustrating the complexity of apoptosis regulation. When many genes are analyzed in a single experiment, microarray technology could assist the detection of genes or gene patterns related to alterations of cellular signaling pathways [12]. The aim of our study was to identify new apoptosis-related genes and to analyze abnormally regulated cell death pathway in AML using the microarray approach.

2. Materials and methods

2.1. Samples

Bone marrow aspirates were obtained from 15 *de novo* AML patients at the time of diagnosis and from five healthy donors. AML were typed according to the FAB classification using conventional morphological analysis, immunophenotype and cytogenetic analysis. Biological and clinical data of the 15 patients are shown in Table 1.

2.2. RNA isolation

Total RNA was extracted from bone marrow samples using the Trizol® Reagent (Gibco BRL, Grand Island, NY, USA) after mononuclear cell isolation using Lymphoprep™ (Progen Biotechnik GmbH, Heidelberg, Germany). DNase treatment of total RNA was performed according to the Atlas cDNA Expression Array's User Manual (Clontech Laboratories Inc., Palo Alto, CA, USA). The quality and integrity of the RNA were checked on 1 % agarose gel after electrophoresis.

2.3. Reference

An RNA pool consisting of 1 μg of total RNA from each of the five healthy donors was used as a reference.

2.4. cDNA array hybridization

Apoptosis gene expression study was performed in ten AML samples (patients 1-10) using the Atlas™ Human Apoptosis Array (Clontech Laboratories Inc.). Each filter contains duplicate spots of cDNA fragments of 217 genes, which include nine human housekeeping gene cDNAs used as positive controls and for data normalization, three non-human genes used as a negative controls, and 205 genes with functions related to cell death and cell cycle. The list of genes is available online: http://www.clontech.com. Three μg of total RNA from each AML patient and from the reference were converted into cDNA and labeled with ^{33}PdATP using the Clontech cDNA Array Labeling Kit. Following hybridization, the filters were washed and exposed to imaging plates (BAS-MP 204OS; Fuji, Kanagawa, Japan). After 2-4 days, the plates were scanned with phosphorimager (Bio-Imaging Analyzer, BAS-2500; Fuji) to obtain high resolution images of 16 bits in tiff format.

2.5. cDNA array data analysis

The intensities of spots were determined by using the Atlas Image Analysis Software 2.0 (Clontech Laboratories Inc.). Local background was subtracted at each point. To minimize the possible effect of differences in the amounts of RNA and efficiencies in the hybridizations, intensity values were normalized so that the mean intensities between samples were the same. To obtain the ratio value of expression, normalized intensity values of spots of each patient were compared to the respective reference values. Ratios were $\log_2$ transformed and a total of 34 genes, in which the expression ratio altered significantly (range from –1.32 to 1.32) in at least three patients, were chosen for further analysis. Hierarchical clustering was applied to both axes with the Cluster Program (Michael Eisen, http://rana.lbl.gov/) using uncentered correlation as a similarity metric. Results were visualized with the TreeView Program (Michael Eisen, http://rana.lbl.gov/).

2.6. Real-time quantitative reverse transcription polymerase chain reaction (QRT-PCR)

Quantitative gene expression analysis of four apoptosis-related genes and beta-actin (*ACTB*) housekeeping gene was performed on 15 AML samples and the reference (Table 2). QRT-PCR was carried out using LightCycler FastStart DNA Master SYBR Green I and LightCycler Instrument (Roche Diagnostics, Mannheim, Germany). Each reaction mixture included 1-2 mM of $MgCl_2$, 0.3-1 μM of each forward and reverse primer, 1 μl of LightCycler-FastStart DNA Master SYBR Green I mix (Taq DNA polymerase, dNTP, $MgCl_2$ and SYBR Green I dye) and 1 μl of diluted cDNA template, in a volume of 10 μl. Four dilutions of beta-globulin gene (DNA Control kit; Roche Diagnostics), rising from 0.015 to 15 ng/μl, were used to acquire the standard curve in each assay. The amplification cycle conditions were optimized according to the specific target requirements following the manufacturer's instructions. In every assay, runs were independently duplicated and a negative control was included. The amplification of PCR products was verified using the melting curve analysis option. Data analysis was performed using the LightCycler Software Version 3.5 (Roche Diagnostics).

Average values of run1 and run2 were calculated for *ACTB* and for all four apoptosis-related genes. Average value of each gene was then normalized based on the average value of *ACTB* in every patient and reference. Relative gene expression ratio in every patient was calculated by dividing the normalized gene value by the normalized median value of the same gene from the reference.

3. Results

3.1. Differentially expressed apoptosis-related genes

To identify genes and pathways that induce or repress apoptosis in AML, we used a cDNA array which contained 205 genes associated with regulation of cell death and cell cycle. The analysis showed 34 genes

differently expressed as compared to the reference gene expression, in at least three patients. Of these, 22 were down-regulated and 12 were up-regulated. The gene expression profile for all the patients is shown in Figure 1, in which hierarchical clustering analysis was used to group the genes on the basis of the similarity in their expression.

The affected genes, listed in Table 3, included BCL2 family proteins (*BAG-1*, *NIP3* and *BCL2A1*); caspase 8; growth factors, cytokines and chemokines (*TNFSF5, LTB, TNFA* and *IGF2*), and their receptors (*IGF1R*); receptor associated proteins (*NSMAF*, *GRB10* and *RIPK*); xenobiotic transporters (*GPX1, GSTP1, GSR, GSTM1* and *MGST*); extracellular transporter (*IGFBP3* and *IGFBP5*); nuclear transport factor (*CSE1L*); kinase network members (*MAPK7*); transcription modulators (*P53, JUN*, *NF-ATC1* and *E2F5*); CDK regulators (*CDC37*, *NEDD5* and *CDKN2A*); stress response proteins (*POR*); metalloproteinase (*ADAM17*); DNA synthesis, recombination and repair protein (*GADD45*); chromatin protein (*RBBP4*); and other apoptosis-associated proteins (*CLU* and *DAD1*). Figure 2 shows an overview of the ratios obtained from the 34 differently expressed genes in AML patients compared to the reference. Furthermore, expression of the nine human housekeeping genes on the cDNA array was similar among samples, with a variance of 0.09.

3.2. Altered apoptotic signaling pathways

Genes involved in common apoptotic pathways were found to be abnormally expressed in our study. The TNF signaling pathway was deregulated in our AML samples, reflected by up-regulation of *ADAM17* and *RIPK1*, and by down-regulation of *TNFSF5, TNFA, NSMAF* and *LTB* (Fig. 3). Moreover, several genes of the IGF signaling pathway were altered in our patients, including up-regulation of *IGF10* and down-regulation of *IGF1R*, *IGF2*, *IGFBP3* and *IGFBP5* (Fig. 4). The glutathione detoxification pathway was abnormally expressed in our samples with down-regulation of *GPX1, GSR, GSTM1, GSTP1* and *MGST1* (Fig. 5). Furthermore, the caspase

cascade was altered by up-regulation of *CASP8*, which is one of its main effectors, and by abnormal expression of the BCL2-related pathway, including up-regulation of *BAG-1* and *BCL2A1*, and down-regulation of *NIP3* and *DAD1* (Fig. 6). The DNA damage signaling pathway was abnormally expressed as tumor suppressor genes *P53* and *CSE1L* were both down-regulated, whereas *GADD45A* was up-regulated (Fig. 7A). Altered pathways known to be associated with cell cycle progression were detected in AML samples (Fig. 7B). *RBBP4* was up-regulated and the G1/S check point was altered by up-regulation of *CDC37* and down-regulation of *CDKN2A,* and the G2/M check point showed up-regulation of *NEDD5.* In addition, the signaling molecule *MAPK7* was down-regulated, whereas the transcription factors *JUN* and *E2F5* were up-regulated at the transcriptional level, and *NF-ATC1* was down-regulated.

3.3. cDNA array versus real-time QRT-PCR

To confirm the data obtained by cDNA array technique and to extend the analysis to a larger set of AML samples, we assessed the expression of four normally regulated apoptosis-related genes by real-time QRT-PCR. The selected genes were myeloid cell leukemia sequence 1 *(MCL1)*, MYC proto-oncogene (*MYC)*, BCL-associated X protein (*BAX)* and death-associated protein kinase 1 (*DAPK1)*. Means of the relative expression values obtained by QRT-PCR in the 15 AML patients as compared to the reference, expressed in $\log_2$, were 6.4 ± 7.7 for *MCL1*, 3.8 ± 5.2 for *MYC*, 3 ± 3.4 for *BAX* and 5.5 ± 7.3 for *DAPK1*. Furthermore, the relative expression ratios from cDNA array and QRT-PCR experiments were compared. Because the ratios were relative, they were not comparable as absolute values. In Figure 8, ratios are depicted in a graph that shows array ratios on the X-axis and QRT-PCR on the Y-axis. The expression values of the genes are closely correlated if spots in the figure are either in the top right corner or in the left bottom corner. The correlation of ratios for *MCL1*, *DAPK1* and *BAX* was high, and in case of *MYC*, the correlation was good in six out of ten patients.

Therefore, the expression values of these four apoptosis-related genes in the ten AML patients were significantly correlated.

4. Discussion

4.1. Differentially expressed genes related to lineage specificity of hematopoietic cells

Mononuclear cells isolated from bone marrow samples of AML patients were analyzed and their gene expression was compared with that of the reference. As no mononuclear cell enrichment was performed during sample preparation, we ensured that gene expression changes between AML samples and reference were not related to differences in lineage specificity or maturation stage of hematopoietic cells by excluding the pseudo-differentially expressed genes for further discussion. Among the 34 differentially expressed genes, *NF-ATC1,* known to be expressed in lymphocytes [13], was down-regulated in our samples. Although the TNF signaling pathway also induces apoptosis in other cell types [14], the differentially expressed genes of this signaling pathway were excluded as they are mainly produced by activated macrophages and T cells through immune responses. At the ligand level, *TNFA*, *LTB* and *TNFSF5* were down-regulated, whereas *ADAM17* was up-regulated [15]. At the cytosolic level, the adapter protein of cell surface death-receptor *NSMAF* was down-regulated, whereas the signaling molecule *RIPK1* was up-regulated [16-19] (Fig. 3).

4.2. Differentially expressed apoptosis-related genes in AML

To identify new apoptosis-related genes in AML, we performed gene expression analysis using a cDNA array, which contained 205 genes related to cell death and cell cycle. After excluding the pseudo-differentially expressed genes, analysis of our array data identified a group of 27 genes whose expression was significantly different, suggesting that they may have an important impact in the regulation of apoptosis and, consequently, in the

pathogenesis of AML. Among these, such tumor suppressors as *P53* and *CDKN2A* were substantially down-regulated, whereas proto-oncogenes, e.g., *JUN* and *GRB10*, were up-regulated.

4.3. Altered apoptotic signaling pathways in AML

Cells mediate apoptosis through multiple independent pathways initiated either by intrinsic or extrinsic pathways [20]. The intrinsic pathways are activated from within the cell itself and involve the release of cytochrome c from mitochondria, which is needed for the activation of caspases. The extrinsic cell death pathways are induced by ligand mediated activation of cell surface receptors [14]. These death receptors belong mainly to the TNF receptor gene superfamily, whose cytosolic signaling pathway induces apoptosis, either dependently or independently of caspase activation. To identify abnormal apoptosis pathways in AML, we grouped the 27 differentially expressed genes according to their cellular pathway.

Several differentially expressed genes of the extrinsic signaling pathways were identified in our AML patients. The insulin-like growth factor system regulates proliferation and differentiation of hematopoietic cells [21-23] (Fig. 4). Our results showed alteration of this potent mitogenic and cell death protection signaling pathway by detecting down-regulation of both ligand IGF2 and receptor IGF1R. The implication of *IGF2* in the development of AML requires further investigation as loss of its imprinting has also been reported [24], whereas reduced expression of *IGF1R* has been observed in childhood acute leukemia [25]. As discussed above, *GRB10,* which is related to cell proliferation and apoptosis [26], was up-regulated in our AML samples. The association of *GRB10* with hematologic malignancies was evidenced when it was defined as a new binding partner for the oncogenic fusion protein BCR/ABL [27]. Furthermore, we detected down-regulation of both *IGFBP3* and *IGFBP5*. In addition to its central role in IGF regulation, *IGFBP3* promotes antiproliferative effects through P53 as well as through BAX independently of P53 [28], whereas *IGFBP5* enhances

cell survival [29]. Despite its unknown implication in AML, *IGFBP5* has been observed to be up-regulated, whereas lower expression of *IGFBP3* has been associated with continuous remission in acute lymphoblastic leukemia patients [30, 31].

Array data identified down-regulation of the glutathione detoxification pathway by showing lower expression levels of *GPX1, GSR, GSTM1, GSTP1* and *MGST1* (Fig. 5). All the enzymes mentioned above are detoxicants of xenobiotics to reduce cellular stress, which is detrimental to cellular health. Down-regulation of the glutathione pathway has been detected by microarray analysis in lymphoma cell lines sensitive to apoptosis [32], whereas up-regulation in breast cancer has recently been found to be associated with different clinical features [33]. Thus, our results reveal that understated apoptosis in AML involves reduction of intracellular glutathione levels by inhibition of glutathione detoxification pathway. Alterations in this pathway have been associated with risk of adult acute leukemia and they have been suggested to confer susceptibility to t-AML after cytotoxic chemotherapy [34, 35].

The study of the equilibrium between pro-apoptotic and anti-apoptotic signals in the mitochondria has been a challenge to cancer research. Indeed, patient prognosis and treatment implications have been found in AML [36, 37]. Although the small number of samples did not allow us to perform correlation analysis, our results showed opposite cell death signals at the level of mitochondria (Fig. 6). In the present study, we detected altered caspase cascade by up-regulation of *CASP8*, which is one of the most important inductors and the endpoint of several cell surface death-receptor cytosolic signals. Some of the BCL2 family members, which are upstream regulators of caspase activation [38], were differentially expressed in our patients. We found up-regulation of anti-apoptotic genes, such as *BCL2A1* [39] and *BAG-1* [40], and down-regulation of the pro-apoptotic *NIP3* [41]. Furthermore, we observed down-regulation of the anti-apoptotic *DAD1* [42],

whose abnormal expression in AML has previously been detected by microarray [43].

In our data the participation of endoplasmic reticulum during apoptosis in AML is shown by down-regulation of *POR*, whose expression is generally deficient in tumor cells and related to treatment implications [44-46].

Our results suggest an alteration of the pathway which contributes to the maintenance of genomic stability by down-regulation of *P53* and *CSE1L,* and by up-regulation of *GADD45* (Fig. 7A). The critical role of P53 is evidenced by the fact that its function is suppressed in the majority of tumors [47]. Transcription factor P53 keeps the cell from progressing through the cell cycle until DNA damage is repaired [48]. Deletion of chromosome 17p arm and mutations in *P53* are relatively uncommon abnormalities in AML karyotypes, especially compared to their frequency in solid tumors [49, 50]. However, we found down-regulation of *P53* in all the patients studied, which agrees with previous *P53* expression observations [51]. Moreover, for the first time in AML, we detected down-regulation of *CSE1L* in all the patients. CSE1L is a nuclear transport factor which is related to the mitotic spindle check point, and it exports proteins, such as P53 and RB1 [52]. In addition, up-regulation of *GADD45*, a component of the P53 pathway, has been observed to act as a link between cell cycle check point and DNA repair [53]. However, it also mediates apoptosis by activation of MAPK14/JNK pathway in response to stress [54]. To date, the involvement of *GADD45* in AML was only observed in cell lines [55-57]. These findings indicate that up-regulation of *GADD45* in leukemic cells could send either DNA repair or cell death signals as a mechanism to overcome the deficiencies in the maintenance of genomic stability produced by down-regulation of *P53* and *CSE1L*.

P53 and RB1 are two of the principal pathways controlling cell proliferation, and CDKN2A is involved in both of them encoding P16 (INK4A), a regulator of RB1 phosphorylation mediated by CDK4 and

CDK6, and P14 (ARF), a modulator of P53 degradation mediated by MDM2 [58] (Fig. 7B). *CDKN2A* was down-regulated in our samples. A defect in CDKN2A can interfere with cell cycle control and contribute to carcinogenesis [59]. *CDC37,* a CDK stability protein which plays a positive role in cell cycle progression, was up-regulated. Moreover, our samples revealed up-regulation of *RBBP4*, whose binding with RB1 regulates its growth suppression function. Additionally, *NEDD5* (the human homolog of yeast CDC10) whose expression is cell cycle-dependent with increased levels found at G2/M phase, was up-regulated [60]. Loss of either *CDKN2A* or *P53*, and overexpression of *CDKs* have similar effects on cell cycle progression as they contribute to activate CDKs, which will be able to phosphorylate RB1 and allow transcription of cell cycle progression genes. It should be noted that the alterations in *P53* will effect both G1/S and G2/M check points, while *CDKN2A* abnormalities only act in G1/S. All told, these specific alterations on cell cycle regulation may represent common pathways to leukemogenesis in AML.

The endpoint of many cellular signaling pathways is direct activation of gene transcription. The MAP kinase transduction cascade is activated by multiple external cell stimuli that transduce the signal to the transcription factors, regulating either proliferation or survival [61]. The down-regulation of *MAPK7* in our AML samples therefore reflects partial blockage of MAPK signaling cascade on leukemic cells, which could influence therapeutic targeting strategies in AML [62]. Furthermore, we found abnormal regulation at the transcriptional level in AML, detecting up-regulation of *JUN* and *E2F5*. JUN directly induces apoptosis downstream of the TNF pathway [14], while E2F5 is related with cell cycle progression and transformation, acting downstream of TGF pathway [63].

Finally, it should be emphasized that *CLU* [64] was down-regulated in all the AML samples. The up-regulation of *CLU* in apoptotic tissue and during cellular senescence is believed to be related to ageing and tumor suppression [65, 66]. In addition, *CLU* expression has recently been

associated with class prediction in AML by microarray analysis [67]. Therefore, our results confirm the possibility that down-regulation of *CLU* during hematopoiesis may contribute to leukemogenesis by resistance to apoptosis.

In conclusion, our study provided an overview of deregulated apoptotic genes in AML patients that allows to detect several altered cell death pathways. To summarize, an alteration was found in pathways which contribute to genomic stability and are related to the regulation of cell cycle progression. This may enhance the promotion of deregulated cell proliferation and accumulation of genomic abnormalities that are likely to lead to leukemogenesis. Furthermore, both intrinsic and extrinsic apoptosis pathways, such as the caspase cascade and IGF signaling pathway, were altered. AML patients with defects in normal apoptosis signaling could be refractory to conventional anti-cancer drugs, which are designed to promote apoptosis in target cells. Our study of apoptosis regulation in AML presents therefore new evidence of altered apoptosis pathways. These abnormalities detected at the time of diagnosis may explain cases of resistance to AML treatment and can further serve to identify therapeutic targets, which can defeat insensibility to apoptosis of leukemic cells. Valuable knowledge on how these pathways are modified by treatment will be obtained in extended studies that will compare the apoptotic changes at diagnosis and after therapy. Furthermore, the present results provide novel molecular insight into the regulation of cell death that may contribute to our understanding of the role of apoptosis in the pathogenesis of AML.

Acknowledgements

This work was supported by the Generalitat de Catalunya in Spain (Grant number 00285 FI/FIAP) and by the Sigrid Jusélius Foundation in Finland.

References

[1] Lichtman MA. The stem cell in the pathogenesis and treatment of myelogeneous leukemia: a perspective. Leukemia 2001;15:1489-94.

[2] Alcalay M, Orleth A, Sebastiani C, Meani N, Chiaradonna F, Casciari C, Sciurpi MT, Gelmetti V, Riganelli D, Minucci S, Fagioli M, Pelicci PG. Common themes in the pathogenesis of acute myeloid leukemia. Oncogene 2001;20:5680-94.

[3] Scandura JM, Boccuni P, Cammenga J, Nimer SD. Transcription factor fusions in acute leukemia: variations on a theme. Oncogene 2002;21:3422-44.

[4] Klampfer L, Zhang J, Zelenetz AO, Uchida H and Nimer SD. The AML1/ETO fusion protein activates transcription of BCL-2. Proc Natl Acad Sci USA 1996;95:11863-68.

[5] Britos-Bray M, Ramirez M, Cao W, Wang X, Liu PP, Civin CI, Friedman AD. CBFbeta-SMMHC, expressed in M4eo acute myeloid leukemia, reduces p53 induction and slows apoptosis in hematopoietic cells exposed to DNA-damaging agents. Blood 1998;92:4344-52.

[6] Pandolfi PP. Oncogenes and tumor suppressors in the molecular pathogenesis of acute promyelocytic leukemia. Hum Mol Genet 2001;10:769-75.

[7] Adler HT, Chinery R, Wu DY, Kussick SJ, Payne JM, Fornace AJ Jr, Tkachuk DC. Leukemic HRX fusion proteins inhibit GADD34-induced apoptosis and associate with the GADD34 and hSNF5/INI1 proteins. Mol Cell Biol 1999;19:7050-60.

[8] Shinobu N, Maeda T, Aso T, Ito T, Kondo T, Koike K, Hatakeyama M. Physical interaction and functional antagonism between the RNA polymerase II elongation factor ELL and p53. J Biol Chem 1999;274:17003-10.

[9] Nagy B, Lundán T, Larramendy ML, Aalto Y, Zhu Y, Niini T, Edgren H, Ferrer A, Vilpo J, Elonen E, Vettenranta K, Franssila K, Knuutila S. Abnormal expression of the apoptosis-related genes *BCL2*, *BAX*, *BCL2L1*, *MCL1*, *DAPK1* and *MYC* in haematologic malignancies. Br J Haematol 2003 (in press).

[10] Marie JP. Drug resistance in hematologic malignancies. Curr Opin Oncol 2001;13:463-69.

[11] Smith BD, Bambach BJ, Vala MS, Barber JP, Enger C, Brodsky RA, Burke PJ, Gore SD, Jones RJ. Inhibited apoptosis and drug resistance in acute myeloid leukaemia. Br J Haematol 1998;102:1042-49.

[12] Zhao R, Gish K, Murphy M, Yin Y, Notterman D, Hoffman WH, Tom E, Mack DH, Levine AJ. Analysis of p53-regulated gene expression patterns using oligonucleotide arrays. Genes Dev 2000;14:981-93.

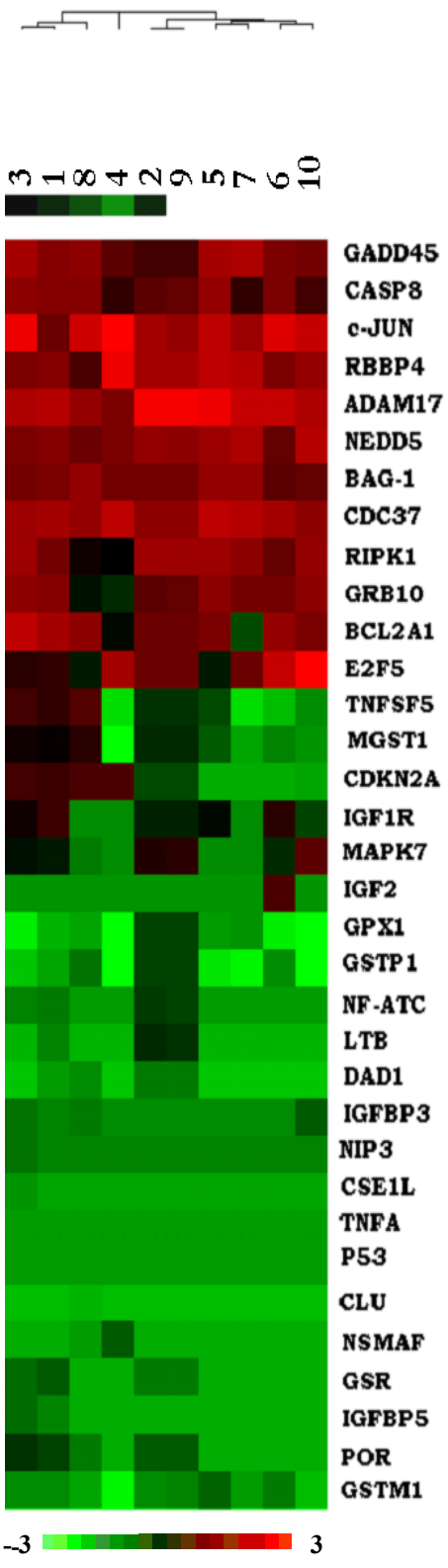
[13] Horsley V and Pavlath GK. NFAT: ubiquitous reglator of cell differentiation and adaptation. J Cell Biol 2002;156:771-74.

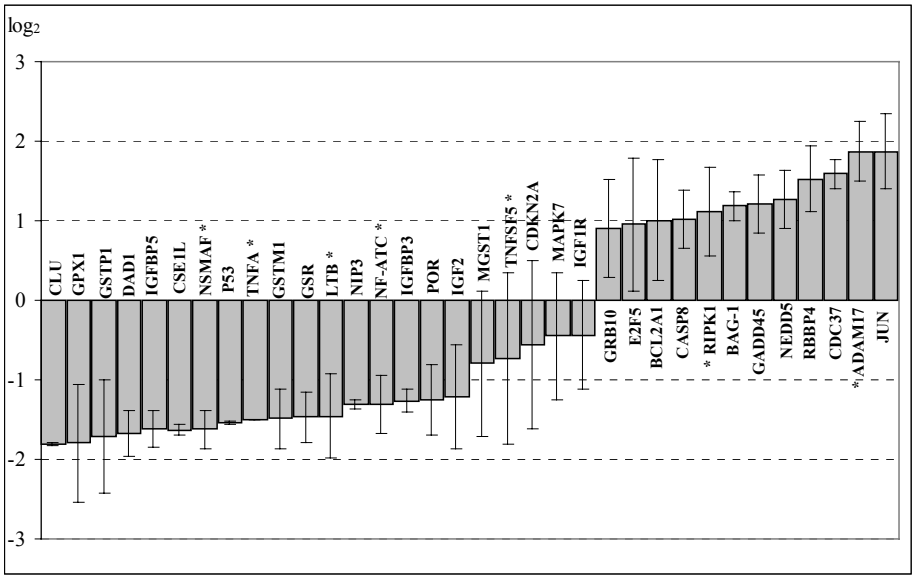
[14] Ashkenazi A and Dixit VM. Death receptors: signalling and modulation. Science 1998;281:1305-8.
[15] Li HD, Havell EA, Brown LC, Cullen JM. Woodchuck lymphotoxin-α, -β and tumor necrosis factor genes: structure, characterization and biological activity. Gene 2002;242:295-305.
[16] Stanger BZ, Leder P, Lee TH, Kim E, Seed B. RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell 1995;81:513-23.
[17] Adam-Klages S, Adam D, Wiegmann K, Struve S, Kolanus W, Schneider-Mergener J, Kronke M. FAN, a novel WD-repeat protein, couples the p55 TNF-receptor to neutral sphingomyelinase. Cell 1996;86:937-47.
[18] Segui B, Andrieu-Abadie N, Adam-Klages S, Meilhac O, Kreder D, Garcia V, Bruno AP, Jaffrezou JP, Salvayre R, Kronke M, Levade T. CD40 signals apoptosis through FAN-regulated activation of the sphingomyelin-ceramide pathway. J Biol Chem 1999;274:37251-8.
[19] Segui B, Cuvillier O, Adam-Klages S, Garcia V, Malagarie-Cazenave S, Leveque S, Caspar-Bauguil S, Coudert J, Salvayre R, Kronke M, Levade T. Involvement of FAN in TNF-induced apoptosis. J Clin Invest 2001;108:143-51.
[20] Strasser A, O'Connor L, Dixit VM. Apoptosis signalling. Annu Rev Biochem 2000;69:217-45.
[21] Baserga R. The contradictions of the insulin-like growth factor 1 receptor. Oncogene 2000;19:5574-81.
[22] Valentis B and Baserga R. IGF-I receptor signalling in transformation and differentiation. Mol Pathol 2001;54(3):133-7.
[23] Zumkeller W. The insulin-like growth factor system in hematopoietic cells. Leuk Lymphoma 2002;43:487-91.
[24] Wu HK, Weksberg R, Minden MD, Squire JA. Loss of imprinting of human insulin-like growth factor II gene, IGF2, in acute myeloid leukemia. Biochem Biophys Res Commun 1997;231:466-72.
[25] Eshet R, Silbergeld A, Zaizov R, Stark B, Freud E, Laron Z, Shamai Y. Decreased insulin-like growth factor-I receptor sites on circulating mononuclear cells from children with acute leukemia. Pediatr Hematol Oncol 2000;17:253-60.
[26] Han DC, Shen TL, Guan JL. The Grb7 family proteins: structure, interactions with other signaling molecules and potential cellular functions. Oncogene 2001;20:6315-21.
[27] Nantel A, Mohammad-Ali K, Sherk J, Posner BI, Thomas DY. Interaction of the Grb10 adapter protein with the Raf1 and MEK1 kinases. J Biol Chem 1998;273:10475-84.
[28] Baxter RC. Signalling pathways involved in antiproliferative effects of IGFBP-3: a review. Mol Pathol 2001;54:145-8.
[29] Perks CM, McCaig C, Holly JM. Differential insulin-like growth factor (IGF)-independent interactions of IGF binding protein-3 and IGF

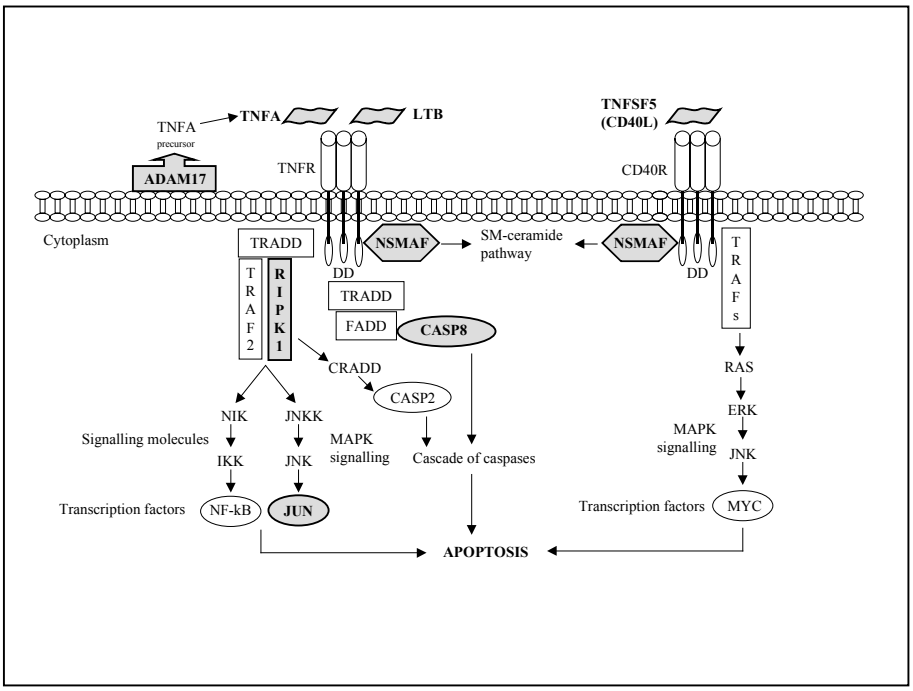
binding protein-5 on apoptosis in human breast cancer cells. Involvement of the mitochondria. J Cell Biochem 2001; 80:248-58.
[30] Petridou E, Skalkidou A, Dessypris N, Moustaki M, Mantzoros C, Spanos E, Trichopoulos D. Insulin-like growth factor binding protein-3 predicts survival from acute childhood leukemia. Oncology 2001;60:252-7.
[31] Vorwerk P, Wex H, Hohmann B, Mohnike K, Schmidt U, Mittler U. Expression of components of the IGF signalling system in childhood acute lymphoblastic leukaemia. Mol Pathol 2002;55:40-5.
[32] Voehinger DW, Hirschlberg DL, Xiao J, Lu Q, Roederer M, Lock CB, Herzenberg LA, Steinman L, Herzenberg LA. Gene microarray identification of redox and mitochondrial elements that control resistence or sensitivity to apoptosis. Proc Natl Acad Sci USA 2000;97:2680-5.
[33] Perquin M, Oster T, Maul A, Froment N, Untereiner M, Bagrel D. The glutathione-related detoxification system is increased in human breast cancer in correlation with clinical and histopathological features. J Cancer Res Clin Oncol 2001;127:368-74.
[34] Allan JM, Wild CP, Rollinson S, Willett EV, Moorman AV, Dovey GJ, Roddam PL, Roman E, Cartwright RA, Morgan GJ. Polymorphism in glutathione S-transferase P1 is associated with susceptibility to chemotherapy-induced leukemia. Proc Natl Acad Sci USA 2001;98:11592-7.
[35] Rollinson S, Roddam P, Kane E, Roman E, Cartwright R, Jack A, Morgan GJ. Polymorphic variation within the glutathione S-transferase genes and risk of adult acute leukaemia. Carcinogenesis 2000;21:43-7.
[36] Del Poeta G, Venditti A, Del Principe MI, Maurillo L, Buccisano F, Tamburini A, Cox MC, Franchi A, Bruno A, Mazzone C, Panetta P, Suppo G, Masi M, Amadori S. The amount of spontaneous apoptosis detected by bax/bcl-2 ratio predicts outcome in acute myeloid leukemia (AML). Blood 2002;prepublished online November 7.
[37] Kornblau SM, Thall PF, Estrov Z, Walterscheid M, Patel S, Theriault A, Keating MJ, Kantarjian H, Estey E, Andreeff M. The prognostic impact of BCL2 protein expression in acute myelogenous leukemia varies with cytogenetics. Clin Cancer Res 1999;5:1758-66.
[38] Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev 1999;13:1899-911.
[39] Lin EY, Orlofsky A, Berger MS, Prystowsky MB. Characterization of A1, a novel hemopoietic-specific early-response gene with sequence similarity to bcl-2. J Immunol 1993;151:1979-88.
[40] Takayama S, Sato T, Krajewski S, Kochel K, Irie S, Millan JA, Reed JC. Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. Cell 1995;80:279-84.
[41] Boyd JM, Malstrom S, Subramanian T, Venkatesh LK, Schaeper U, Elangovan B, D'Sa-Eipper C, Chinnadurai G. Adenovirus E1B 19 kDa

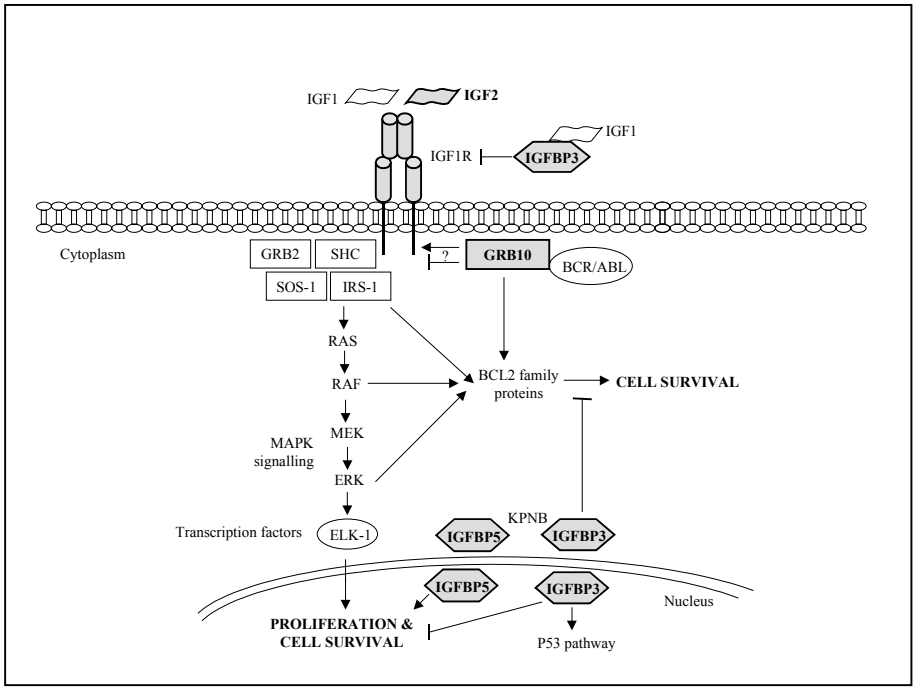
and Bcl-2 proteins interact with a common set of cellular proteins. Cell 1994;79:341-51.
[42] Nakashima T, Sekiguchi T, Kuraoka A, Fukushima K, Shibata Y, Komiyama S, Nishimoto T. Molecular cloning of a human cDNA encoding a novel protein, DAD1, whose defect causes apoptotic cell death in hamster BHK21 cells. Mol Cell Biol 1993;13:6367-74.
[43] Virtaneva K, Wright FA, Tanner SM, Yuan B, Lemon WJ, Caligiuri MA. Expression profiling reveals fundamental biological differences in acute myeloid leukemia with isolated trisomy 8 and normal cytogenetics. Proc Natl Acad Sci USA 2001;98:1124-9.
[44] Haniu M, McManus ME, Birkett DJ, Lee TD, Shively JE. Structural and functional analysis of NADPH-cytochrome P-450 reductase from human liver: complete sequence of human enzyme and NADPH-binding sites Biochemistry 1989;28:8639-45.
[45] Bustamante J, Bersier G, Badin RA, Cymeryng C, Parodi A, Boveris A. Sequential NO production by mitochondria and endoplasmic reticulum during induced apoptosis. Nitric Oxide 2002;6:333-41.
[46] Waxman DJ, Chen L, Hecht JE, Jounaidi Y. Cytochrome P450-based cancer gene therapy: recent advances and future prospects. Drug Metab Rev 1999;31(2):503-22.
[47] Harris CC and Hollstein M. Clinical implications of the p53 tumor-suppressor gene. N Engl J Med 1993;329:1318.
[48] Levine AJ, Momand J, Finlay CA. The p53 tumor suppressor gene. Nature 1991;351:453.
[49] Fenaux BP, Jonveaux P, Quiquandon I, Lai JL, Pignon JM, Locheux-Lefebvre MH, Bausters F, Berger R, Kerckaert JP. P53 mutations in acute myeloid leukemia with 17p monosomy. Blood 1991;78:1652-57.
[50] Hu G, Zhang W, Deisseroth AB. P53 gene mutations in acute myelogenous leukaemia. Br J Haematol 1992;81:489-94.
[51] Schottelius A, Brennscheidt U, Ludwig W-D, Mertelsmann RH, Herrmann F, Lübbert M. Mechanisms of p53 alteration in acute leukemias. Leukemia 1994;8:1673-81.
[52] Brinkmann U, Brinkmann E, Gallo M, Pastan I. Cloning and characterization of a cellular apoptosis susceptibility gene, the human homologue to the yeast chromosome segregation gene CSE1. Proc Natl Acad Sci USA 1995;92:10427-31.
[53] Smith ML, Chen IT, Zhang Q, Bae I, Chen CY, Gilmer TM, Kastan MB, O'Conor PM, Fornance AJ Jr. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science 1994;266:1376-80.
[54] Takekawa M and Saito H. A family of stress-inducible GADD45-like proteins mediate activation of stress-repsponsive MTK1/MEKK4 MAPKKK. Cell 1998;95: 521-30.
[55] Chin PL, Momand J, Pfeifer GP. In vivo evidence for binding of p53 to consensus binding sites in the p21 and GADD45 genes in response to ionizing radiation. Oncogene 1997;15:87-99.

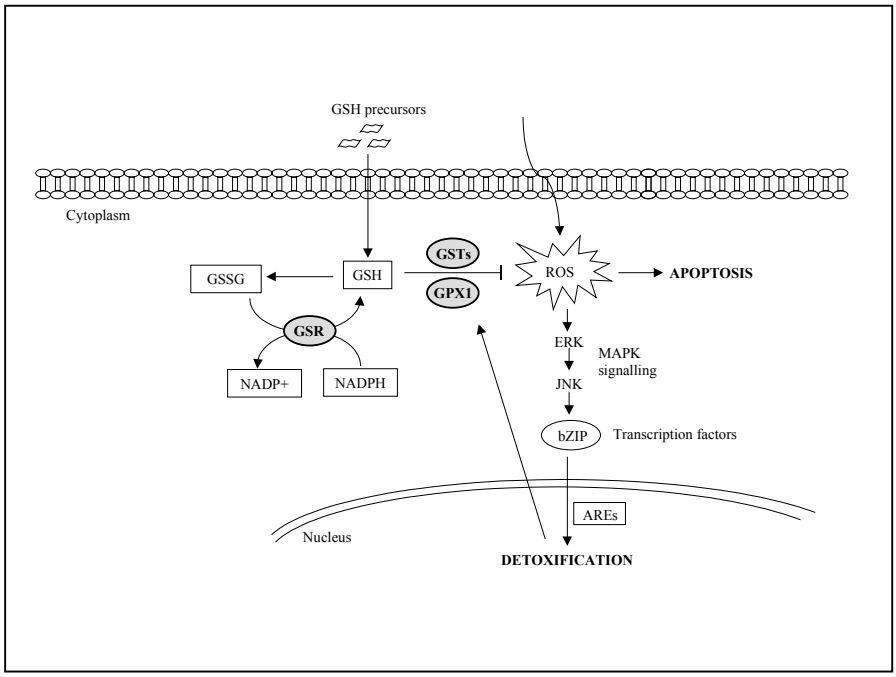
[56] Santucci MA, Ripalti A, di Paola MC, Mianulli AM, Iacurti E, Campanini F, Gamberi B, Tura S. Procedure for the quantification of Gadd45 expression levels in clonal hematopoietic progenitors cells by competitive polymerase chain reaction. Clin Biochem 1999;32:1-8.
[57] Zhang W, Hoffman B, Liebermann DA. Ectopic expression of MyD118/Gadd45/CR6 (Gadd45beta/alpha/gamma) sensitized neoplastic cells to genotoxic stress-induced apoptosis. Int J Oncol 2001;18:749-57.
[58] Zhang Y, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell 1998;92:725-34.
[59] Serrano M, Hannon G J, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature 1993;366:704-7.
[60] Mori T, Miura K, Fujiwara T, Shin S, Inazawa J, Nakamura Y. Isolation and mapping of a human gene (DIFF6) homologous to yeast CDC3, CDC10, CDC11, and CDC12, and mouse Diff6. Cytogenet Cell Genet 1996;73:224-7.
[61] Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature 2001;410:37-40.
[62] Milella M, Kornblau SM, Estrov Z, Carter BZ, Lapillonne H, Harris D, Konopleva M, Zhao S, Estey E, Andreeff M. Therapeutic targeting of the MEK/MAPK signal transduction module in acute myeloid leukemia J Clin Invest 2001;108:851-9.
[63] Chen CR, Kang Y, Siegel PM, Massague J. E2F4/5 and p107 as Smad cofactors linking the TGF-beta receptor to c-myc repression. Cell 2002;110:19-32.
[64] Purrello M, Bettuzzi S, Di Pietro C, Mirabile E, Di Blasi M, Rimini R, Grzeschik KH, Ingletti C, Corti A, Sichel G. The gene for SP-40, human homolog of rat sulfated glycoprotein 2, rat clusterin, and rat testosterone-repressed prostate message 2, maps to chromosome 8. Genomics 1991;10:151-6.
[65] Jones SE and Jomary C. Clusterin. Int J Biochem Cell Biol 2002;34:427-31.
[66] Trougakos I and Gonos E. Clusterin/apolipoprotein J in human aging and cancer. Int J Biochem Cell Biol 2002;34:1430-48.
[67] Schoch C, Kohlmann A, Schnittger S, Brors B, Dugas M, Mergenthaler S, Kern W, Hiddemann W, Eils R, Haferlach T. Acute myeloid leukemias with reciprocal rearrangements can be distinguished by specific gene expression profiles. Proc Natl Acad Sci U S A 2002;99:10008-13.
[68] Pearson LL, Castle BE, Kehry MR. CD40-mediated signaling in monocytic cells: up-regulation of tumor necrosis receptor-associated factor mRNA and activation of mitogen-acivated protein kinase signalling pathways. Int Immunol 2001;13:273-83.

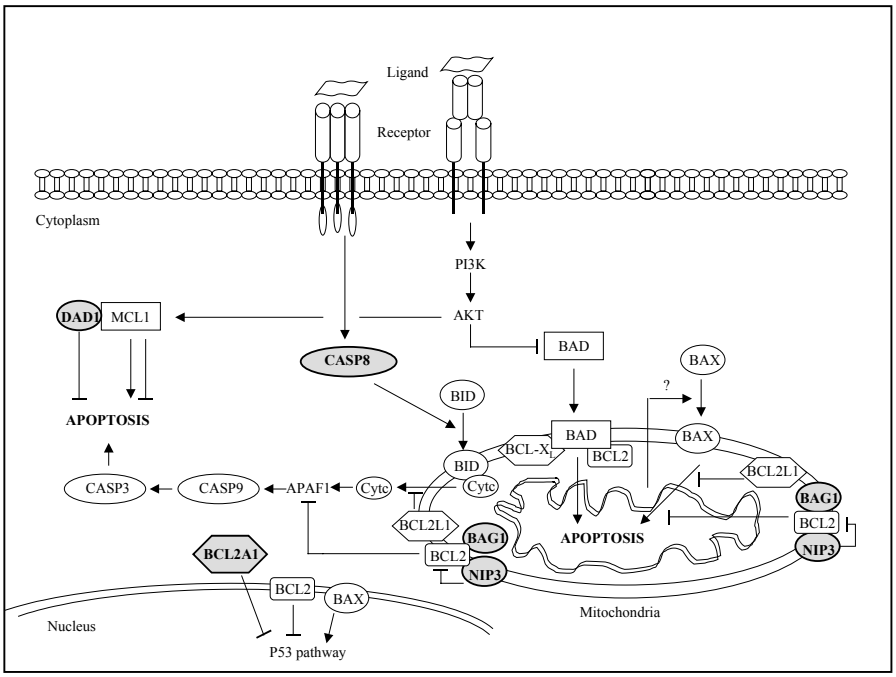

3 1 8 4 2 9 5 7 6 10
GADD45
CASP8
c-JUN
RBBP4
ADAM17
NEDD5
BAG-1
CDC37
RIPK1
GRB10
BCL2A1
E2F5
TNFSF5
MGST1
CDKN2A
IGF1R
MAPK7
IGF2
GPX1
GSTP1
NF-ATC
LTB
DAD1
IGFBP3
NIP3
CSE1L
TNFA
P53
CLU
NSMAF
GSR
IGFBP5
POR
GSTM1
-3
3

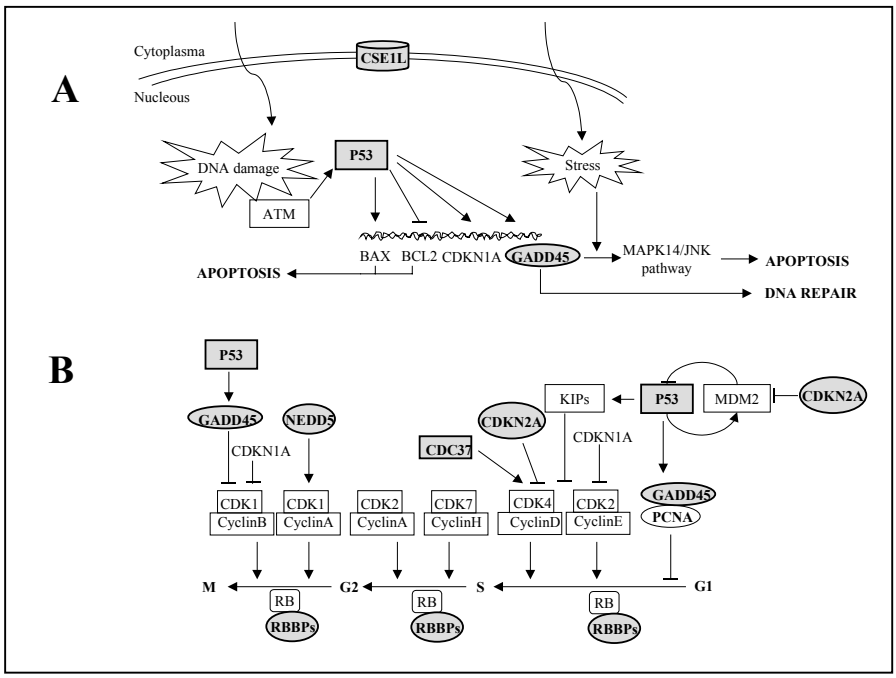

log2
3
2
1
0
-1
-2
-3
CLU
GPX1
GSTP1
DAD1
IGFBP5
CSE1L
NSMAF *
P53
TNFA *
GSTM1
GSR
LTB *
NIP3
NF-ATC *
IGFBP3
POR
IGF2
MGST1
TNFSF5 *
CDKN2A
MAPK7
IGF1R
GRB10
E2F5
BCL2A1
CASP8
* RIPK1
BAG-1
GADD45
NEDD5
RBBP4
CDC37
*ADAM17
JUN

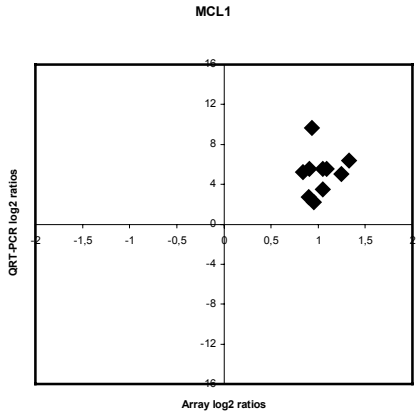

TNFA
precursor
TNFA
LTB
ADAM17
TNFR
TNFSF5
(CD40L)
CD40R
Cytoplasm
TRADD
NSMAF
SM-ceramide
pathway
NSMAF
DD
DD
TRADD
FADD
CASP8
TRAF2
RIPK1
TRAFs
CRADD
CASP2
RAS
ERK
NIK
JNKK
Signalling molecules
MAPK
signalling
MAPK
signalling
JNK
IKK
JNK
Cascade of caspases
Transcription factors
NF-kB
JUN
Transcription factors
MYC
APOPTOSIS

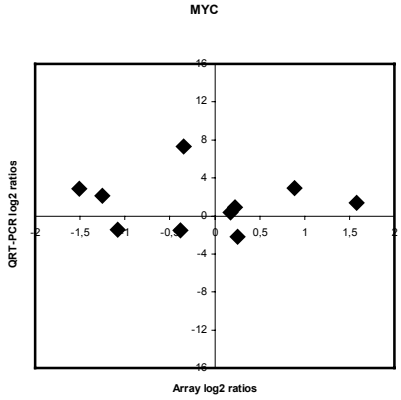

IGF1
IGF2
IGF1
IGF1R
IGFBP3
Cytoplasm
GRB2
SHC
?
GRB10
BCR/ABL
SOS-1
IRS-1
RAS
RAF
BCL2 family proteins
CELL SURVIVAL
MAPK signalling
MEK
ERK
Transcription factors
ELK-1
KPNB
IGFBP5
IGFBP3
IGFBP5
IGFBP3
Nucleus
PROLIFERATION & CELL SURVIVAL
P53 pathway

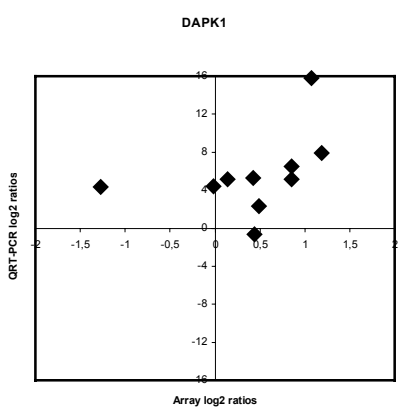

GSH precursors
Cytoplasm
GSSG
GSH
GSTs
GPX1
ROS
APOPTOSIS
GSR
NADP+
NADPH
ERK
MAPK
signalling
JNK
bZIP
Transcription factors
AREs
Nucleus
DETOXIFICATION


Ligand
Receptor
Cytoplasm
PI3K
AKT
DAD1
MCL1
APOPTOSIS
CASP8
BAD
BAX
?
BID
BAD
BAX
BCL-X$_L$
BCL2
BID
BCL2L1
CASP3
CASP9
APAF1
Cytc
Cytc
BAG1
BCL2L1
BCL2
APOPTOSIS
BAG1
BCL2
NIP3
NIP3
BCL2A1
BCL2
BAX
Nucleus
Mitochondria
P53 pathway


A
Cytoplasma
Nucleous
CSE1L
DNA damage
ATM
P53
Stress
BAX
BCL2
CDKN1A
GADD45
MAPK14/JNK
pathway
APOPTOSIS
APOPTOSIS
DNA REPAIR
B
P53
GADD45
NEDD5
CDKN1A
CDK1
CyclinB
CDK1
CyclinA
CDK2
CyclinA
CDK7
CyclinH
CDC37
CDKN2A
KIPs
CDK4
CyclinD
CDK2
CyclinE
CDKN1A
P53
MDM2
CDKN2A
GADD45
PCNA
M
G2
S
G1
RB
RBBPs
RB
RBBPs
RB
RBBPs


MCL1
QRT-PCR log2 ratios
Array log2 ratios


MYC
QRT-PCR log2 ratios
Array log2 ratios


DAPK1
QRT-PCR log2 ratios
Array log2 ratios

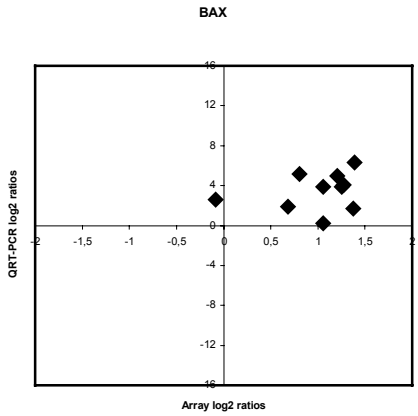

BAX
QRT-PCR log2 ratios
Array log2 ratios

Figure legends

Figure 1. Expression analysis of apoptosis-related genes of ten AML patients. Hierarchical clustering analysis was performed for all the patients on the basis of 34 differentially expressed genes. Patient numbers are listed above, with gene names on the right. Red color indicates up-regulated genes and green down-regulated genes. The intensity of the color correlates with the degree of expression, ranging from –1.32 to 1.32 in $\log_2$. Branch nodes connect closely related samples, branch's length correlates with the degree of relationship.

Figure 2. Overview of 34 differentially expressed apoptosis-related genes in at least three of the AML patients compared to the reference. Bars illustrate the mean ± sd of $\log_2$ transformed gene expression ratios. Left to right: highest under-expressed to over-expressed genes. Asterix marks differentially expressed genes that may result from lineage specificity and/or maturation stage of the hemopoetic cell samples.

Figure 3. TNF signaling pathway. Disintegrin and metalloproteinase domain 17 (ADAM17) cleaves tumor necrosis factor-a (TNFA) precursor to its mature form. Ligand (TNFA/LTB) trimerizes receptor (TNFR/LTBR) after binding. TNFR-associated death domain (TRADD) then binds to the receptor, allowing the recruiting of several signaling molecules to the death domain (DD). TNFR-associated factor-2 (TRAF2) and receptor interacting protein (RIPK1) mediate signaling pathways to activate NF-kB and JUN, whereas FAS-associated via death domain (FADD) stimulates apoptosis through caspase 8 (CASP8). RIPK1 interacts with CRADD and leads to the activation of CASP2. The signal transduction pathway of CD40 has not been fully described. Upstream events include RAS activation, followed by MAP kinase cascade and activation of transcription factors. Neutral sphingomyelinase activation-associated factor (NSMAF) regulates TNF and

CD40 signals through activation of SM-ceramide pathway [15, 18, 19, 68]. The differentially expressed apoptosis-related genes found in our AML samples are shaded.

Figure 4. IGF signaling pathway. Insulin like growth factors (IGFs) and their receptor (IGF1R) provide a proliferative signal and block apoptosis. After ligand interaction, Src homology 2 domain containing (SHC), son of sevenless homolog 1 (SOS-1) and growth factor receptor-bound protein 2 (GRB2) are associated with IGF1R to activate RAS and the MAP kinase cascade (RAF, MEK, ERK). The endpoint is the activation of transcription factors, such as ELK-1, to transcript proliferation and cell survival genes. Insulin receptor substrate-1 (IRS-1) contributes to mitogenic signaling by interaction with the BCL2 family proteins. Growth factor receptor-bound protein 10, partner of the oncogenic fusion protein BCR/ABL, interacts with IGFR1 and inhibits/enhances IGF1 mediated cell proliferation. In addition, GRB10 shows anti-apoptotic function interacting with BCL2. Insulin-like growth factor binding proteins (IGFBPs) transport and stabilize IGFs in the circulation. IGFBP3 inhibits the access of IGF to IGF1R. IGFBP3 and IGFBP5 are also located in the nucleus transported by b-importin (KPNB), inducing apoptosis or cell survival, respectively [21, 26, 28]. The differentially expressed apoptosis-related genes found in our AML samples are shaded.

Figure 5. Glutathione detoxification pathway. Reactive oxygen species (ROS) are detrimental to cellular health. Antioxidant response elements (AREs) regulate the expression of genes which protect cells from oxidative stress. Glutathione peroxidase 1 (GPX1) and glutathione S-transferases (GSTs) catalyze the conjugation of glutathione (GSH) with xenobiotics. Glutathione reductase (GSR) keeps GSH in its reduced form by generating $NADP^+$ (Biocarta, http://www.biocarta.com) [32]. The differentially expressed apoptosis-related genes found in our AML samples are shaded.

Figure 6. Caspase cascade and BCL2 related signaling pathway. Ligand engaging on cell surface receptors activates caspase-8 (CASP8). CASP8 translocates BH3 interacting domain death agonist (BID) to the mitochondria. Cytochrome *c* (CYTc) then leaves from mitochondria and activates both apoptotic protease activating factor 1 (APAF1) and the effector caspases. Death stimuli from the mitochondria translocate BCL2 associated X protein (BAX) to the external membrane as a homodimer. After external stimuli, murine thymoma viral oncogene homolog (AKT) keeps BCL2 antagonist of cell death (BAD) in the cytosol and stimulates myeloid cell leukemia-1 (MCL1). Cooperative dimerization between antagonist and agonist proteins results in different apoptotic function. Homodimers of both BCL2 and BCL2-like 1 (BCL2L1) generate strong anti-apoptotic signals by inhibition of BAX function and CYTc relase. Heterodimers of both of them with BAD enhance pro-apoptotic BAD function. BCL2-associated athanogene (BAG-1) increases the anti-apoptotic function of BCL2, while BCL2/adenovirus E1B 19kDa interacting protein 3 (NIP3) inhibits it. Defender against cell death 1 (DAD1) needs MCL1 interaction to induce pro-apoptotic signal. Several BCL2 family proteins, such as BCL2, BAX and BCL2-related protein A1 (BCL2A1) could regulate the P53 pathway [39-41]. The differentially expressed apoptosis-related genes found in our AML samples are shaded.

Figure 7. A: P53 signaling pathway. The ataxia telangiectasia-mutated gene (ATM) is activated by DNA damage to induce P53 pathway. P53 pathway stimulates DNA repair and blocks progression through the cell cycle, by transcription of growth arrest and damage-inducible protein (GADD45) and cyclin-dependent kinase inhibitor 1 A (CDKN1A). If DNA damage can not be repaired, P53 sends apoptotic signal by transcription activation of BAX and transcription inhibition of BCL2. GADD45 is also an apoptotic inducer in response to stress through the MAPK14/JNK pathway. **B:** Cell cycle

regulation In the regulation of cell cycle, cyclins drive the stages in combination with cyclin-dependent kinases (CDKs), and dissociation of the retinoblastoma (RB)-repressor complex permits transcription. CDKs are inhibited by CDK inhibitors (CDKNs) and activated by cell division cycle proteins (CDCs). RB is regulated by several RB binding proteins (RBBPs) during cell cycle. Neutral precursor cell expressed developmentally downregulated 5 (NEDD5), the human homolog of yeast CDC10, acts at G2/M check point. CSE1L acts as a nuclear transport factor which exports important proteins to the nucleus, such as P53 and RB1 [48, 52-54, 57, 60]. The differentially expressed apoptosis-related genes found in our AML samples are shaded.

Figure 8. Validation of gene expression data. Depicted are the comparisons of gene expression values from QRT-PCR and cDNA array studies. Log_2 gene expression ratios from microarray study are on the X-axis and QRT-PCR ratios are on the Y-axis. Dots represent gene expression ratio values obtained by QRT-PCR and microarray. The most highly correlated gene expression ratios are on the top right corner or bottom left corner.

Table 1. Biological and clinical data of 15 AML patients

	Sex	Age	Date of diagnosis	FAB	Karyotype	Immunophenotype	*WBC (x109/l)*
1	F	51	12/06/01	M4	46,XX,t(3;12)(q21;q24),t(8;21)(q22;q22)/46,XX	CD13+,CD33+,CD34+,CD56+,CD64-, CD14-,CD7-,CD19-,CD2-,CD117+	18
2	F	57	13/07/01	M5b	46,XX	CD13+,CD33+,CD34+,CD56-,CD64+, CD14-,CD7-,CD19+,CD2-,CD117+	31.2
3	F	26	20/08/01	M2	48,XX,t(8;21)(q22;q22),del(11)(q21q23),+15,+21c	CD13+,CD33+,CD34+,CD56-,CD64-,CD14-,CD7-,CD19-,CD2-,CD117+	94
4	M	1	07/08/01	M5	46,XY,del(10)(p12p15)/46,XY	CD13-,CD33+,CD34-,CD56+,CD64+,CD14-,CD7-,CD19-,CD2-,CD117+	-
5	M	53	22/08/01	M5	45,XY,t(5;19)(q11;p12),der(7)t(7;?)(p15;?), der(12)t(12;18)(p?;q?),del(16)(q13),−17, der(18)t(17;18)(q21;q?),dup(22)(q?)	CD13+,CD33+,CD34+,CD56-,CD64+, CD14+,CD7-,CD19+,CD2-,CD117+	61
6	F	40	9/10/01	M2	46,XX	CD13-,CD33+,CD34+,CD56+,CD64-, CD14-,CD7+,CD19-,CD2-,CD117+	12
7	F	36	17/10/01	M1	47,XX,+4	CD13+,CD33+,CD34-,CD56-,CD64-, CD14-,CD7-,CD19-,CD2-,CD117+	28.6
8	F	59	22/10/01	M2	ND	CD13+,CD33+,CD34+,CD56-,CD64-, CD14-,CD7-,CD19-,CD2-,CD117+	1.26
9	M	38	4/11/01	M1	47,XXYc	CD13+,CD33+,CD34+,CD56-,CD64+, CD14-,CD7+,CD19+,CD2+,CD117+	5
10	M	35	2/11/01	M5	45,XY,-7	CD13+,CD33+,CD34+,CD56-,CD64-, CD14-,CD7-,CD19-,CD2-,CD117+	73
11	M	34	9/05/01	M4	46,XY	CD13+,CD33+,CD34+,CD56-,CD64-, CD14-,CD7-,CD19-,CD2-,CD117+	1.6
12	M	58	29/06/01	M5b	46,XY	CD13+,CD33+,CD34-,CD56-,CD64-, CD14-,CD7-,CD19-,CD2-,CD117+	14.6
13	F	60	9/08/01	M5b	ND	CD13+,CD33+,CD34+,CD56+,CD64+, CD14+,CD7+,CD19-,CD2-,CD117+	26

14	F	58	4/09/01	M5b	46,XX	CD13+,CD33+,CD34+,CD56+,CD64+, CD14+,CD7-,CD19+,CD2-,CD117+	37.8
15	F	46	1/11/01	M2	46,XX,inv(11)(p15q23)	CD13+,CD33+,CD34+,CD56-,CD64-, CD14-,CD7-,CD19-,CD2-,CD117+	110

M, male; F, female; WBC, white blood cell count; ND, not done

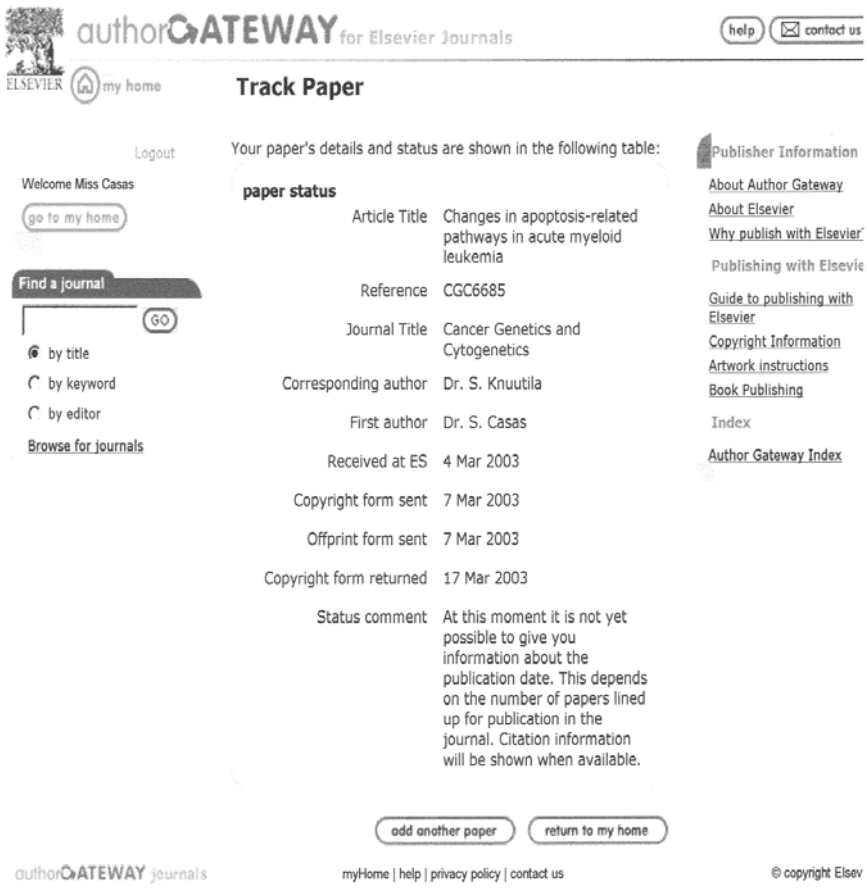
Table 2. Primers used for real-time quantitative reverse transcription polymerase chain reaction

Gene		*Sequence 5′ - 3′*	*Length (bp)*	*CG (%)*	*Tm (°C)*	*Product size (bp)*
MYC	F	GGCAAAAGGTCAGAGTCTGG	20	55	63.7	209
	R	GTGCATTTTCGGTTGTTGC	19	47.4	64.1	
DAPK1	F	CAGTGTTGTTGCTCTAGGAAG	21	47.6	57.2	196
	R	GGGACTGCCACAAATGATGAG	21	52.4	58.2	
BAX	F	TGCTTCAGGGTTTCATCCAG	20	50	56.9	169
	R	GGCGGCAATCATCCTCTG	18	61.1	57.3	
MCL1	F	GATGATCCATGTTTTCAGCGAC	22	45.5	56.4	205
	R	CTCCACAAACCCATCCCAG	19	57.9	57.7	
ACTB	F	AGCCTCGCCTTTGCCGA	17	64.7	68.5	174
	R	CTGGTGCCTGGGGCG	15	80	64.1	

F, forward; R, reverse; bp, base pair; Tm, melting temperature

Table 3. General information about the 34 differently expressed apoptosis-related genes in our AML samples

	Array location	Gene symbol	Gene name	Gene Bank #	Chromosome location
1	11J	*CASP8*	Caspase 8	U60520/U58143/ X98172	2q33-q34
2	11B	*BAG-1*	BCL2-binding athanogene 1	S83171/Z35491	9p12
3	12H	*RIPK1*	Receptor-interacting serine/threonine kinase 1	U25994/U50062	6p24.3
4	8J	*GRB10*	Growth factor receptor-bound protein 10	U69276	7p12.p11.2
5	16C	*IGF1R*	Insuline-like growth factor I receptor	X04434/M24599	15q25-q26
6	6L	*MAPK7*	Mitogen-activated protein kinase 7	U25278	17p11.2
7	16B	*IGF2*	Insuline-like growth factor II	M29645	11p15.5
8	22E	*NIP3*	BCL2/adenovirus E1B 19-kDa-interacting protein 3	U15174	8p21
9	9F	*P53*	P53 cellular tumor antigen	M14694/M92424	17p13.1
10	12O	*TNFA*	Tumor necrosis factor alpha	X01394	6p21.3
11	20B	*NF-ATC1*	Cytoplasmatic nuclear factor of activated T-cells 1	U08015	18q23
12	10K	*BCL2A1*	BCL2-related protein A1	U29680/Y09397	15q24.3
13	7L	*RBBP4*	Retinoblastoma-binding protein 4	X74262	1p34.3
14	6F	*CDC37*	Cell division cycle 37	U6131	19p13.2
15	8M	*JUN*	JUN proto-oncogene	J04111	1p32-p31
16	14D	*ADAM17*	A disintegrin and metalloproteinase domain 17	U69611	2p25
17	5O	*NEDD5*	Neutral precursor cell expressed developmentally downregulated 5	D63878	2q37
18	22C	*GADD45A*	Growth arrest and damage-inducible protein	M60974	1p31.2-p31.1
19	8E	*E2F5*	E2F transcription factor 5	U15642	8q22-q21.13
20	16G	*IGFBP3*	Insulin-like growth factor-binding protein 3	M31159/M35878	7p13-p12
21	21E	*GPX1*	Glutathione peroxidase 1	Y00483/M21304	3p21.3
22	21C	*GSTP1*	Glutathione S-transferase pi	X08058/M24485	11q13
23	20C	*DAD1*	Defender against cell death 1 protein	D15057	14q11-q12
24	14C	*LTB*	Lymphotoxin-beta	L11015	6p21.3
25	20D	*CLU*	Clusterin	M74816	8p21-p12
26	19B	*CSE1L*	Chromosome segregation 1-like	U33286	20q13
27	23B	*NSMAF*	Neutral sphingomyelinase activation-associated factor	X96586	8q12-q13
28	20F	*GSR*	Glutathione reductase	X15722	8p21.1
29	17B	*IGFBP5*	Insulin-like growth factor-binding protein 5	M65062	2q33-q36
30	21G	*POR*	Cytochrome p450 reductase	S90469	7q11.2
31	21B	*GSTM1*	Glutathione S-transferase m1	X68676/S01719	1p13.3
32	22F	*TNFSF5*	Tumor necrosis factor superfamily member 5	L07414	Xq26
33	20G	*MGST1*	Microsomal glutathione S-transferase 1	J03746	12p12.3-p12.1
34	5H	*CDKN2A*	Cyclin-dependent kinase inhibitor 2A	L27211	9p21



authorGATEWAY for Elsevier Journals

help | contact us

my home

Track Paper

Logout

Welcome Miss Casas

go to my home

Find a journal

GO

by title

by keyword

by editor

Browse for journals

Your paper's details and status are shown in the following table:

paper status

Article Title	Changes in apoptosis-related pathways in acute myeloid leukemia
Reference	CGC6685
Journal Title	Cancer Genetics and Cytogenetics
Corresponding author	Dr. S. Knuutila
First author	Dr. S. Casas
Received at ES	4 Mar 2003
Copyright form sent	7 Mar 2003
Offprint form sent	7 Mar 2003
Copyright form returned	17 Mar 2003
Status comment	At this moment it is not yet possible to give you information about the publication date. This depends on the number of papers lined up for publication in the journal. Citation information will be shown when available.

add another paper | return to my home

Publisher Information

About Author Gateway

About Elsevier

Why publish with Elsevier

Publishing with Elsevie

Guide to publishing with Elsevier

Copyright Information

Artwork instructions

Book Publishing

Index

Author Gateway Index

authorGATEWAY journals

myHome | help | privacy policy | contact us

© copyright Elsev

3.5 Anàlisis d'expressió dels gens HOXA9, DEK, CBL i CSF1R (VI)

Casas S, Nagy B, Elonen E, Aventín A, Larramendy ML, Sierra J, Ruutu T, Knuutila S. Aberrant expression of *HOXA9*, *DEK*, *CBL* and *CSF1R* in acute myeloid leukemia. Leukemia and Lymphoma 2003;acceptat, pendent de publicació.

3.5.1 Quantificació de l'expressió relativa dels gens

Mitjançant QRT-PCR a temps real, la quantificació dels nivells d'expressió dels gens *DEK, CBL, CSF1R* i *HOXA9* en les mostres de MO de pacients de LMA comparada amb la de les mostres de referència, va permetre les següents observacions (Taula 4):

Dels 41 pacients analitzats, *HOXA9* estava sobreexpressat en 32 casos (78 %), sotaexpressat en 6 casos (14,6 %) i presentava nivells normals del transcrit en els 3 casos restants (7,3 %). La mitjana dels valors d'expressió relativa comparada amb les mostres de referència va ser 3000 ± 11595. Pel què fa a *DEK*, es va detectar sobreexpressió del gen en la majoria de pacients estudiats (97,6 %). En aquest cas, la mitjana dels valors d'expressió relativa comparat amb les mostres normals va ser 1415 ± 3094. En l'anàlisi quantitativa d'expressió de *CSF1R* es va observar sobreexpressió del gen en 7 pacients (17,1 %), sotaexpressió en 17 casos (41, 5 %) i una expressió normal en els 17 pacients restants (41,5 %). La mitjana dels valors d'expressió relativa en relació a les mostres control va ser de 7,5 ± 28,3. Per últim, es va detectar sobreexpressió de *CBL* en 8 casos (19,5 %), sotaexpressió en 8 casos (19,5 %), i una expressió normal en els 25 pacients restants (61 %). La mitjana dels valors d'expressió relativa del gen *CBL* en relació a les mostres de referència va ser 3,9 ± 131.

3.5.2 Anàlisi d'associació entre expressió gènica i paràmetres d'interès

Els resultats d'expressió dels gens *DEK, CBL, CSF1R* i *HOXA9* es van comparar amb certs factors clínics i biològics d'interès en relació a la LMA (Taula 5).

Els pacients amb t(8;21)(q22;q22) al cariotip presentaven una menor expressió de *HOXA9* (p=0,004). Així mateix, es va observar una associació significativa entre mostres CD34- i s'obreexpressió de *DEK* (p=0,01) i *HOXA9* (p=0,005). No obstant, no es va detectar associació significativa per l'expressió immunològica dels marcadors CD56 i CD64. Al considerar la classificació FAB, es va identificar sotaexpressió de *CSF1R* (p=0,034) i de *HOXA9* (p=0,045) associat a LMA de subtipus M2, així com sobreexpressió de *CBL* (p=0,012) i *CSF1R* (p=0,021) en M5. No es va detectar cap més associació significativa amb els altres factors analitzats, excepte la sotaexpressió de *CBL* en pacients majors de 60 anys (p=0,034).