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STUDY OF RELATED SENESCENCE PATHWAYS IN SOFT TISSUE TUMORS DEVELOPMENT

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STUDY OF RELATED SENESCENCE PATHWAYS IN SOFT TISSUE TUMORS DEVELOPMENT

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«Ever tried. Ever failed. No matter. Try again. Fail again. Fail better»

Samuel Beckett

INDEX

SUMMARY

Background: Oncogenic-induced senescence (OIS) is a growth-arrest mechanism demonstrated in cells and in some types of human cancers and it is considered a tumor suppressor process against malignant transformation. Different molecular pathways are involved in this process, such as RAS/RAF/MAPK or ARF/p53 and $p16^{INK4a}$/pRb. Peripheral nerve sheath tumors (PNSTs) are soft tissue neoplasms arising in nerve sheaths that can be sporadic or associated to clinical syndromes such as neurofibromatosis (NFs). Schwannomas are benign PNSTs and only very rarely undergo malignant changes that similarly to nevus are composed of cells derived from the neural crest. In nevus RAS/RAF/MAPK the pathway is activated mainly due to BRAF V600E mutations that cause the expression of senescence-associated markers in these lesions. This alteration is explained by changes in NF1 or NF2 genes in those cases associated to NF1 or NF2 Neurofibromatosis. However, the pathogenesis of genetic alteration of sporadic PNST is less well known. This study analyzes senescence markers in PNSTs to demonstrate the possible role of senescence in their tumorigenesis, together with the presence or absence of BRAF V600E mutations in these tumors, especially in sporadic ones.

Methods: A retrospective immunohistochemical study was done in 39 schwannomas and 18 malignant peripheral nerve sheath tumors (MPNSTs). Staining for $p16^{INK4a}$, Ki67, p53 and Cyclin D1 was performed in all the cases. Additionally, SA-β-Gal staining was done in those cases where frozen tissue was available (n=8). Moreover, BRAF exon 15 and KRAS exons 2 and 3 polymerase chain reaction (PCR) sequencing was performed in formalin-fixed/paraffin-embedded samples of 59 schwannomas, 16 neurofibromas and 24 MPNSTs, related and non-related to NF types 1 and 2.

Results: In schwannomas we found high expression of $p16^{INK4a}$, low or absent levels of Ki67 and positivity of SA-β-Gal activity. An opposite pattern was found in MPNSTs. Oncogenic BRAF V600E mutations were observed in 4/40 schwannomas and 1/13 MPNST (not associated with NF). A KRAS G12S mutation was also evident in one sporadic schwannoma.

Conclusions: Our results support the senescence nature of schwannomas and the absence of senescence phenotype in MPNSTs. Moreover, the finding of BRAF V600E

and KRAS mutations in a subset of PNSTs not related to NF represents an important event, especially for the development of novel specific treatment for these tumors.

INTRODUCTION

1. SENESCENCE

1.1. INTRODUCTION

Cellular senescence is a growth-arrest mechanism that protects the cell from hyperproliferative signals and various forms of stress[1]. The process of cellular senescence was first described by Hayflick and Moorhead (1961) observing that normal human fibroblasts were able to enter into a state of irreversible growth arrest after serial cultivation *in vitro*[2].

Cell senescence can be defined as a biological program that causes *permanent* and *irreversible* arrest of the cell cycle. Senescence can be divided into two subtypes: *replicative senescence* and *premature senescence*. *Replicative senescence* is a consequence of the erosion or the shortening of telomeres due to the repeated replication of the DNA in the absence of telomerase. *Premature senescence* is a form of senescence that is activated in response to various types of stress. Stress such as DNA damage, oxidative or genotoxic stress or drugs, damage to the chromatin structure or overexpression of oncoproteins[3-5] (Figure 1).

Upon exposure to these triggers, cells engage a coordinated network of effector pathways, that regulates senescence process and converges on two key phases of this process; a stable proliferation arrest and an altered secretory pathway connected with inflammation, the *senescence associated secretory phenotype* (SASP)[6].

Although senescence has been implicated in the promotion of aging[7, 8], it has been observed that this process serves as a potent tumor suppression mechanism. [9-11] A number of studies have shown that premalignant lesions from human and mice are

enriched in senescent cells. For instance, benign melanocytic nevi, which frequently carry the BRAFV600E oncogene, show features of senescence, including SA-β-Gal activity and high $p16^{INK4a}$ expression[12]. Moreover, senescence markers were found in human dermal neurofibromas, murine lung adenomas, human and murine prostatic adenomas, murine pancreatic intraductal neoplasias and murine lymphomas[9, 11, 13-15]. Both proliferation arrest and SASP are thought to act in concert to mediate tumor suppression. The proliferation arrest restrains tumor growth, and the SASP recruits innate immune cells to clear the damaged premalignant cells[16-19].

In summary, senescence is a complex process activated by different triggers in association with varied physiological end-points that restricts the proliferative capacity of impaired or damaged cells, as well as normal cells, protecting them of different types of damage.

1.1.1. Quiescence versus senescence

In contrast to senescence, quiescence is a *reversible* G0/G1 phase cell cycle arrest, which is induced by the absence of mitogens or growth factors, nutrient starvation, or increasing cell density[20, 21]. In general, quiescence is characterized by low metabolism and protein synthesis, lack of cellular growth, and, the absence of global heterochromatin structures[22-24]. While downregulated expression of genes is involved in cell division, quiescent cells upregulate genes (such as HES1) that inhibit senescence, differentiation and apoptosis[25, 26]. Unlike senescence, which can occur either in the G1 or G2 phase of the cell cycle, (depending on when the damage is detected and the

efficiency of the checkpoints[27, 28]) quiescence essentially takes place in G1, prior to the restriction (R) checkpoint[29]. Both quiescence and senescence are characterized by modifications of cell cycle regulators such as CDKs inactivation. But, unlike senescence, where CDKs are inhibited by p21 or/and $p16^{INK4a}$, the major regulator involved in quiescent arrest is p27[30-32]. Unlike p21, p27 induction is independent of the p53 pathway[31] and its levels/activity is primarily regulated by translation, phosphorylation, and Skp2-mediated degradation[33]. Although p53 and p21 were also implicated in the cell cycle arrest following growth factor removal, p53 integrity does not seem to be essential for quiescence[34-36].

1.2. SENESCENT CELL FEATURES

Although quiescent and senescent cells share similar metabolic features, senescent cells show specific and characteristic morphologic and molecular changes.

Cellular morphology could be indicative of senescence. Senescence cells show an increase of their volume, flat and vacuole-rich cytoplasm, nuclear pleomorphism and chromatin changes. This behavior is observed in a wide variety of normal cells, and it is widely accepted that normal human somatic cells have an intrinsically limited proliferative lifespan, even under ideal growth conditions[37].

Moreover, senescent cells show an altered genetic expression, especially in cell cycle regulation genes, inducing the cessation of DNA synthesis. Modifications of the ARF/p53 and $p16^{INK4a}$/pRb pathways have been observed, or of the expression of genes coding for proteins related with cell proliferation, such as c-FOS, Cyclin D1, Cyclin A, Cyclin B and PCNA[38] and increase in other cytoplasmic and nuclear proteins.

One of the most important features of senescent cells is their irreversible arrest of the cell cycle, usually with a DNA content characteristic of the G1 phase[39]. Once stopped, they cannot start the replication of DNA, even if they have adequate growing conditions.

Senescent features involve most of the physiological aspects of the cell. These features are listed below:

- *Morphological features of senescence*: Senescent cells show flat, enlarged morphology and are commonly multinucleated[40].

- *Metabolic alterations*: There is an increase of SA-β-Gal activity. SA-β-Gal is a popular biomarker of senescence[41] because it is easy to detect. This modification is indicative of expansion of lysosomal compartment. This results in the eradication of lysosomal recycling capacity for proteins, lipids, and mitochondria. Consequently, there is an elevated reactive oxygen species (ROS) production with oxidative damaged enzymes accumulated in the cytosol. Finally, oxidative stress results in DNA damage as well as in the damage of other molecular species, including proteins and lipids[42, 43].

- *Molecular pathways involved*: Senescent cells are terminally arrested at G1, showing increased levels of many cell cycle inhibitors[44, 45]. OIS is a consequence of the activation of a complex network of pathways mostly involving the ARF/p53, and/or $p16^{INK4a}$/pRb pathways. Stabilization of p53 can be acquired through ARF, a negative regulator of MDM2 that targets p53 for proteosomal degradation. Activation of p53 induces senescence in part by transactivating the CDKI p21 which, amongst other activities, inhibits phosphorylation and therefore suppression of pRb by the CDK2/cyclin E complex. PRb is also activated via up-regulation of the CDKI $p16^{INK4a}$,

which inhibits phosphorylation of pRb by CDK4/6/cyclin D complexes. Subsequently, pRb inhibits E2F-dependent gene transactivation and promotes cell-cycle arrest in G1. Stable suppression of E2F-responsive genes is achieved through senescence associated heterochromatic foci (SAHF) formation, which is thought to contribute to the apparent irreversibility of senescence[46, 47].

- *Modification of chromatin structure*: The initiation of senescence triggers and the generation and accumulation of distinct heterochromatic structures, known as *SAHF*. SAHF formation and promoter repression depend on the integrity of the pRb pathway[46]. In replicative senescence the telomerase gene is deactivated in many adult human cells. As a result, these cells lose small portions of the ends (telomeres) of their chromosomes each time they divide. This process appears linked to their finite replicative lifespan in cell culture (The Hayflick Limit). However, oncogene- or culture stress-induced senescence does not rely on telomere shortening.

- *Senescence-associated secretory phenotype (SASP)*: Senescent cells undergo widespread changes in protein expression and secretion, which ultimately develops into the SASP[48, 49]. Senescent cells upregulate the expression and secretion of several matrix metalloproteinases that comprise of a conserved genomic cluster and interleukins that promote the growth of premalignant epithelial cells. A limited number of cell culture and mouse xenograft studies support the idea that senescent cells secrete factors that can disrupt tissue structure, alter tissue function and promote cancer progression[7, 50, 51]. Recent studies on the SASP of human and mouse fibroblasts show it is conserved across cell types and species; moreover, specific secreted factors are strong candidates for stimulating malignant phenotypes in neighboring cells[48, 52]. The idea that a biological

process, such as cellular senescence, can be beneficial (tumor suppressive) and deleterious (pro-tumorigenic) is consistent with a major evolutionary theory of aging termed antagonistic pleiotropy[48]. The SASP is possibly the major reason for the deleterious side of the senescence response[49]. A complex cocktail of factors including proinflammatory cytokines, chemokines, extracellular proteases, matrix components and growth factors, regulate the initiation and maintenance of senescence. IL-8, its receptor CXCR2 and IL-6 are considered the most important cytokines secreted by senescent cells[53-55].

Table 1 reports the details of senescent cells.

1.3. ONCOGENIC-INDUCED SENESCENCE (OIS)

The cancerous transformation begins with an accumulation of mutations that produce more aggressive cells, which are further selected by the tissue and/or tumor microenvironment[56]. Neoplastic transition is characterized by an increase in the expression of oncogenes, which control different biological processes, such as cell proliferation and apoptosis. Oncogenic mutations typically cause excessive cell proliferation, leading to the disruption of normal tissue microanatomy and impaired tissue function.

Interestingly, oncogenes such as RAS, RAF, E2FS, STAT5, and AKT can induce cellular senescence in normal cells by activating diverse tumor supressors. This mechanism is called oncogene-induced senescence (OIS)[57, 58]. OIS is considered a special form of premature senescence, in which this irreversible cell cycle arrest is

activated in response to oncogenic stimulation, independently of telomere length[37]. Table 2 illustrates the oncogenes that are known to induce senescence.

OIS was observed for the first time by Serrano et al. in 1997, when an irreversible cell cycle arrest was induced in cultured fibroblast by HRAS[4]. Replicative senescence and OIS share several characteristics, such as induction of various tumor suppressor pathways, including ARF-p53 and $p16^{INK4a}$/pRb. Additional hallmarks in telomere- and oncogene-associated senescence programs include persistent cell cycle arrest, morphological transformation, induction of SA-β-Gal activity, emergence of SAHFs, activation of SASP, increased production of ROS, and in some settings; DNA damage, endoplasmatic reticulum stress, and autophagy[11, 59].

Currently there is a lot of evidence that the OIS is an event that occurs not only in cell lines, but also in animal models and *in vivo*, such as in some human tumors[60-62]. Recent studies have reported the detection of senescence biomarkers in different lesions, in mice, and in humans [63, 64].

In recent times, nevus has become the main model to study OIS, due to the ability of melanocytic cells to enter in senescence in response to oncogenic stimulation[12]. Human nevi are benign tumors of melanocytes that frequently harbor oncogenic mutations. The most frequent is the substitution of a valine for glutamic acid in BRAF gene (V600E)[65]. Nonetheless, nevi typically remain in a growth-arrested state for decades and only rarely progress into malignancy (melanoma). In the nevus-melanoma model, the induction of cell cycle arrest by BRAF (V600E) mutation has been seen, and is associated to the overexpression of $p16^{INK4a}$ and the SA-β-Gal activity, a process independent of

telomerase action[12, 66]. It has been explained that senescent melanocytes show a phenotype characterized by large, flat, pigmented cells that express senescence markers, accompanied by low expression of the proliferation marker Ki67, dephosphorilation of pRb, downregulation of CDK2 and CDK4 kinase activity, reduced expression levels of p21, p27 Cyclin D1 and Cyclin E and presence of SAHF[67-69].

It has been seen that a malignant transformation of nevus to melanoma is only possible when senescence induction pathways are unharmed. Therefore, based on the senescence studies, benign nevi are considered the first step of this model and to be potential precursors of melanoma[12, 70-72]. The next stage of melanoma progression is dysplastic nevi in which escape from $p16^{INK4a}$/pRb senescence has been hypothesized. This kind of lesion shows reduced expression levels of $p16^{INK4a}$, p53 and p21, associated with chromosomal abnormalities[72]. Activation of telomerase in dysplastic nevi could lead to a radial growth phase (RGP) lesion, in which melanomas are thin, growing only in or near the epidermis[73]. Tumor cells consist of immortal but keratinocyte dependent cells[69, 71] and it seems likely that melanocyte immortalization is a mandatory step in early melanoma development, requiring both telomerase activation and deficiency of the $p16^{INK4a}$/pRb pathway. The final stage of melanoma progression is called the vertical growth phase (VGP), in which melanomas invade more deeply and are competent for metastasis[73]. Alterations in PTEN/PIK3/AKT/mTOR and p53 pathways or other mechanisms seems to be implicated in bypassing senescence, and promoting transformation to malignant melanoma[74, 75].

1.4. SENESCENCE REGULATION

1.4.1. RAS implicated in the mechanisms of OIS

Senescence is a complex mechanism activated by the cells in response to different types of stress. This cellular response is aimed at stopping the proliferation of cells that have suffered damage and prevent the transmission of mutations to daughter cells. Different mechanisms of senescence regulation have been reported.

OIS *in vitro* has been described in the context of oncogenic stimulation by the *RAS/RAF/MAPK pathway*[4, 12]. RAS activation triggers a number of downstream signaling pathways. Serrano et al. first showed that, despite an initial hyperproliferative response, RAS-induced proliferation in normal cells leads to cell cycle arrest and senescence[4]. Moreover it has been observed that RAS effectors are able to induce OIS. *In vivo*, expression of KRAS in the mammary gland causes senescence[62] and the mutated BRAF-V600E induces senescence in thyroid tumors and melanocytic nevi[12, 63, 76]. In addition to the RAS oncogenes and their proximal downstream kinases, distal effectors of the RAS pathway, such as the E2F family of transcription factors, can also induce senescence[77]. RAS-induced senescence is accompanied by overexpression of both $p16^{INK4a}$ and $p19^{ARF}$, and the consequent activation of pRb and p53[4, 78-80]. Conversely, in the absence of $p16^{INK4a}$, $p19^{ARF}$, or p53, RAS can induce cellular transformation directly in mouse embryonic fibroblasts[4, 81], thus underscoring the importance of these tumor suppressors in the RAS-induced senescence response.

Moreover RAS pathway activation can lead to senescence stimulating other mechanisms that include *ROS*, *DDR*, and the p53 and PTEN/PI3K/AKT/mTOR pathways[82, 83].

In vitro studies have demonstrated the constitutive activation of RAS gene results in an increase in intracellular and, in particular, mitochondrial ROS. These results suggest that in normal diploid cells, RAS proteins regulate oxidant production, and that a rise in intracellular H_2O_2 represents a critical signal mediating replicative senescence[82, 84]. However, it has also been observed that activation of the PTEN/PI3K/AKT/mTOR pathway can induce senescence, although it is less effective compared to activated RAS-senescence[85, 86]. Nevertheless, alterations in these downstream effectors of RAS, especially the genetic inactivation of PTEN (an inhibitor of PI3K/AKT/mTOR), are involved in suppressing the senescent mechanism induced by activation of RAS and these promote tumorigenesis[13, 87, 88].

The activation of the *SASP* has also been implicated in induction and maintenance of senescence in an autocrine manner. It has been shown that knockdown of CXCR2 (which is a receptor for IL8 and CXCL) leads to the bypass of senescence in human diploid fibroblasts, and overexpression of CXCR2 causes p53-dependent senescence[54, 89]. Moreover, it has also been seen that IL-6 and IL-8, and their receptors, play a causative and necessary role in the establishment and maintenance of senescence and that has a relation with RAS and p53[54, 89, 90].

1.4.2. Cellular senescence and tumor suppressor gene $p16^{INK4a}$

Although there are many mechanisms related to senescence regulation, it is known that this process is established and maintained through two major regulating pathways, ARF/p53 and $p16^{INK4a}$/pRb. These pathways can interact or be activated in independent ways, depending on the kind of stress on the different species or the cell types.

As described in our previous review article, one of the most important mechanisms involved in the induction of senescence is $p16^{INK4a}$ overexpression[91]. $P16^{INK4a}$ is the principal member of the Ink4 family of CDK inhibitors. It is codified by a gene localized on chromosome 9p21 within the INK4a/ARF locus [92]. It is well known that $p16^{INK4a}$ contributes to the regulation of cell cycle progression by inhibiting the S phase. Briefly, $p16^{INK4a}$ binds to CDK4/6, inhibiting cyclin D–CDK4/6 complex formation and CDK4/6-mediated phosphorylation of pRb family members. Expression of $p16^{INK4a}$ maintains the pRb family members in a hypophosphorylated state, which promotes binding to E2F1 and leads to G1 cell cycle arrest[92]. However, this classically known function seems to be just a simplified scheme of the global role of $p16^{INK4a}$, and many aspects of its function and regulation are still partially unresolved.

It has been demonstrated that an elevated level of expression (upregulation) of $p16^{INK4a}$, induced by oncogenes such as RAS, DNA damage response, or aging, is involved in cellular senescence. Expression of $p16^{INK4a}$ is markedly increases with ageing in most mouse tissues and in human skin and kidney tissues[93, 94], suggesting the importance of this tumor suppressor in ageing and senescence. In addition, $p16^{INK4a}$ overexpression has been reported in senescent fibroblasts in response to oxidative stress, DNA damage

and changes in chromatin structure[95]. The molecular mechanism by which $p16^{INK4a}$/pRb pathway is involved in senescence regulation is shown in Figure 2.

While genetic inactivation of the $p16^{INK4a}$ gene by deletion, methylation and point mutation has been found in nearly 50% of all human cancers[96, 97], the overexpression of $p16^{INK4a}$ has been observed in benign and premalignant lesions, *in vitro* and *in vivo*, as an activator of senescence to prevent proliferation of potentially dangerous cells[98]. $P16^{INK4a}$ overexpression has been also observed in some malignant tumor development, associated with aggressive behavior and poor prognosis of disease. This occurrence seems to be related to a deregulation of $p16^{INK4a}$/pRb pathway. pRb loss is frequent in several neoplasms, which results in increased $p16^{INK4a}$ expression in tumor cells and cancer tissue due to positive feedback in cells with uncontrolled cell proliferation[99-101].

1.4.3. P53 as regulator of senescence

P53 is a tumor suppressor gene, activated by DNA damage, such as ionizing radiation or telomere dysfunction. This response is mediated by the activation of ARF/p14, encoded by the Ink4a/ARF gene. The transcription of this locus generates two transcripts corresponding to ARF and $p16^{INK4a}$, but with a different promoter[92]. ARF increase p53 activity, inhibiting the E3 ubiquitin ligase HMD2, which mediates the degradation of p53. P53 activation causes multiple consequences in gene expression, but the most relevant is the activation of p21, an inhibitor of Cyclin E/CDK2 complex, which produces cell cycle arrest. The p53 tumor plays a crucial role in the integration of stress signaling and the coordination of cellular responses to stress and is one of the most important determinants of cellular senescence. Depending on the kind of stress stimuli,

stress strength and cellular context, activation of the tumor suppressor p53 can induce reversible quiescence, cellular senescence or apoptosis[102].

However, determinants of whether the cell cycle arrest is reversible or induced senescence continues being, is an unanswered question. It is hypothesized that fast and efficient repair of DNA damage inhibits the p53-p21 signaling, while an incorrect, incomplete or slow repair of the damage promotes the induction of senescence[103]. Recent studies indicate that the crosstalk between p53 and mTOR in normal cells plays an important role in the transition of cells from quiescence to senescence, an irreversible step called *geroconversion*. It seems that p53 induces a cell cycle arrest and the RAS-dependent hyperactivation of mTOR and its substrate S6K represent the irreversible step to drive quiescent cells to senescence[104, 105].

1.5. SENESCENCE MARKERS

Several markers have been described as useful to identify senescent cells *in vivo* and *in vitro*, but none have been demonstrated as exclusive of senescence.

An obvious marker is represented by the *loss of DNA replication* that can be determined through the incorporation of 5-bromodeoxyuridine o timidin-H^3 in cultivate cells or by immunohistochemical staining of the proliferation index Ki67. However, these markers are unable to discriminate between senescent, quiescent or differentiated cells.

One of the most important senescent markers is represented by the increase of the *SA-β-Gal activity*, detected at an acidic pH6. β-galactosidase is a lysosomal enzyme related to the expansion of the lysosomal compartment, observed during senescence[106]. Although considered the major marker of senescence, (both *in vivo* and *in vitro*), the increase of

SA-β-Gal activity related to a lysosomal expansion, suggests that it is not an exclusive marker of senescence, otherwise all of those specific alterations would cause an increase in the lysosomal compartment[107].

Expression of proteins related with the *ARF/p53* and *$p16^{INK4a}$/pRb pathways*, such as p21, p53, $p16^{INK4a}$ or pRb, are also indicated as senescence markers. These proteins are not exclusive of senescence, because a p21 increase has been also found in quiescent cells, or $p16^{INK4a}$ is overexpressed in some tumors, especially those with an alteration of the pRb gene.

Some evidence demonstrates that, during senescence, extensive changes in the structure of chromatin are developed, called *SAHF*. This alteration can be visualized by the appearance of clusters of DAPI-stained nuclear foci. However, these foci are not universally found in all human tissues and are not considered reliable markers of *in vivo* senescence[38].

Recently, new efforts to find new markers of senescence cells have been made, identifying two genes, *topoisomerase IIa* and *HDAC9*, whose expression was specifically altered under several conditions associated with senescence[108] and a pool of novel senescent-specific proteins associated with the plasma membrane of senescent bladder cancer cell line[109].

At any rate, a universal specific senescence marker has not yet been found and a combination of different actual markers is used at this present time for studying senescent cells.

1.6. SENESCENCE AND CANCER

Cancer development is an event that requires the accumulation of multiple genetic alterations in the cells, but in many cases the mechanism of malignant transformation remains unclear.

One of the most fascinating hypotheses is that OIS represents a possible mechanism to prevent proliferation of potentially dangerous cells and an important suppressor of tumorigenesis. It has been thought that OIS provides an initial barrier to the development of malignancies, whereas telomere-based senescence may attenuate tumor progression[11, 110, 111].

This hypothesis is supported by the fact that senescence cells have been found in a few number of benign and premalignant lesions, but not in malignant ones[10, 11, 72, 112]. For example, human neurofibromas, melanocytic nevi, prostatic intraepithelial neoplasia, and colon adenomas, display some of the features of senescence, along with low or negative proliferation markers and increased expression of proteins related with senescence, such as $p16^{INK4a}$ [12, 113, 114]. As previously described (paragraph 1.4.2.), $p16^{INK4a}$ overexpression has been found in premature senescence, and particularly in OIS, and has consequently been associated with senescence in benign and premalignant lesions[67, 115, 116]. In addition to oncogenic signaling, inactivation of tumor suppressors such as PTEN[13], pRb[117], and VHL[118, 119] also led to premalignant tumors that expressed markers of senescence[120]. In Table 3 we report mouse models and human tumors with evidence of senescent cells.

1.6.1. Mechanisms of evasion of OIS

Several *in vitro* and animal model studies have demonstrated the cells ability to bypass senescence. This represents the main molecular mechanism and the first step of human cancer development[9, 115, 121]. According to recent theories the mechanisms that lead the cells into tumor progression could occur via one of two possible scenarios:

- A subset of cells may primarily bypass tumor suppression altogether, never undergoing senescence due to loss of key components of the senescence pathway (e.g. p53, $p16^{INK4a}$,etc.). These cells would then continue to proliferate and acquire further mutations with transforming properties, eventually leading to tumor progression;
- Some of the cells that have exited the cell cycle and acquired features of senescence may at some point re-enter the cell cycle and regain the ability to proliferate, due to genetic, epigenetic, or microenvironmental changes that disrupt the molecular pathways essential for maintenance of senescence.

These two mechanisms could not be mutually exclusive, and conceivably could both contribute to tumor progression depending on the setting and context.

It is well known that the activation of oncogenes, loss of tumor suppressors, or other genetic mutations could drive normal cells into malignant transformation. This process is due to several cellular mechanisms, providing properties to the cells such as proliferative advantage, resistance to cell death, induction of angiogenesis and activation of invasion and metastasis[122]. New evidences suggest that additional hallmarks of cancer are involved in the pathogenesis of some and perhaps all malignant

tumors. These mechanisms include the capability to modify cellular metabolism in order to most effectively support neoplastic proliferation, evasion of immunological destruction, genomic instability and the tumor-promoting inflammation[123-125].

As previously described, the majority of cells in a lesion may undergo senescence in response to oncogenic stimuli. However, it is thought that a few cells are able to bypass the molecular pathways leading to senescence, resulting in tumor emergence within a background of senescent cells. Multiple studies on premature senescence induced *in vitro*, (in human and mouse cells) established that senescence can be bypassed with different mechanisms[126-129].

- *Inactivation of senescence regulatory pathways*, with the disruption of the p53 tumor suppressor pathway alone[130], the $p16^{INK4a}$/pRb pathway alone[46], while in other settings both the p53 and pRb pathways needed to be disengaged[131] or alterations of PTEN/PIK3/AKT/mTOR pathway[132, 133].

  Alterations in the $p16^{INK4a}$/pRb pathway represent the most important mechanism involved in bypassing senescence. Loss of $p16^{INK4a}$ is one of the most frequent events in human tumors and allows pre-cancerous lesions to bypass senescence. Close to half of all human cancers show $p16^{INK4a}$ inactivation[134, 135]. Whereas $p16^{INK4a}$ overexpression in benign lesions is associated with senescence induction in response to oncogenic stimuli, malignant transformation seems to be associated with a loss of $p16^{INK4a}$[91]. Different mechanisms are involved in the inactivation of $p16^{INK4a}$ gene, such as homozygotic deletions, loss of heterozygosity, point mutations and promoter methylation[97, 136, 137]. Moreover $p16^{INK4a}$ inactivation has been reported to be an early and critical event in tumor progression in some types

of tumors[137-139], suggesting a relevant role in the development of some preneoplastic lesions.

On the other hand, pRb loss is a frequent event in many neoplasms, and it seems to be independent from $p16^{INK4a}$ inactivation. Indeed, infrequent overlap between CDKN2A and pRb gene deletion are described, implying that these are often mutually exclusive tumorigenic events[140]. It has been seen that tumors with early defects in pRb signaling continue to express $p16^{INK4a}$ and that the deregulation of pRb results in increased $p16^{INK4a}$ expression in tumor cells and cancer tissue due to positive feedback[101, 141]. Inactivation of the pRb checkpoint can be caused by different mechanisms, such as mutation, deletion, or methylation of the RB1 gene or HPV viral oncoproteins and the ability to inactivate pRb protein leading to increases in $p16^{INK4a}$ expression[142-146].

Alteration of other senescence regulators and their role in bypassing senescence may contribute to tumor transformation. For example, in prostate cancer development, p53 loss and the activation of the PTEN/PIK3CA/AKT/mTOR pathway seems to be implicated in this process, leading to an accelerated tumorigenesis[4, 13, 104, 132]. However, in the model nevus-melanoma, inactivating mutations of PTEN, Lkb1, and CDKN2A in post-arrested melanocytes results in elevated mTORC1/2 signaling and enhanced proliferation, bypassing senescence, and promoting transformation to melanoma[75, 147].

- *Disruption of components of the DNA damage response (DDR)*. It has been seen that alteration of components of DDR, such as ATM or CHK2, could lead to bypass of senescence: depending on the cell type and the oncogenic signal, bypass

occurred when the DDR pathway alone was disrupted, or required also disruption of the pRb pathway[83, 148, 149].

- *Role of microenvironment and SASP*. Senescent cells are metabolically active and show changes in protein expression and secretion. This phenotype has also been termed "the senescence messaging secretome"[6, 150, 151]. SASP factors can be globally divided into the following major categories: Soluble signaling factors (interleukins, chemokines, and growth factors), secreted proteases, and secreted insoluble proteins/extracellular matrix components (ECM). The activities of these factors provide potent mechanisms by which senescent cells can modify the tissue microenvironment. In addition, some of these factors may play a paracrine role in tumor promotion, despite their autocrine tumor-suppressive effects. However, it has been observed that factors secreted by senescent cells can promote tumor development. These effects have been observed in a number of tissues, including breast, skin, prostate, pancreas, and oropharyngial mucosa[7, 48, 51]. Senescent cells secrete growth factors and cytokines, that are able to create a local tissue environment that promotes the proliferation of epithelial or mesenchimal cells, cell migration and invasion, alter the differentiation status of neighboring cells and modify leukocyte infiltration and tumor immunology[48, 49, 152].

- *Importance of heterochromatin formation*. It has been observed that in certain contexts the absence or knockdown of proteins needed for formation of SAHF, such as HMGA proteins or macroH2A in human fibroblasts, or the

methyltransferase SUV39h1 in mouse splenocytes, can result in primary bypass of senescence[9, 153, 154].

The factors implicated in senescence by-pass are summarized in Figure 3.

1.7. MORPHOLOGY AND PATHOGENESIS OF PERIPHERAL NERVE SHEATH TUMORS (PNSTS)

Proliferative lesions of peripheral nerves are a group of tumors that originate from the elements that compound the sheath of peripheral nerves. They include non-neoplastic lesions (such as traumatic neuroma), benign tumors and malignant ones. The group of benign lesions comprises of schwannomas, neurofibromas, perineuriomas, granular cell tumors and nerve sheath myxoma. The malignant neoplasms are represented by the MPNSTs and the malignant granular cell tumor. These lesions are relatively frequent and show identifiable features, but in many cases, the differential diagnosis may be difficult. Moreover, although well-defined subtypes of peripheral nerve sheath tumors were described early in the history of surgical pathology, controversies regarding the classification and grading of these tumors continue[155, 156].

1.7.1. Schwannomas

Schwannomas are peripheral nerve sheath tumors consisting entirely of a clonal proliferation of Schwann cells. These lesions occur usually sporadically as solitary tumors in about 90% of cases, with a pick of incidence in the fourth to sixth decade of life. They can affect all ages without predisposition of sex and race. These lesions may

also be multiple and/or associated with other tumors as a manifestation of clinical syndromes such as type 2 neurofibromatosis (NF2), schwannomatosis or Carney's complex[157-159].

The most frequent sites of origin are peripheral nerve sheaths localized in the skin and subcutaneous tissue of head and neck region or extremities. Intracranial nerves are also involved with a significant frequency, the majority of them localized in the cerebellopontine angle region, emanating from the vestibular division of the 8th cranial nerve[160, 161]. It is rare that these lesions affect the visceral organs, bones, spinal intramedullary region or central nervous system. Clinically, the majority of schwannomas are asymptomatic, showing a slow growth, but some deep-seated tumors, such as retroperitoneal and mediastinal lesions, may become symptomatic by local compression or bony erosion.

Microscopically, schwannomas are well circumscribed masses, with a surrounding capsule, and contain areas composed of fascicles of Schwann cells that have a spindle cell morphology (Antoni A pattern) and more loosely textured and microcystic areas (Antoni B pattern). Schwannomas show areas of nuclear alignment or palisading, often forming parallel nuclear arrays or Verocay bodies. Some tumors show extensive degenerative change, also known as "ancient schwannomas," presenting pleomorphic cells with marked degenerative nuclear atypia, which should not be confused with malignant change[162, 163]. Immunohistochemically schwannoma tumor cells show uniform strong nuclear and cytoplasmic positivity for S100; they may also show immunoreactivity to glial fibrillary acidic protein (GFAP)[164], and in few cases express cytokeratins[165].

1.7.1.1. Histological variants of schwannomas

Morphologically distinct variants of schwannomas have been described:

- *Plexiform schwannoma*. These lesions are usually located in dermal superficial sites and are more frequent in young adults. Plexiform schwannomas tend to occur sporadically, but a minority of cases are associated with NF1 and NF2 or schwannomatosis[166]. These tumors show typical histological features of schwannomas, with predominantly Antoni A-type tissue and sometimes Verocay body formation, but they show an intraneural nodular pattern of growth. These kind of schwannoma do not have the capacity to metastasize, and respond well to surgical excision, so these should not be confused with MPNSTs[167, 168].
- *Cellular schwannomas*. Cellular schwannomas commonly occur in the spinal and paraspinal regions, about 10% are intracranial. The majorities of cases are sporadic, and are not associated with clinical syndromes. Morphologically, this variety of schwannomas is composed predominantly of cellular Antoni A-type tissue with a higher cellularity and mitotic rate than conventional schwannomas; occasionally containing small foci of necrosis, they may be locally erosive but are benign tumors without metastatic potential. Moreover, cellular schwannomas have a significant local recurrence rate of up to 40%, depending on the extent of resection and location[169-171]. With these features, this benign variant of schwannoma may be confused with a MPNST or other malignant tumors[172-174]. Useful features to distinguish cellular schwannomas from malignant peripheral nerve sheath tumors are; a relatively high cellularity for the mitotic rate, good circumscription, perivascular hyalinization, uniformly diffused S100 protein immunoreactivity, and

variable GFAP immunoreactivity[168]. In a recent report, a combination of morphological and immunohistochemical features have been studied, to find useful criteria to distinguish between malignant peripheral nerve sheath tumor and cellular schwannoma[175].

- *Melanocitic schwannoma*. This entity is an uncommon variant of schawannoma, occurring mainly in adults, with a slight predominance in females. These entities are usually deep seated and involve the head and neck nerves. Occasionally, they may involve the gastrointestinal tract, soft tissues, skin, liver and heart[156]. Histologically the most important feature is the presence of pigmented Schwann tumoral cells, which have a spindle cell and epithelioid morphology. Multinucleated cells and cells with vesicular nuclei and prominent eosinophilic nucleoli could be present and half of the cases show psammoma bodies. Melanotic schwannomas are S-100, Melan-A and HMB-45 immunoreactive, but negative with antibodies to GFAP[176-178]. Although different S100 protein subtypes seem to be expressed in PNSTs and in melanoma[179]. There are no clear-cut histological criteria for malignancy, although features such as large nuclei, prominent nucleoli, mitoses and necrosis are common. These lesions may occur sporadically, but generally arise in association with Carney's complex. A significant proportion of melanotic schwannomas show a malignant behavior, with a rate of local recurrence of 35% and metastatic spread of 44%[180]. Especially in young patients with Carney's complex, metastasis in lung, liver, stomach, adrenal glands and brain can occur in the later life, and they could be potentially lethal with a mortality rate of about 15% of patients.

- *Minor variants*. Although most schwannomas demonstrate classic histology or most common variants, other curious morphologic variations are occasionally encountered. The *reticular schwannoma* is characterized by abundant myxoid change, microcysts, and a tendency to arise in viscera[181]. Rare findings in schwannomas include large cellular palisades resembling neuroblastic rosettes[182], pseudoglandular structures[183], benign epithelioid change[184], and lipoblastic differentiation[185].

1.7.2. Neurofibromas

Neurofibromas are benign, heterogeneous PNSTs that occur in association with NF1, but may also occur sporadically. They are relatively common, particularly at superficial cutaneous sites, but can be found anywhere within the central or peripheral nervous system, especially in the neck, thorax, cranium, retroperitoneum, and flexor surfaces of the extremities[186]. Histologically, the Schwann cell represents the primary neoplastic cell component of neurofibroma, but they also incorporate a mixture of non-neoplastic peripheral nerve components, including axons, perineurial cells, fibroblasts, and variable inflammatory elements, such as mast cells and lymphocytes. In addition, a population of CD34 positive cells of unclear histogenesis is present. Neurofibromas are usually easy to distinguish from schwannomas by their lack of a capsule, mixed population of cells, cells with wavy nuclei, and absence of Antoni A and B patterns[187, 188]. In any case, morphological variants of neurofibromas may make it more difficult to diagnose of these neoplasms.

1.7.2.1. Histological variants of neurofibroma

Specific clinicopathologic subtypes based on architectural growth patterns include localized, diffuse, plexiform and atypical neurofibromas.

- *Cutaneous neurofibromas*. This variant can occur in two forms; localized and diffuse. These tumors grow from small nerves in the skin or in hipodermis and arise as small masses. Typically they appear in puberty, but may increase in number with advancing of age. The localized cutaneous form is the most common variant of neurofibromas, and occurs sporadically in the majority of cases. Only a 10% of this variant is associated with NF1. Clinically, these tumors are asymptomatic and they can be nodular, sessile or pedunculated masses. Cutaneous neurofibromas remain benign throughout life and do not become malignant cancers, even though they may cause significant esthetic problems[168, 186, 189].
- *Localized intraneural neurofibromas*. Localized intraneural neurofibromas are the second most frequent form of neurofibromas and they can be sporadic or arise in NF1[168]. They can affect a wide variety of sizes, and involve a minor or a major nerve. Frequently they can arise from the large nerves of brachial, cervical or lumbosacral plexuses or involve visceral nerves[190, 191]. Unlike the cutaneous variant, these lesions show a capacity to malignant transformation, and together with plexiform variant they are considered precursors of MPNSTs[186].
- *Diffuse neurofibromas*. Diffuse neurofibromas occur primarily in children and young adults and they are associated with NF1 in up to 20%-30% of cases. Histologically, they are characterized by a plaque-like enlargement of spindle cells that contain elongated ovoid to curved nuclei and are surrounded by a stroma with collagenous

fibers rather than myxoid[192]. They show a specific immunohistochemical feature represented by S100 positive pseudo-meissnerian corpuscles[193, 194]. Malignant transformation is extremely rare, but, even after complete excision, clinical recurrences may develop because of the infiltrative growth pattern[195, 196].

- *Plexiform neurofibromas*. Plexiform neurofibromas are defined by the involvement of numerous adjacent nerve fascicles or multiple components of a nerve plexus. These lesions affect about 50% of individuals with NF1 and are virtually pathognomonic of the disease. They tend to involve single or multiple nerve fascicles that frequently arise from the cranial and large peripheral nerve[197-199]. Clinically, plexiform tumors may be discrete, homogeneous, and well circumscribed or diffuse, heterogeneous, and infiltrative. Microscopically, plexiform neurofibromas often show an admixture of areas resembling localized and diffuse-type neurofibromas. Plexiform neurofibroma has a potential for malignant degeneration, and it is a recognized precursor for MPNST in NF1 patients[200, 201].
- *Other variants*. Other less common morphological findings in neurofibroma include the presence of melanin pigment[202], metaplastic bone and glandular differentiation[203]. Massive soft tissue neurofibroma, a very rare subtype, is characterized by large size, infiltration of soft tissue and skeletal muscle, often involving large anatomical regions, and histologically demonstrating the presence of a cellular component[204]. They may contain plexiform components, but usually do not undergo malignant degeneration.

1.7.2.2. Histologic atypia and malignant changes of neurofibroma

Some neurofibromas show unusual features such as degenerative cytological atypia (neurofibroma with ancient change, atypical neurofibroma) and/or increased cellularity (cellular neurofibroma).

Neurofibromas can show degenerative nuclear atypia, containing scattered cells with markedly enlarged, hyperchromatic nuclei with smudgy chromatin and occasional nuclear inclusions; however, they lack increased cellularity, fascicular growth, or mitotic activity[205-208]. These features are considered benign atypical changes.

However, an increase of atypical neurofibroma cells with hyperchromatic and pleomorphic nuclei without smudged chromatin, in the absence of mitoses with a pronounced fascicular growth pattern, are considered early changes in malignant transformation. In neurofibromas with these characteristics, molecular changes have been found, especially the deletion of the CDKNA/B gene that encodes for $p16^{INK4a}$ protein[209-211].

Clear atypical changes in neurofibromas, indicating a malignant transformation, could appear in two forms, both presenting as localized moderate hypercellularity. The first is characterized by diffuse hypercellularity of uniformly hyperchromatic spindle cells with at least a three-fold nuclear enlargement compared with the size of ordinary neurofibroma nuclei. In this form, mitosis need not be present. In the second type, groups of elongated or ovoid cells are present and show ample cytoplasm and bizarre hyperchromatic pleomorphic nuclei[212].

1.7.3. Malignant peripheral nerve sheath tumors (MPNSTs)

MPNSTs are rare, aggressive neoplasms that represent about of 2-5% of all sarcomas[213]. They have an incidence of 0.001% in the general population[214]. They can occur sporadically or in patients with NF1 and arise either *de novo* or from a preexisting neurofibroma[215]. In extremely rare cases, these tumors develop from a preexisting benign schwannoma, (especially associated with NF2 and with intracranial localization[216, 217]).

These tumors are most frequently localized in the extremities, particularly proximally, followed by the trunk and head and neck. Clinically, they form a heterogeneous group of neoplasms with a range of morphology, showing a high metastatic potential and poor prognosis, especially if associated with large tumors (>5 cm), truncal location, NF1, high grade, high mitotic index (> 6/10 HPF) and incomplete resection[218-220].

Microscopically, MPNSTs are usually highly infiltrative lesions that display a varied range of cell morphologies (including spindle, epithelioid, pleomorphic, or small round cell) and architectural patterns. They present mitoses, hemorrhage, and necrosis that may be extensive, with islands of viable tumors condensed around vessels. Immunohistochemically, there are no diagnostic markers of MPNST. Expression of S-100 protein is variable and often incomplete, of limited diagnostic utility because diffuse expression of S-100 protein is not typical of MPNST. Malignant peripheral nerve sheath tumors can express other markers, such as pancytokeratin AE1/AE3 focally, epithelial membrane antigen (EMA), Transducin-like enhancer of split 1 (TLE1) in 30% of cases; CD34 and nestin are expressed in about a quarter of tumors [221, 222].

Although there are no well-defined criteria, grading can be applied according to the grading system of the French Federation of Cancer Centres Sarcoma Group, based on cellular differentiation, mitosis count and tumor necrosis[223-225]. Approximately 10% to 15% of MPNST are considered of "low-grade" morphology[203, 225], and patterns vary from cellular/atypical neurofibroma like, low grade fibromyxoid sarcoma like, low grade epithelioid to hemangiopericytoma like[226, 227].

The differential diagnosis of these tumors in peripheral nerve and soft tissue is wide; it includes a variety of sarcomas, primarily adult-type fibrosarcoma, synovial sarcoma, rhabdomyosarcoma, leiomyosarcoma, de-differentiated liposarcoma, and clear cell sarcoma[228]. Moreover, a subset of MPNSTs shows areas of divergent differentiation. Heterologous differentiation in the form of mesenchymal (cartilage, bone or skeletal muscle) or epithelial changes may be present in these tumors, especially in patients with NF1[227, 229, 230]. Molecular techniques, including FISH[231] and array comparative genomic hybridization[209] may be helpful tools in differential diagnosis.

1.7.4 Genetic syndromes associated with PNSTs and pathogenesis of sporadic tumors

PNSTs can arise sporadically or they can be the clinical manifestation of some genetic syndromes, the most frequent of these are the two type of neurofibromatosis (NF1 and NF2).

1.7.4.1 Neurofibromatosis type 1 (NF1) and type 2 (NF2)

NF1 is a common autosomal dominant disease with a birth incidence of 1:3000 and a minimum prevalence of 1:5000[232-235]. Clinical manifestations are represented by the formation of neural crest-derived tumors, especially plexiform neurofibromas, MPNSTs and optic nerve gliomas, and those associated to skin, bone or cardiovascular abnormalities[236-238]. NF2 is an autosomal dominant disorder, with a birth incidence of about 1:25000, clinically characterized by the development of multiple schwannomas, especially vestibular subtype, meninigiomas, pilocytic astrocytomas, ependymomas and skin and ocular alterations[238, 239]. In childhood, initial signs and/or symptoms may often be unrecognized, and the most frequent clinical manifestations are cranial meningiomas, spinal tumors, cutaneous schwannomas, facial mononeuropathy and retinal hamartomas[240, 241].

For both NF1 and NF2, genetic and environmental factors seem to modulate phenotypic and clinical variability. It has been found that in neurofibromatosis familiar syndromes, molecular alterations of specific tumor suppressor genes are involved in their pathogenesis: *NF1 gene*, for neurofibromatosis type 1 and *NF2 gene*, in

neurofibromatosis type 2. The *NF1 gene*, localized on chromosome 17q11.2, comprises more than 350 kb of genomic DNA and 60 exons, and code for a protein of 327 kDa, denominated *neurofibromin*[242-244]. *Neurofibromin* is a cytoplasmic protein that contains a GAP-related domain that acts by downregulating RAS via stimulation of intrinsic GTPase[245, 246]. RAS is a guanosine triphosphate (GTP)-binding protein that is active in the GTP-bound state and inactive in the guanosine diphoshate (GDP) bound state. GAP proteins help maintain RAS in the inactive GDP form by accelerating the conversion of GDP-RAS[242, 247]. Increased GTP-RAS leads to increased signaling through RAF kinase, which activates a kinase cascade involving MEK kinase and the ERK1 and ERK2 isoforms of MAPK resulting in cell proliferation[248, 249]. Increased GTP-RAS also protects cells from apoptosis by activating mTOR[249, 250]. Studies confirm that *neurofibromin* negatively regulates PIK3CA/AKT/mTOR pathway[251, 252]. These pathways are involved in the regulation of cell proliferation, differentiation, motility, growth, apoptosis, and cell senescence[253].

The *NF2 gene* is located in chromosome 22q 11.2 an has 17 coding exons. The gene encodes a 595 amino acid protein known as *merlin* (or schwannomin), structurally related to the moesin/ezrin/radixin proteins (ERM family), which link the actin cytoskeleton to cell surface glycoproteins that control cellular growth and remodeling[254]. *Merlin* interacts with numerous molecules, such as proteins involved in cytoskeletal dynamics and proteins involved in regulating ion transport[255-257]. This protein is considered a tumor suppressor, because of its involvement in several different signal transduction pathways[255, 258-260]. In fact, *merlin* is able to activate the tumor suppressor Salvador/Warts/Hippo pathway, negatively regulate RAS/RAF/MAPK

pathway, suppress RAC-PAK signaling, restrain activation of mTORC1 independently of AKT, and inhibit PI3K-AKT and FAK-SRC signaling. All the pathways that play an important role in regulating proliferation and apoptosis[261-264].

In NF syndromes, inactivating mutations of *NF1* and *NF2* genes and consequent loss of activity of their proteins (*neurofibromin* and *merlin*, respectively) occur. The genes *NF1* and *NF2* are found to have high rates of different mutations. These consist of deletions, insertions, nonsense mutations, missense mutations, and intronic mutations. It has been observed that the type of mutation can determinate or influence the different phenotypes of the syndromes[265-267]. These two proteins, *neurofibromin* and *merlin*, are very different in structure and probable mechanisms of action, but loss of both molecules in NF may constitutively activate a common molecular pathway, (RAS/RAF/MAPK), leading to uncontrolled cell proliferation, loss of cellular adhesion and tumor transformation[268, 269].

1.7.4.2 Pathogenesis of sporadic PNSTs

As described, molecular alterations involved in the tumorigenesis of PNSTs arisen in NF syndromes have been well studied. Contrarily, pathogenesis of sporadic PNSTs is not clear and the involvement of *NF* gene mutations remains poorly investigated.

Sporadic neurofibromas are rare entities that appear as isolated lesions without other signs of NF1. Very few studies have analyzed molecular alterations in these tumors, founding a biallelic somatic inactivation of *NF1* gene in two cases of sporadic neurofibromas[270, 271]. To the best of our knowledge, these two reports represent the only

demonstration of molecular alteration in neurofibromas not associated with NF1 syndrome.

Conversely, some other studies have been made in relation to sporadic schwannomas. It has been demonstrated that in up to 45% of sporadic vestibular schwannomas, a loss of the chromosome 22q has been identified[272]. As well as this, studies revealed a copy number addition on chromosome 9q34 in 10% of cases and an addition on chromosome 17q in 5% of samples[273]. The *NF2* gene mutation and loss of *merlin* production have also been observed, with the inactivation of the *NF2* gene being an essential step in tumorigenesis[274, 275]. Other alterations have been found in sporadic vestibular schwannomas such as upregulation of p53 and MDM2, loss of ARF and p21 or alterations of MET and associated genes, such as integrin, and caveolin-1 (CAV1), androgen receptor downregulation and an upregulated osteopontin gene (SPP1)[276-278].

MPNSTs can arise *de novo,* or from the malignant transformation of a sporadic or NF1-associated neurofibroma. The genetic mechanisms that lead the conversion of a benign neurofibroma in an aggressive sarcoma are still unclear. It is acknowledged that mutations of *NF1* gene have been found in NF1-associated MPNSTs, but also the majority of patients with sporadic MPNST show this molecular alteration[187]. In addition, other genes like CDKN2A, KIP 1 and TP53 may show mutations or inactivation of their products $p16^{INKa}$, p27 and p53 in sporadic MPNSTs. Further, overexpression of EGFR, loss of PTEN expression and alterations of the PIK3/AKT/mTOR pathway has been described[279-282].

Recently, it has been observed that loss-of-function (LOF) of the Polycomb repressive complex 2 (PRC2) core components; EZH2, EED or SUZ12 are present in sporadic,

NF1-associated and radiotherapy-associated MPNSTs, an analogy that they share with melanomas[283, 284]. In MPNSTs, these mutations are significantly linked with somatic alterations of CDKN2A and *NF1*. This demonstrates that a loss of PRC2 function and loss of CDKN2A are important for tumorigenesis, and may be a critical cooperative event in addition to *NF1* loss in MPNSTs[285]. Moreover it has been observed that there is a strong connection between PRC2 and RAS activation. In the study of De Raedt et al, the authors observed that PRC2 loss function amplifies *RAS* activation mediated by *NF1* loss in MPNSTs cell lines[286]. However, the relationship between these mutations and malignant transformation of neurofibromas into MPNSTs remains unclear.

PNSTs constitute a clinically, phenotypically and molecularly heterogeneous group. Although well-defined subtypes of PNSTs were described, controversies regarding the classification and grading of these tumors continue. Moreover, the pathogenesis of the majority of sporadic tumors remains elusive and few details are known about the molecular and genetic alterations driving their arising and/or malignant transformation. Recent advances in molecular biology have provided new insights into the nature of PNSTs, but a specific effective target of treatment is not yet available. Therefore, studying other pathways of malignant development in these tumors, may lead to the discovery of new and different biological mechanisms that can drive to novel targeted therapeutic approaches.

HYPOTHESIS

- Considering that both melanocytes and Schwann cells share a common origin from neural crest cells during embryogenesis, and that both lesions, nevi and schwannomas, have a very low proliferative potential, the hypothesis of this study is that benign schwannomas could be senescence lesions, like melanocytic-nevi. Contrarily, the malignant counterpart of schwannomas, MPNSTs loses the senescence phenotype, with absent or low expression of senescence markers and alteration of senescence regulatory pathways.

- Because, it is known that the RAS/RAF/MAPK pathway is involved in the genesis of PNSTs and that BRAF V600E is a frequent mutation in nevus, we can hypothesize that BRAF V600E mutation could also be a frequent event in sporadic schwannomas.

OBJECTIVES

The main objective is:

1. To demonstrate the role of senescence in PNSTs and to evaluate the presence of BRAF and KRAS mutations in these tumors.

Secondary objectives are:

1. To describe the expression of senescence markers in schwannomas and the loss of them in MPNSTs.
2. To analyze the SA-β-gal activity, as the most important senescence marker, in all cases in which frozen tissue was available.
3. To examine the presence of KRAS and BRAF mutations in schwannomas, neurofibromas and in MPNSTs.

MATERIAL AND METHODS

REFERENCES

Article nº1:

BRAF V600E and KRAS G12S mutations in peripheral nerve sheath tumours.

Serrano C, **Simonetti S**, Hernández-Losa J, Valverde C, Carrato C, Bagué S, Orellana R, Somoza R, Moliné T, Carles J, Huguet P, Romagosa C, Ramón y Cajal S.

Histopathology. 2013 Feb;62(3):499-504. doi: 10.1111/his.12021.

Article nº2:

Schwannomas, benign tumors with a senescent phenotype.

Simonetti S, Serrano C, Hernández-Losa J, Bagué S, Orellana R, Valverde C, Lleonart ME, Aizpurua M, Carles J, Ramón y Cajal S, Romagosa C.

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DISCUSSION

1. JUSTIFICATION OF THE STUDY IN PNST

PNSTs are a group of relatively frequent benign and malignant lesions, sporadic, or associated with specific clinical syndromes.

Neurofibromas, schwannomas and MPNSTs show a common origin of Schwann cells, but these tumor types have distinct pathologies and clinical behaviors, indicating that they arise via distinct pathogenic mechanisms. Current knowledge of the biological pathways in these tumors has greatly expanded over the past two decades. Nonetheless, the molecular mechanisms responsible for the pathogenesis and malignant transformation of PNSTs remains an intricate enigma[287, 288]. Molecular alterations such as increased expression or downregulation of $p16^{INK4a}$, p27 or p53[289] or dysfunction of oncogenic pathways like RAS/RAF/MAPK, PTEN/PIK3/AKT/mTOR or PRC2 in PNSTs have been described[285, 290-293], but a relation with the mechanisms of senescence and their implication in malignant transformation of these tumors have not yet been studied. The poor prognosis of MPNSTs, lack of results with conventional systemic drugs and the difficulty in a correct diagnosis of some variants of schwannomas and neurofibromas, warrants a desperate need for further studies of potential diagnosis and treatment in these lesions.

The diagnostic difficulties of MPNSTs are associated with treatment issues of these[286] tumors. MPNSTs are highly aggressive tumors and carry a poor prognosis. Overall 5- and 10-year survival rates are 34% and 23%, respectively[219]. The 5-year survival rates have been reported to range from 34% to 52% with a median survival ranging from 44 to 66 months[294, 295]. Patients with a paraspinal disease have a substantially worse

prognosis[296]. Due to their aggressiveness, the high frequency of local recurrence and resistance to radio- and chemotherapy means that surgical eradication with wide negative margins remains the gold standard in the treatment of these tumors. However, in many cases, the complete excision of the tumor may not be feasible, depending on different variables, such as tumor size, location, and metastatic presentation[297, 298]. Radiotherapy may be used to control local disease and reduce recurrences, but it appears to have little effect on long-term survival[299, 300]. Chemotherapy is generally not effective[301], although some studies have shown that it may benefit patients with high-grade histology[302, 303] or children with unresectable tumors[304]. The role of adjuvant treatment is still unclear[305, 306].

Therefore our study has the objective to analyze the mechanisms of senescence for identifying novel biomarkers and molecular processes that can bring a significant advance in the diagnosis and therapy of PNSTs.

2. EXPRESSION OF P16^{INK4a}, SA-β-GAL ACTIVITY AND LOW EXPRESSION OF KI67 IN SCHWANNOMAS SUPPORT THE HYPOTHESIS OF A SENESCENCE PHENOTYPE OF THESE TUMORS

Schwannomas are benign tumors which only rarely undergo malignant transformation[307]. Sporadic or NF2 associated tumors are molecularly related to defects in the *NF2* gene and deregulation of its product, *merlin*[287]. *Merlin* loss seems to drive numerous cellular alterations, especially activation of growth factors, signaling RAS/RAF/MAPK and PTEN/PI3K/AKT/mTOR pathways, resulting in increased proliferation *in vitro* and *in vivo*[263]. Other alterations found in these lesions have a relation with cell cycle regulation, such as upregulation of p53 and MDM2, loss of p14ARF and p21 or alterations of MET[276-278].

Although a lot of effort has been made to understand the pathogenesis of these lesions, to our knowledge of the literature, a relationship between senescence and the pathogenesis of schwannomas has not been yet described. Our study demonstrates that schwannomas show a senescent-like phenotype and, the senescence mechanisms could be involved in the control and block of their malignant transformation. In our cases all schwannomas available for SA-β-Gal activity analysis (the most important marker of senescence) resulted positive. This data was associated with a significantly high expression of p16^{INK4a} and an absent or very low expression of Ki67 in all lesions. Previous studies have demonstrated that p16^{INK4a} overexpression has been found in premature senescence, and particularly in OIS, and was consequently associated to

senescence in benign and premalignant lesions[68, 72, 116]. Moreover, our data confirms that expression of Ki67 in benign PNSTs has nuclear staining < 5%, even if vestibular and NF2 associated schwannomas seem to exhibit a slightly higher increase than the sporadic[308, 309].

The senescence nature of schwannomas had been previously suggested by Yokoo et al[310, 311]. These authors observed that eosinophilic hyaline droplets (EHD); autophagic lysosomial vacuoles, preferentially appear after oxidative stress in acoustic schwannomas and distribute around areas of Antoni B. The hypothesized production of EHDs represents a cause or a result of growth arrest of schwannoma cells, and may be related to a senescent phenotype. Our data clearly supports the role of senescence in benign schwannomas that can be activated by oncogenes, but also by other mechanisms, such as DNA damage or oxidative stress. It is well known that Antoni B areas in schwannomas are hypocellular zones, contrasting with hypercellular areas named Antoni A. Yokoo at al. suggested that the nature of the hypocellular areas is mainly degenerative and that it shows lower proliferative indexes. We confirmed the lower number of Ki67 positive cells in Antoni B areas, but expression of $p16^{INK4a}$ did not show significant differences between Antoni A and B areas, being slightly higher in Antoni B. These results cannot confirm the role of senescence in differences between Antoni A and B areas, both of them showing a senescence phenotype. However, the presence of oxidative stress in Antoni B areas, demonstrated by Yokoo et al, could contribute to the delicate difference in the senescence phenotype that seems to be between Antoni A and B areas.

3. AN INTERESTING EXPRESSION PATTERN OF CYCLIN D1 STRENGTHENS THE HYPOTHESIS OF THE SENESCENCE NATURE OF SCHWANNOMAS

Cyclin D1 is an important cell cycle regulator which promotes progression from the G1 phase to the S phase of the cell cycle, activating CDKs 4 and 6, which in turn, inactivates the pRb gene, leading to increased cell proliferation[312, 313]. Several studies have established Cyclin D1 as a proto-oncogene, revealing that its amplification and overexpression may contribute to uncontrolled cell growth in many premalignant and malignant human tumors[314, 315]. Cyclin D1 is a nuclear protein and in the majority of neoplasms, its overexpression is localized in the nucleus of the cells[316, 317].

In our study, an interesting pattern of expression of Cyclin D1 was observed in schwannomas. In fact, we found a statistically significant higher level of Cyclin D1 in schwannomas compared with MPNSTs ($p=0.0001$). Moreover, surprisingly, in schwannomas, we found a nucleo-cytoplasmic location of this protein, whereas most of MPNSTs showed only nuclear staining ($p=0.004$). Lassaletta et al. found positive expression for Cyclin D1 in more than half of cases in a series of 21 vestibular schwannomas[318, 319]. They described the positive immunostaining of Cyclin D1 as nuclear, but in the figures of the article, a low, concurrent, cytoplasmic expression may be observed, similarly to our cases.

While the nuclear localization seems to be involved in tumor progression, cytoplasmic sequestration of Cyclin D1 seems to be related to the cell cycle arrest, as previously

described in post-mitotic neurons and senescent cells[320-322]. In senescence cells, the accumulation of Cyclin D1 has been associated with the formation of Cyclin D1-CDK2 (unphosphorylated form) complexes[323, 324]. Recent evidence demonstrates that hyperinduction of Cyclin D1 represents the earliest markers of geroconversion and is the most persistent marker of senescence[324-335]. In fact senescent cells have extremely high levels of Cyclin D1 and it seems to be related to activation of RAS/RAF/MAPK and PTEN/PI3K/AKT/mTOR pathways[328, 336]. Although little is known about the mechanisms and the role of Cyclin D1 in the cytoplasm, together, this evidence suggests a relationship between cytoplasmic sequestration of Cyclin D1 and irreversible cell cycle arrest.

In our cases of schwannomas, the concomitant elevated expression of $p16^{INK4a}$, activity of SA-β-Gal, low or absent proliferation index Ki67and high levels and the nucleo-cytoplasmic expression of Cyclin D1, seem to support the senescence nature of these lesions.

4. MPNSTS SHOW A LOSS OF THE SENESCENT PHENOTYPE PRESENTING A CONTRARY PROFILE TO THAT OF SCHWANNOMAS

Contrary to benign tumors, malignant lesions show a loss of senescent phenotype, with alteration of senescence regulatory pathways. In fact, in MPNSTs, we found an opposite pattern compared with schwannomas, with significantly lower or absent expression of

p16^{INK4a} and high levels of Ki67. Concordant with these findings, the three cases of MPNST in which frozen tissue was available, resulted negative for SA-β-Gal activity.

MPNSTs are aggressive soft tissue sarcomas, derived from Schwann cells, that occur in association with NF1, or spontaneously. Generally these tumors result from the malignant transformation of a neurofibroma, often of the plexiform subtype, even if rare examples develop from conventional schwannomas or ganglioneuromas[337]. These tumors show a poor prognosis and a high Ki67 labelling index (LI) (>25%) which is corrolated with a reduced survival rate[338, 339].

Regarding the mechanisms involved in malignant transformation of PNSTs, several molecular alterations have been found in these lesions. In NF1, the loss of *NF1* gene product, *neurofibromin,* leads to activation of proliferation via RAS/RAF/MAPK pathway and inhibition of protection of apoptosis process by inactivation of mTOR[251]. *NF1* mutations are also found in in the majority of patients with sporadic MPNST[187]. In addition, other genes mutations such as CDKN2A, KIP 1 and TP53 and alterations of PIK3CA/AKT/mTOR pathways have been described in these tumors[279, 292].

In the majority of MPNSTs, equally distributed into NF1-related and sporadic groups[293], various studies have previously suggested an association with p16^{INK4a} downregulation[340, 341]. Several reports, in fact, show that there is a high frequency of inactivation of the 9p21 locus in MPNSTs, imputing as the predominant cause the homozygous deletion of CDKN2A gene[293, 341, 342]. Our study confirms that MPNSTs show loss of p16^{INK4a}, supporting the relevance of p16^{INK4a} downregulation in malignant transformation of PNSTs. Recently Lee et al have identified loss of function of two core

components of the PRC2, EED and SUZ12 as an recurrent event in MPNSTs. Polycomb group proteins are epigenetic transcriptional repressors that function through recognition and modification of histone methylation and chromatin structure. This group of proteins maintains, by posttranslational modification of histones, the silenced state of genes involved in critical biological processes, including cellular development, stem cell plasticity, and tumor progression[343, 344]. It has been observed that PRC2 components, like EZH2, may influent the activity and the expression of cell cycle regulators, such as $p16^{INK4a}$, $p14^{ARF}$ and p21, and they control the replication timing of the INK4a/ARF locus during senescence[345-347]. There is some evidence that EZH2 is involved in melanoma progression and metastasis[284] and it has been observed that a direct transcriptional activation of EZH2 promotes senescence bypassing, regulating the tumor suppressor genes p21 and $p16^{INK4a}$[348, 349].

In MPNSTs, alterations of the PRC2 components could be related to a deregulation of senescence tumor suppressor control and a malignant transformation of these tumors.

P53 mutations and loss of heterozygosity (LOH) have also been described in MPNSTs[350, 351]. Point mutations and LOH of TP53 gene and nuclear overexpression of p53 protein have been observed in these tumors, mainly restricted to sporadic presentation[351, 352]. In the present study, although the association between p53 overexpression and malignant transformation has been mainly observed in the intensity of staining and not in the percentage of positive cells, our results do not refute the possible role of p53 mutations in the genesis of these tumors.

Surprisingly, the only case included in the study showing malignant transformation from a schwannoma, showed an atypical pattern compared to other MPNSTs. In this case there was an overexpression of $p16^{INK4a}$, while the expression of nuclear Cyclin D1 was dramatically lost. This single result is difficult to interpret due to the fact that it could be an isolated event. However, it could be illustrating differences in molecular pathways between malignant transformation of schwannomas and neurofibromas, or pathways involved in the genesis of primary MPNSTs.

5. BRAF V600E AND KRAS G12S MUTATIONS REPRESENT AN IMPORTANT EVENT IN A SUBSET OF PNSTS NOT RELATED TO NF

As previously reported, alterations of KRAS or BRAF pathways had been found in PNSTs in cases associated to NF1 or NF2. It is known, in fact, that somatic inactivation of *NF1* gene leads to a complete loss of *neurofibromin* function and subsequently, RAS/RAF/MAPK pathway activation, giving rise to NF-associated benign and malignant tumors[353]. Inactivation of both *NF1* gene alleles is thought to be sufficient to initiate the development of a benign neurofibroma, but not of a MPNST[354]. Nearly all NF 1 patients develop benign neurofibromas, with a risk of malignant transformation to MPNST[200, 238]. Consequently, MPNST are found at an increased frequency in patients with NF, with a lifetime risk of 8–13%. Similarly, NF 2 is caused by a functional loss of the RAS-regulator *merlin* due to inactivating mutations of the *NF2* tumor suppressor

gene[263, 355]. A loss of function in the *NF1* and *NF2* genes leads to RAS/RAF/MAPK pathway activation which, in turn, promotes the growth of the PNSTs associated with these inherited disorders.

None the less, an important number of benign and malignant PNSTs arise as sporadic lesions, and are not associated with the NF1 or NF2 syndromes[356, 357]. Moreover, malignant transformation from benign schwannomas to their malignant counterparts is extremely rare[358, 359]. Accordingly, subsequent key genetic changes underlying tumorigenesis and the progression mechanisms of sporadic PNSTs are yet to be determined. Considering the concept that both melanocytes and Schwann cells share a common embryological origin from neural crest cells[360] and that most cutaneous melanomas harbor mutations in either BRAF or RAS[61, 361], we hypothesized that activating BRAF and/or KRAS mutations may play a role in the pathogenesis of benign and malignant PNSTs, not related to NF. The presence of BRAF or KRAS mutations in PNSTs has been reported only in very rare occasions of malignant tumors. In a previous study, Bottillo et al. found the rarer BRAF mutation (c.1742A > G, N581S) in one patient with a sporadic MPNST, harboring two somatic mutations in the *NF1* gene[362]. Additionally, the most frequent BRAF mutation V600E, has been studied in a large series of neuronal tumors and it has been found that it is not a frequent event in PNSTs[363]. To the best of our knowledge, there are no reports identifying KRAS and BRAF mutations in benign PNSTs. In fact, the absence of BRAF mutations in these lesions has been reported in one series with a small number of neurinomas[363]. This data has been confirmed in a recent report in which no mutations of BRAF, EGFR, PIK3CA,

and KRAS have been seen, in 48 sporadic vestibular schwannomas[364]. Also neurofibromas do not harbor BRAF V600E mutations[363].

In our study, for the first time, we described BRAF V600E mutation in four of 40 cases of sporadic schwannomas (10%), including one vestibular neurinoma, and in one of 13 sporadic MPNSTs (7.7%). Furthermore, oncogenic KRAS exon 2 G12S mutation was found in one case of schwannoma, not associated with NF syndrome. BRAF or KRAS mutations were found in neither sporadic nor NF-related neurofibromas, confirming the data of literature. Although the number of cases included in this series was small, current evidence from our research and other series published so far suggests that BRAF mutations might be absent in neurofibromas. Part of our data has been confirmed in two recent articles, both published in 2014. Dubbink et al.[365] identified BRAF V600E mutations in 3 % (3/86) of MPNSTs not associated to NF. Also, Hirbe et al.[366] described BRAF V600E in 20% (5/25) of sporadic and 2.7% (1/37) NF1-associated MPNSTs, employing the BRAFV600E mutation-specific antibody for immunohistochemistry and a confirmatory sequencing technique; moreover, they confirm the absence of BRAF mutations in neurofibromas.

The presence of BRAF and KRAS mutations in these subsets of tumors may play a role in the pathogenesis of benign and MPNSTs not related to NF. Moreover BRAF mutation screening in sporadic MPNSTs may have an important clinical impact, given the striking efficacy shown by the BRAF inhibitor *vemurafenib* (PLX4032) in BRAF-mutant advanced melanomas[367].

6. IMPORTANCE OF SENESCENCE BY-PASS IN CANCER TRANSFORMATION

The discovery of BRAF mutations in a subset of MPNSTs opens new directions in the pathogenesis of the malignant transformation of these tumors. It is known that inactivation of both *NF1* alleles is thought to be sufficient to initiate the development of a benign neurofibroma[368, 369], but it is almost certainly insufficient for transformation into a MPNST[354]. Therefore it is logical to think that other genetic alterations must occur for benign tumors to progress to malignancy.

In the last two decades, one of the most stimulating assumptions in human cancer development is represented by the cells ability to bypass senescence[9, 115, 121]. Several mechanisms are involved in the regulation of this process and their alterations may lead to bypass senescence, contributing to tumor transformation. Among the pathways engaged in the control of senescence, $p16^{INK4a}$/pRb, ARF/p53, PTEN/PI3K/AKT/mTOR play the most important role, especially in the regulation of OIS, activated by oncogenes like BRAF or KRAS[13, 132]. In the model nevus/melanoma, melanocytes in nevi are able to activate OIS as a tumor suppressor mechanism, arresting cellular proliferation and progression into melanoma, in response to the presence of activating mutations of BRAF[12]. Although the vast majority of nevi never undergo malignant transformation, a significant fraction of melanomas are believed to arise from progression within precursor benign lesions. Recent studies have demonstrated that PTEN inactivation and/or activation of the PI3K/AKT/mTOR pathway, associated to

loss of p16,^{INK4a} are sufficient events to abrogate OIS in melanocytes expressing activated BRAF mutations, implying that melanocytes can escape from senescence and progress to melanoma and metastasis[12, 75, 370].

Our study suggests that in MPNSTs a deregulated control of senescence pathways may be the key of their transformation into tumor and aggressive lesions, in a similar way to melanomas. Contrary to what happens in benign lesions like neurofibrmas, loss of $p16^{INK4a}$ is a frequent finding in these tumors and it is associated with malignant transformation[210, 371]. Reduced or absent expression of $p16^{INK4a}$ protein and mRNA has been described in a high percentage of cases of MPNSTs, as far as homozygous deletion of CDKN2A gene[231, 293, 342]. Moreover, these alterations are associated with aggressive behavior and poor prognosis[341]. It is well known that $p16^{INK4a}$ overexpression has been found in premature senescence, and particularly in OIS, and that senescent cells have been shown in a number of different benign and premalignant lesions, but not in malignant ones[10, 11, 72]. Some authors have demonstrated that atypical neurofibromas are precursor lesions of MPNSTs in NF1, showing a deletion with a minimal overlapping region (MOR) in chromosome band 9p21.3[209]. However, unlike MPNSTs, atypical neurofibromas present high level of $p16^{INK4a}$ expression, especially in cases with lamellar growth, negativity for p53 and a very low or absent proliferation index Ki67[205]. In our series of MPNSTs, we found that low or absent $p16^{INK4a}$ expression was associated to high levels of proliferation index Ki67 and a loss of activity of SA-β-Gal, suggesting that a by-pass of senescence mechanisms could be an important event in the malignant transformations of MPNSTs.

7. FUTURE PERSPECTIVES IN CANCER THERAPY

The discovery of molecular alterations linked to senescence in PNSTs could open new frontiers to different and more effective therapeutic strategies for these tumors.

As previously discussed, MPNSTs are very aggressive cancers, which commonly develop local recurrence and distant metastasis. Complete surgery remains the only therapeutic option, but many cases it cannot be fully resected and the 5-year survival ranges from 35 to 50%[294, 372]. Despite current multimodality treatments, radio and chemotherapy are not effective curative options and targeted molecular treatments are not yet available, although currently, several preclinical and clinical studies with molecular therapies are in development[373]. However, expanded knowledge of MPNST molecular pathobiology will be needed to meaningfully apply such approaches for the benefit of afflicted patients.

Study of senescent markers in human tumors may result in a useful tool in clarifying their pathogenesis and in the development of the most effective targeted treatment. Based on the hypothesis that senescence represents a tumor-suppressor mechanism, the concept of pro-senescence therapy has emerged over the past few years as a novel therapeutic approach to treat cancers.

7.1. MECHANISMS OF OIS INDUCTION

As previously discussed, cellular senescence can also be induced by oncogenes or different kind of stress or drugs and it is well known that OIS is independent of

telomere attrition[374]. OIS has been proposed as a tumor suppressor mechanism in premalignant lesions such as dysplastic melanocytic nevus, neurofibromas, oral precancer lesions and Barrett's esophagus[60, 375-377], giving rise to the hypothesis that pro-senescence therapy could be an effective option for cancer therapy. Several tumors treated with chemotherapy or radiotherapy have clearly been shown to produce a senescent state; termed *therapy-induced senescence* (TIS), which principally involves the p53/p21 and $p16^{INK4a}$/pRb pathways[378, 379]. Several agents, along with ionizing radiation are able to induce a senescence phenotype in different human and murine tumor cells, such as DNA-interactive agents *doxorubicin* and *cisplatin* or other chemotherapeutic drugs such as tyrosine kinase inhibitor *sunitinib* and the alkylating agent *busulfan*[380-386].

Some authors have demonstrated that senescent induction through direct restoration of senescence-regulation genes could be a useful therapeutic appliance to control altered proliferation and malignant transformation. One option for inducing senescence in tumor cells may be p53 reactivation, by the use of small molecules that have been demonstrated to reverse mutated p53 activity in murine models[387-392]. Drugs which competitively displace p53 from the binding on MDM2, inhibiting their coupling, such as *nutlins, RITA (Reactivation of p53 and Induction of Tumor Cell Apoptosis)* or *MI-63*, *MI-219*, and *MI-319* result in the stabilization of p53 and promote the normal function of the protein in causing its senescence response[393-398]. Nowadays, due to its promising anticancer properties, the use of molecule that restore the p53 function has been

transferred to clinical trials for the treatment of human tumors, that are in their early phase of development[399, 400].

Another pro-senescence therapeutic strategy is the modulation of cell cycle regulators. It is well known that $p16^{INK4a}$, p27 and the inhibition of some CDKs activity can promote senescence induction[38, 401]. Over the past 20 years, several CDK inhibitors have been developed as potential cancer therapies, and recent studies suggest that targeting specific CDKs or CDKIs in the appropriate genetic context can result in synthetic lethal interactions promoting a tumor-specific pro-senescence response with a therapeutic benefit[401-404]. Several first and second generation of CDK inhibitor compounds, such as *flavopiridol*, *roscovitine*, *dinaciclib*, *palbociclib* or *ribociclib*, have been tested in numerous trials and in several tumor types, with satisfactory results, but with the onset of drug resistance[405-411]. New drugs capable of restoring $p16^{INK4a}$ function could be introduced into the clinical practice to induce senescence and arrest cancer development. *In vitro*, studies have reported that demethylating agents in tumors showing promoter hypermethylation of $p16^{INK4a}$ are able to restore this gene, resulting in induction of premature senescence in cancer cells[412-416]. Inactivation of PTEN activity in the tumor cells in synergy with Skp2 inhibition induces a senescence response and suppresses tumorigenesis in murine cancer[417, 418]. Experiments in mouse models suggested that targeting Skp2 could trigger senescence in tumors driven by PTEN inactivation, and the development of *MLN4924* results in Skp2 inhibition, preventing the formation of tumors in a PC3 human prostate cancer cell xenograft model by inducing senescence that was independent of p53[419]. Loss of PTEN causes senescence,

also through a signaling short circuit that is driven by hyperactivation of the PI3K/AKT/mTOR pathway and the development of direct inhibitors of PTEN, such as *VO-OHpic*, can drive senescence[420-423].

7.2. CURRENT AND FUTURE TREATMENT OPTIONS FOR SCHWANNOMAS AND MPNSTS.

Schwannomas represents benign tumors arising at the level of peripheral nerves that generally show a good prognosis after complete surgery. Although histologically benign, in some occasions, these lesions can progress or recur after surgery or radiotherapy, especially those with a deep or problematic localization or those related with NF2 syndrome[424-427]. In recent years, new molecular approaches for the treatment of schwannomas have been made, such as the antiangiogensis drugs against the vascular endothelial growth factor (VEGF) such as *bevacizumab*[428-431]. Relatively, to the oncogenes and their signaling, the expression and molecular alterations of some of them have been studied in schwannomas and upregulation of EGFR and ErbB2 mRNA and protein and alterations of mTOR have been found, predominantly, related to NF2 syndrome, but not gene amplification or point mutations of BRAF, EGFR, PIK3CA, and KRAS[262, 364, 432-435]. Moreover, the attempt to use specific targeted therapy with *everolimus*, *eroltinib* or *lapatinib* has been contradictory in terms of success[436-441]. In our study, we described for the first time BRAF V600E and KRAS G12S mutations in a subset of sporadic schwannomas, opening new perspectives in the treatment of these tumors.

At the present time, in MNPSTs, the conventional (and not always effective) therapy is radical surgery, with a complete extirpation of the tumors and free margins, despite the fact that in many patients there is a high risk of local relapse or metastasis. With increased understanding in the molecular pathogenesis of these tumors, several experimental therapies have been performed. For example anti mTOR drugs, *everolimus* alone or coupled with *bortezomib* have showed antitumor activity on MPNSTs[279, 442-444]. Our findings of BRAF V600E mutations in a subset of these tumors, confirmed by a later published article, could open new future perspectives in their treatment[366, 445]. In 2008 Ambrosini et al. characterized MPNST human cell lines for RAS and BRAF activation and treated them with *sorafenib*, resulting in a G1 cell cycle arrest, due to suppression of cyclin D1, inhibition of phospho-MEK, phospho-ERK and hypophosphorylation of pRb.

Clinically, the use of BRAF inhibitors has been proposed in two studies, where it has been reported a modest effect of *sorafenib* and a dramatic response to *vemurafenib* in a little series of MPNSTs, in which the BRAF V600E mutation was not demonstrated[446, 447]. These findings reinforce the idea that patients with MPNST should be screened for BRAF V600E mutations and treated with BRAF inhibitors, when these mutations are found. Moreover, the study of senescence induction and escape mechanisms may lead to the development of specific targeted treatments helping to fight against these aggressive tumors.

CONCLUSIONS

1. In our study, we analyzed schwannomas and demonstrated that these lesions show a senescent-like phenotype with high levels of $p16^{INK4a}$ expression, a very low proliferation index and SA-β-Gal activity. Contrarily to benign tumors, MPNSTs showed loss of senescent phenotype with low levels of $p16^{INK4a}$ expression, high proliferation index and SA-β-Gal activity not present.

2. For the first time, we described BRAF V600E and KRAS G12S mutations in a subgroup of sporadic PNSTs; BRAF V600E mutation was found in schwannomas and MPNSTs, but not in neurofibromas and KRAS G12S mutation was found in a sporadic schwannoma. A screening of these mutations may be adopted routinely in these tumors and those cases of PNSTs harboring BRAF V600E mutation could benefit from specific target therapy, for example, in metastatic melanomas (ex. v*emurafenib*).

3. A thorough study of the mechanisms of induction and escape of senescence in PNSTs could bring new perspectives in the pathogenesis and treatment of these tumors, with the use of specific drugs that act on the mechanisms of regulation of cellular senescence.

FIGURES AND TABLES

Figure 1

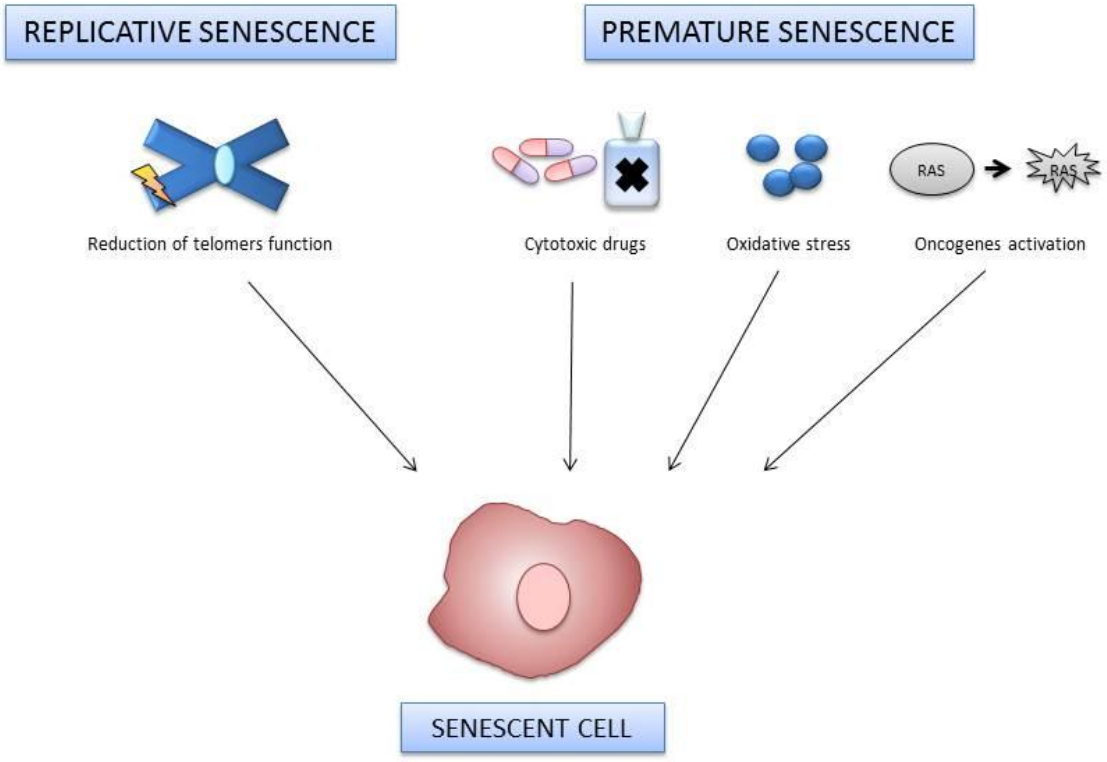
REPLICATIVE SENESCENCE
PREMATURE SENESCENCE
RAS
RAS
Reduction of telomers function
Cytotoxic drugs
Oxidative stress
Oncogenes activation
SENESCENT CELL

Figure 2

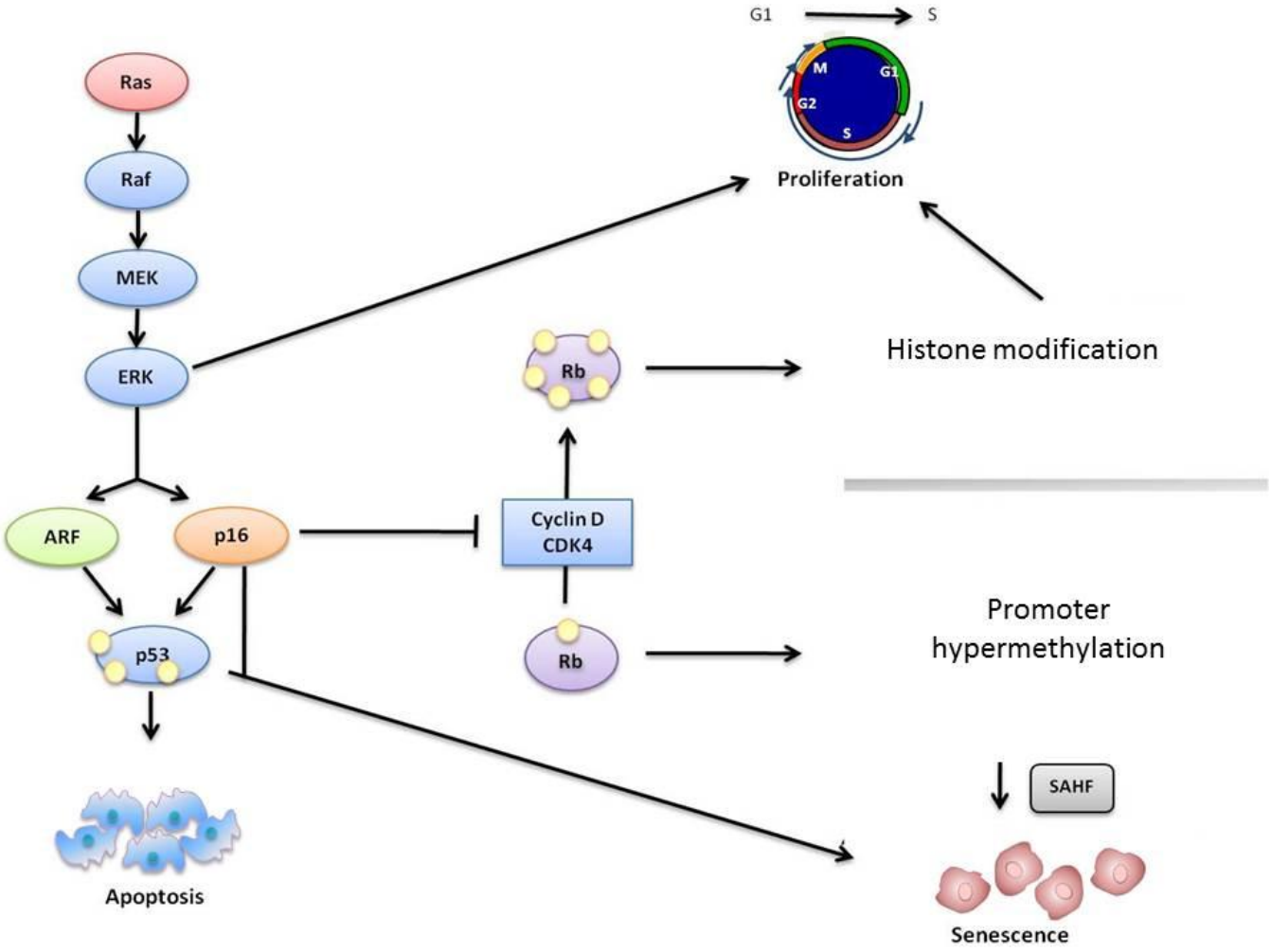
G1
S
M
G1
G2
S
Proliferation
Ras
Raf
MEK
ERK
Histone modification
Rb
ARF
p16
Cyclin D
CDK4
Promoter
hypermethylation
p53
Rb
SAHF
Apoptosis
Senescence

Figure 3

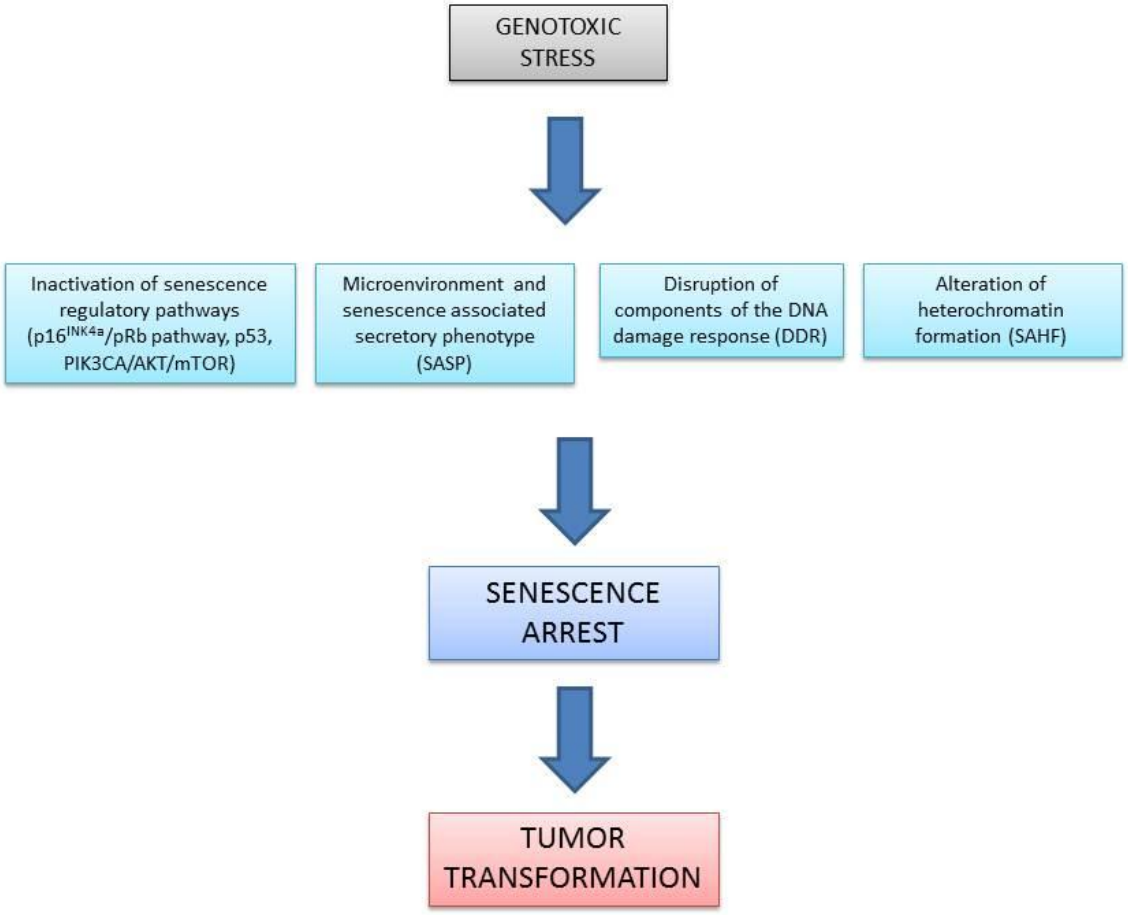
GENOTOXIC STRESS
Inactivation of senescence regulatory pathways (p16INK4a/pRb pathway, p53, PIK3CA/AKT/mTOR)
Microenvironment and senescence associated secretory phenotype (SASP)
Disruption of components of the DNA damage response (DDR)
Alteration of heterochromatin formation (SAHF)
SENESCENCE ARREST
TUMOR TRANSFORMATION

Table 1. Features of senescence cells. Modified from Schmitt C et al. 2007.

Characteristic	Detail	Reference
Morphology	Large flattened cells, with vacuole rich cytoplasm and large multilobulated nuclei	DeNicola et al. 2009
Biomarkers expression	Increased perinuclear activity of SA-β-galactosidase (expension of lysosomal compartment).	Caldwell et al. 2012
Molecular pathways involvement	Increase expression of various oncogene-induces tumor suppressor proteins and associated loci (p16, p15, p21, CDKN2a). Activation of p53 an pRb.	Bardeesy et al. 2006; Vogelstein et al. 2000; Romagosa et al. 2011
Chromatin structure	Senescence associated heterochromatin foci (SAHFs), Focal histone H3 lysine 9 trimethylation. Focal HP1 recruitment.	Narita et al. 2003
Senescence associated secretory phenotype (SASP)	Cells develop altered secretory activity whilst in a senescent state, resulting in a protumorigenic effect through an increase in proteins, interleukins, growth factors and proteases within the tumor microenvironment.	Coppé 2008; Penfield et al. 2013

Table 2. Oncogenes involved in senescence. Modified from Gorgoulis et al 2010.

Action	Gene	Pathway - Function
In vivo		
Activation	TGFb	Promotes Smad signaling
	H-RAS V12	Ras signaling
	K-RAS G12V	Ras signaling
	N-RAS G12D	Ras signaling
	BRAF E600	Promotes Ras signaling
	c-Myc	Effector of Ras signaling — transcription and chromatin remodeling factor
	b-Catenin	Promotes Wnt signaling
	Akt	PI3K/Akt signaling
	Rheb	Promotes PI3K/Akt/mTOR signaling
	E2F3	Promotes G1 to S phase — transcription factor
Inactivation	PTEN (TSG)	Downregulates PI3K/Akt/mTOR signaling
	VHL (TSG)	Targets HIF for degradation
	Hsp72	Promotes PI3K/Akt (p53-dependent) and ERK (p53-independent) signaling
	Rb (TSG)	Regulates E2F activity
Restoration	p53 (TSG)	Effector of various signaling pathways — transcription factor
In vitro		
Activation	TGFb	Promotes Smad signaling
	INFb	Promotes STAT signaling — activates p53
	CXCR2 (IL8RB)	Angiogenic CXC chemokine receptor
	Rac1	Modulates Rho signaling
	Smurf2	Upregulation by telomere attrition; promotes p53/pRb senescence
	Runx1, Runx2, Runx3	Transcription and chromatin remodeling factors
	PTEN	Downregulates PI3K/Akt/mTOR signaling
	Sprouty 2	Downregulates Ras signaling
	EGFR	Promotes Ras signaling
	H-RAS V12	Ras signaling
	N-RAS G12D	Ras signaling
	Raf	Ras signaling
	BRAF E600	Promotes Ras signaling
	Mos	Promotes Ras signaling
	MEK	Promotes Ras signaling
	c-Myc	Ras signaling effector — transcription and chromatin remodeling factor
	IGFBP3, IGFBP5	Modulates IGF1 signaling pathway
	IGFBP7	Modulates IGF signaling; downregulates Ras signaling
	p38a-D176A, p38g-D179A	Promotes p38MAPK signaling
	STAT5	Promotes JAK–STAT signaling
	Cyclin E	Activated cyclin-dependent kinase-2: promotes G1 to S phase
	E2F1	Transcription factor: promotes G1 to S phase
	E2F3	Transcription factor: promotes G1 to S phase
	Cdc6	Replication licensing factor: promotes S phase progression
	Cdt1	Replication licensing factor: promotes S phase progression
	p16INK4A	Cyclin-dependent kinase inhibitor: inhibits G1 progression
	PML	Ras signaling effector; induces p53
	p53	Effector of various signaling pathways–transcription factor
	PAI-1	p53 effector
	DEC1	p53 effector
Inactivation	Rac1	Modulates Rho signaling
	NF1	Downregulates Ras signaling
	PTEN	Downregulates PI3K/Akt/mTOR signaling
	VHL	Targets HIF for degradation

Table 3. Mouse models and human tumors in which oncogenic senescence has been studied. Modified from Collado and Serrano 2010.

Mouse models of tumor cell senescence	
Gene	**Tissue or tumor**
Oncogene activation	
HRAS G12V	Mammary tumours, bladder tumours, and DMBA and TPA-induced skin papillomas
KRAS G12V	Lung adenomas and pancreatic intraductal neoplasias
NRASG12D	Lymphoproliferative disorders
BRAFV600E	Nevi and lung adenomas
Rheb	Prostate intraepithelial neoplasia
E2F3	Pituitary hyperplasia
AKT1	Prostate intraepithelial neoplasia
Ctnnb1	Thymus
Oncogene inactivation	
Myc	Lymphoma, osteosarcoma, livercarcinoma and lung carcinoma
Tumour suppressor inactivation	
PTEN	Prostate intraepithelial neoplasia
Rb1	Thyroid C cell adenomas
Vhl	Kidney
Tumour suppressor activation	
P53	Sarcomas and liver carcinomas
Human tumors showing cell senescence	
Associated oncogenic event	**Human tumor**
NF1 inactivation	Dermal neurofibromas
BRAFV600E mutation	Nevi
Not determined	Prostate intraepithelial neoplasia Colon adenomas

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