Review

The role of key genes and pathways involved in the tumorigenesis of Malignant Mesothelioma

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ARTICLE INFO

ABSTRACT

Malignant Mesothelioma (MM) is a very aggressive cancer with low survival rates and often diagnosed at an advanced stage. Several players have been implicated in the development of this cancer, such as asbestos, erionite and the simian virus 40 (SV40). Here, we have reviewed the involvement of erionite, SV40, as well as, the role of several genes ($p16^{INK4a}$, $p14^{ARF}$, NF2, LATS2, SAV, CTNNB1 and among others), the pathways (RAS, PI3K, Wnt, BCL and Hippo), and their respective roles in the development of MM.

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Contents

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1. An introduction to Malignant Mesothelioma

Malignant Mesothelioma (MM) is a slow-growing solid tumor arising from mesothelial cells that can develop in the pleural space, pericardium, peritoneum, tunica vaginalis testis and ovarian epithelium. In its early stage, it is not common for these tumors to suffer metastasis [1]. MM is divided into three categories according to its histological morphology: epithelial, biphasic, and sarcomatoid with median survival period of 18, 11, and 8 months, respectively [2]. Moreover, MM displays a long latency period that can take up to 40 years [3]. During this period, there is an accumulation of mutations on several key genes [4]. In the US alone, it is expected 70,000 new cases of MM over the next 20 years [5].

The Malignant Pleural Mesothelioma (MPM) is an aggressive form of cancer that affects the pleura. MPM is known to be very aggressive, and it is often diagnosed at a very advanced stage, which contributes to its very poor prognosis with a median survival of 11 months [6].

It has been more than 50 years since the first study correlating asbestos to the development of MM [7]. Evidence has shown a strong relationship between asbestos exposure and MM [8]. Exposure to simian virus 40 (SV40) [9], erionite [10], and genetic predisposition have been also implicated in the development of MM [11].

1.1. An overview of molecular biology of MM

MM frequently displays chromosomal loss involving the chromosomes 1, 3, 4, 6, 9, 13, 14 and 22 [12]. The most common genetic alterations in MM are the homozygous deletion of $p16^{INK4a}$ and $p14^{ARF}$ genes. It was found a homozygous deletion of $p16^{INK4a}$ and $p14^{ARF}$ in 72% of MM [13]. In addition, homozygous deletion of the $p16^{INK4a}$ was present in approximately 75% of MM, and it was associated with a more aggressive cancer, and with a reduction on survival [14].

The tumor suppressor Neurofibromatosis type II (NF2) has been reported to be altered in MM. NF2 inactivation is a frequent event in MM with rates ranging from 20% to 60% [15]. The NF2 gene encodes for the Merlin protein that is associated with the inhibition of several mitogenic signaling pathways [16].

The guardian of the DNA, the protein p53, is encoded by the TP53. The p53 plays a crucial role in the cellular response to DNA damage, and its expression is lost in many advanced cancers [17]. However, in MM only 20–25% of the tumors display mutations on TP53, a fairly low rate when comparing to other cancers [18]. In recent studies using MM samples, it was reported an overexpression of p53 in 58.2% [19], and in 81% of the cases [20].

The phosphatase and tensin homolog (PTEN), also known as MMAC (mutated in multiple advanced cancers) is a dual lipid and protein phosphatase encoded by the PTEN, which is a tumor suppressor gene (TSG) located on chromosome 10q23. PTEN is known to negatively regulate the AKT pathway; thus the loss of PTEN expression increases AKT pathway activation, which ultimately leads to an uncontrolled cell growth [21–23].

New studies have demonstrated that other genes play important roles in MM. Germline mutation of BAP-1 has been identified in a cancer syndrome predisposing individuals to cancer, including MM; furthermore, it has also been shown BAP-1 somatic mutations in MM samples [24]. Likewise, the LATS2 has been recently implicated in the development of MM [25]. DNA methylation and MicroRNA (miRNA) expression have exhibited significant roles in MM, as it is described later on.

The PI3K/AKT/mTOR pathway is altered in MM and plays an important role in cell proliferation, survival and motility in many cancers. In 62% of MM cell lines, AKT activation was reported [26]. In another study, it was shown that 65% of human MM species displayed elevated levels of AKT activity [22].

Furthermore, other pathways are dysregulated in MM. The Receptor Tyrosine Kinases (RTKs) drive cell proliferation, survival, differentiation and cell cycle control. Several mechanisms can activate this pathway in cancer providing a good therapeutic option. The overexpression of the epidermal growth factor receptor (EGFR) plays an important role in the progression of several cancers [27]. In a study, the EGFR was present in 44% of MM samples; however, it is not found to be an independent prognostic factor [28].

The Vascular Endothelial Growth Factor Receptors (VEGF) are a potent inducer of the angiogenesis, and its role in the cancer is well established [29]. High levels of VEGF in MM have been demonstrated, being associated with a worse patient survival [30]. Moreover, the importance of this receptor in regulating the angiogenesis, and tumor progression was established; thus making this pathway as a therapeutic target in MM [31].

The retinoblastoma protein (pRb) pathway plays an important role in apoptosis and cell cycle regulation. Mutation on pRb is common in many cancers, but not in MM [32]. Nevertheless, the pRb and p53 pathways play an important role in MM. The $p16^{INK4a}$ and $p14^{ARF}$ exert effects on the pRb and on p53 pathways. The p16^{INK4a} inhibits the cyclin dependent kinases (CDks), preventing the inactivation of pRb; on the other hand, the p14^{ARF} promotes degradation of MDM2, leading then to the stabilization of p53 [33]. Indeed, mutations on TP53 and on RB are not a common event in MM; however mutations and/or alterations on $p16^{INK4a}$/$p14^{ARF}$ are very common. Thus, alterations and/or mutations on $p16^{INK4a}$/$p14^{ARF}$ have the potential to disrupt key cell cycle control pathways.

The BCL-2 family of genes exerts a critical role in the apoptosis process. There are several proteins, which are divided into proapoptotic and antiapoptotic proteins. The proapoptotic proteins are thought to promote the permeability of the mitochondrial membranes, thus promoting the apoptosis; on the other hand, the antiapoptotic proteins are thought to inhibit cells from undergoing programmed death. Studies have found that BCL-2 expression is inversely associated with apoptosis; however this protein is not frequently expressed in MM [34,35]. High levels of BCL-XL are a common event in MM; however, downregulation of BCL-XL increases apoptosis and the cystostatic effects of cisplatin and gemcitabine [36].

The hippo pathway controls cell proliferation, growth, differentiation and death [37], and it has been implicated in the development of MM [38,39]. The Wnt pathway plays a fundamental role in the determination of cell fate, proliferation, polarity, and cell death during embryonic development [40]. The Wnt signaling pathway has been reported in MM [41]. Therefore, it is clear that there are several players, genes, and pathways involved in MM, all of which are described in depth in the following sections.

1.2. Asbestos and MM

We have recently reviewed the role of asbestos in MM and its carcinogenic mechanisms, which are summarized in Fig. 1. In this same work, we have also reviewed the roles of *PTEN* and *TP53* in the development of MM [42].

1.3. SV40 and MM

The Simian virus 40 (SV40) is a DNA monkey virus that was present in contaminated polio vaccines produced from 1955 to 1978. It is believed, that this is the most likely route of SV40 transmission into humans [43]. Furthermore, the SV40 has been implicated in MM [44]. It was observed that specific SV40 viral sequences were present in 57% of epithelial invasive MM [45]. In another study, initially, it was reported the presence of SV40 in 60% of MM samples; then later on, it was shown that these findings were incorrect due to plasmid contamination, and that only 6% of the positive samples had the presence of SV40 DNA [46]. Recently, an Italian study in a hyperendemic area of MM has detected SV40 DNA in 22% of the MM tumors with a low viral load [47].

Negative results have also been published. In a study, the authors have reported that SV40 was absent in 69 (100%) MM tumors [9]. Another study has reached the similar conclusion [48]. Recently, no SV40 was detected in Korean MM samples [49], and similar conclusions has reached a recent study in Slovenia [50]. Thus, these contradictions in the literature have caused a huge controversy regarding the role of SV40 in MM. Until now, at least 50 laboratories have detected the presence of SV40 in human tumors using a great variety of molecular biology techniques; and thus raising even more the controversy about the role of SV40 in MM [51]. For instance, 100% of animals injected with SV40 in the pleural tissue developed MM within 6 months [52], thus showing a relationship between SV40 and MM, at least in animal studies. Besides, the relationship and the controversy between SV40 and MM have been extensively addressed by Qi et al. [51].

However, an animal study has shown that SV40 alone was not able to cause MM, only asbestos exposure caused 20% of MM, and remarkably, asbestos and SV40 together caused 90% of MM in hamsters. This study has shown that lower amounts of asbestos may cause MM in animals infected by SV40 [43], and similar conclusions have been found by another study [53]. Therefore, these studies indicate that the levels of asbestos exposure that are considered "safe" for the whole population, may not be for those who were previously exposed to SV40. Lastly, a recent study has shown that long-term exposure to asbestos in SV40 infected cells generates resistance to chemotherapy-induced apoptosis [54].

The mechanisms behind SV40 carcinogenic activity are indeed complex, and not fully understood. The SV40 oncogenic activity rests on the production of two major proteins: the small t antigen (tag) and the large T antigen (TAG). It is known that TAG is able to inactivate several tumor suppressor genes, such as *TP53* and *RB*. These genes encode key proteins to the cell cycle checkpoints, and the loss of these proteins leads to uncontrolled cell proliferation [53]. Furthermore, the tag protein inhibits the cellular phosphatase 2A (PP2A), which is involved in the dephosphorylation of many protein substrates, including elements of the MAPK pathway (Fig. 1). Consequently, the loss of PP2A by tag may alter the activity of several phosphoproteins [55]. In addition to these classical mechanisms, it was recently shown that TAG-p53-pRb-p300 complex regulates the transcription of the insulin-like growth factor I (IGF-1) gene by binding to the IGF-1 promoter. In other words, there
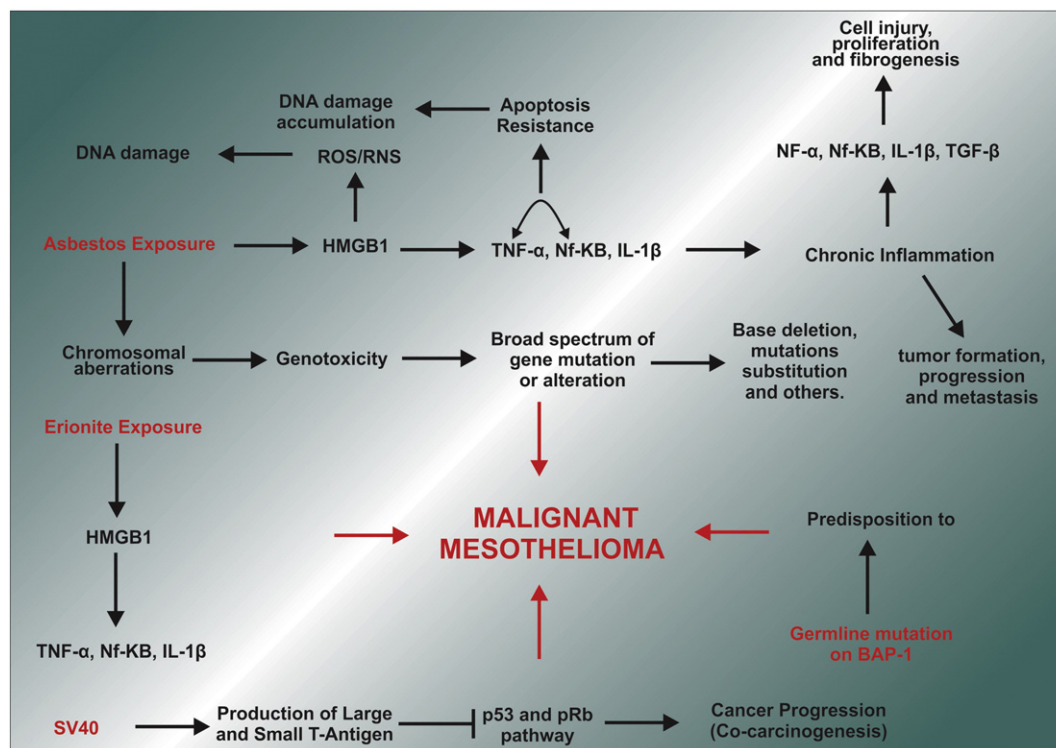
Fig. 1. It is represented the most common players involved in the development of MM. Asbestos causes DNA damage, apoptosis resistance, chronic inflammation and genotoxicity. Erionite plays an important role in the development of MM through the involvement of TNF-α, Nf-Kb and IL-1β. The oncoproteins of SV40 interact with p53 and pRb pathways playing an important role as a co-carcinogenic factor in MM. Lastly, germline mutation on *BAP*-1 confers predisposition to MM.

is an increase of IGF-1 production, which leads to enhanced cell growth [56]. Moreover, it has also been shown the involvement of SV40 in the expression of VEGF [57], and in the increase of telomerase activity in MM cells [58].

Taken altogether, it is still not clear the direct carcinogenic effects of SV40 in MM in humans; however, it is widely accepted the role of SV40 as a co-carcinogenic player in association with asbestos in the development of MM [51,59].

1.4. Erionite and MM

It has been reported cases of MM without any previous known contact with asbestos particles, in other words, not all MM cases are etiologically related to asbestos exposure [60]. Indeed, it is often wrongly assumed that only asbestos causes MM, but in fact, other agents have been implicated in the development of MM, among these agents, the mineral erionite.

The erionite is a fibrous form of the zeolite group of minerals, which is several times more carcinogenic than crocidolite asbestos in causing MM [10,61]. The relationship between erionite and MM was first studied in some villages in Turkey, where a strong correlation between erionite exposure and MM incidence was found [62,63]. The names of the villages first described were Karain, Tuzköy and Sarihidir, and at that time, there were approximately 5000 people living in these villages. The mortality rate due to MPM among Karain village was 8 deaths/ 1000 inhabitants/year; in addition to 52% of deaths related to MM from 1970 to 1994 [64]. Therefore, these studies have shown that erionite is a strong inducer of MM in humans.

Until recently, erionite exposure was believed to be a health problem only in Turkey; however, this has changed dramatically with the discovery of the first erionite related MM case in the US [65], and also with the first reported case of a patient with erionite-associated pleural mesothelioma in North America [66]. Astonishing evidence has shown that over the past 30 years more than 300 miles of road was surfaced with erionite-containing gravel in Dunn County, North Dakota, USA. In this same study, it has been reported that the airborne concentration of erionite in several places was equal or exceeded the concentrations found in Turkish villages known to have a high incidence of MM induced by erionite [67].

Consequently, asbestos and erionite likely share the mechanisms of toxicity and carcinogenesis [68,69]; in addition, erionite is able to induce the transformation of human mesothelial cells (MET5-A), but on the other hand, asbestos is not able to cause such transformation [70]. Furthermore, it has been speculated that the HMGB1 (High mobility group box 1) (Fig. 1) is a critical initiator of the chronic inflammation in erionite exposed individuals with the release of IL-1β and TNF-α [69]. It has been reported that erionite activates NLRP3 inflammasome in human mesothelial cells, which is associated with the release of IL-1β, IL-6, IL-8 and VEGF, and with the activation of an autocrine feedback loop modulated via the IL-1 receptor. Likewise, it has been shown that IL-1 receptor blocking may play an important role in inhibiting MM growth and progression [71].

2. Genes and MM

2.1. p16^{INK4a}/p14^{ARF}

Located at the 9p21 chromosome, the *p16^{INK4a}/p14^{ARF}* (also known as *CDKN2A/ARF*) are important tumor suppressor genes, which encode two functionally unrelated proteins, the p16^{INK4a} and the p14^{ARF} (also known as p19^{ARF} in mice). These proteins have unique first exons (1α and 1β), but share exons 2 and 3, which are translated from an alternative reading frame with no amino acid homology [72]. The same locus harbors another tumor suppressor gene (TSG) called *p15^{INK4B}* (also known as *CDKN2B*), which encodes the protein p15^{INK4B}, a CDK (cyclin-dependent kinase) inhibitor known to be induced by TGF [73].

The p16^{INK4a} is a CDK inhibitor, and acts by inhibiting the CDK-mediate hyperphosphorylation that leads to pRb inactivation, while p14^{ARF} regulates p53 function by inhibiting p53 degradation through MDM2 interaction [27,74]. p16^{INK4a} maintains pRb in its active hypophosphorylated form by disrupting the CDK4/6-cyclin D complex, leading to G$_1$-phase cell cycle arrest (Fig. 2). Thus, both p16^{INK4a} and
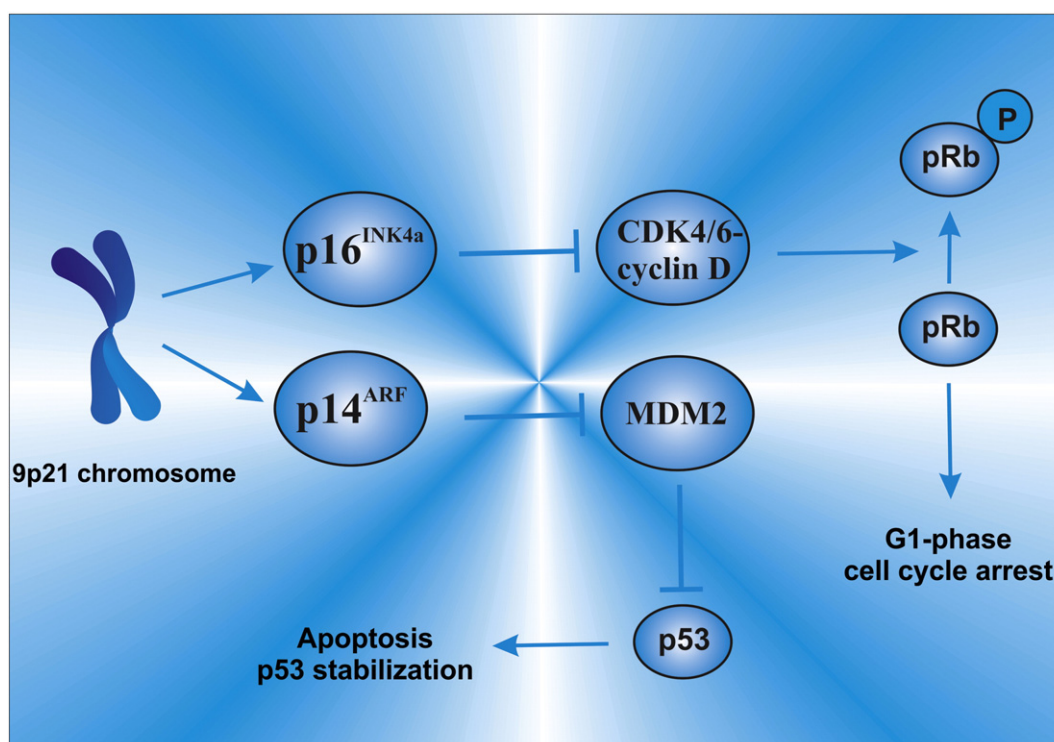

Fig. 2. The role and function of p16^{INK4a}/p14^{ARF} in the cell cycle control, apoptosis and their interaction with other key regulatory cell cycle proteins.

p14[ARF] are key cell cycle regulators due to their role in the p53 and the pRb pathways [75]. Therefore, genetic defects in the $p16^{INK4a}$/$p14^{ARF}$ are able to lead to loss of function on both p53 and Rb pathways, which are key players to a regulated cell cycle control (Fig. 3).

2.1.1. Role of $p16^{INK4a}$/$p14^{ARF}$ and MM

The $p16^{INK4a}$/$p14^{ARF}$ have been implicated in the development of human cancers [76–78]. This is no different in MM, in which these genes have been extensively shown to be inactive. It has been shown homozygous deletions of $p16^{INK4a}$ in 85% of mesothelioma cell lines [79], abnormal p16[INK4a] expression in all primary mesothelioma specimens and cell line [32], and codeletion of $p16^{INK4a}$ and $p15^{INK4B}$ in 72% of primary mesotheliomas [13]. Regarding the histological type, MM epithelioid samples have shown approximately 70% of $p16^{INK4a}$/$p14^{ARF}$ homozygous deletions; the sarcomatoid and biphasic have shown approximately 100% of homozygous deletions [80–83]. Loss of the 9p21 locus was observed in 32% of MM cases. Furthermore, intermediate methylation values were observed in the promoter region of the $p16^{INK4a}$/$p14^{ARF}$ in MM samples with no changes on the prognosis [84]. Further studies are summarized in Table 1.

Genetic engineering has been a great asset in better understanding the functions of these genes. Thus, a knockout mouse for $p19^{ARF}$, but expressing $p16^{INK4a}$ develops tumors early in life [85]. Similar results have been found using knockout mice for $p16^{INK4a}$ [86]. Not surprisingly, knockout mice for both $p16^{INK4a}$/$p19^{ARF}$ were more prone to the spontaneous development of tumors at an early age, and highly sensitive to carcinogenic treatments [87].

Although, several studies have shown loss of $p16^{INK4a}$/$p14^{ARF}$ in MM, only recently a knockout mice model for $p19^{ARF}$ have been developed, showing that the inactivation of this gene plays a significant role in driving MM pathogenesis [88]. Furthermore, an interesting study has shed more light on the role of both genes in the development of MM related to asbestos exposure. In this study, mice deficient for $p16^{INK4a(+/-)}$, $p19^{ARF\ (+/-)}$, and those with double deficiency ($p16^{INK4a(+/-)}$/$p19^{ARF\ (+/-)}$) were exposed to asbestos. The mice $p16^{INK4a\ (+/-)}$/$p19^{ARF\ (+/-)}$ displayed accelerated asbestos-induced MM in comparison to $p16^{INK4a(+/-)}$ or $p19^{ARF\ (+/-)}$ mice alone. The $p16^{INK4a\ (+/-)}$ mice displayed bi-allelic inactivation of $p16^{INK4a}$, loss of the $p14^{ARF}$ or p53 expression, and frequent loss of $p15^{INK4b}$; on the other hand, mice $p19^{ARF\ (+/-)}$ exhibited loss of $p19^{ARF}$ expression, but no loss of $p16^{INK4a}$ or $p15^{INK4b}$ [89].

Thus, this study clearly shows that both genes play significant, and not redundant roles in MM, and their inactivation increases the tumorigenesis caused by asbestos exposure. Another interesting finding of this study was that p53 remained functional even in the absence of $p19^{ARF}$, thus showing that $p19^{ARF}$ loss contributes to MM progression via p53-independent pathway(s) [89], as it has been confirmed by other studies [36,88].

2.1.2. $p16^{INK4a}$/$p14^{ARF}$ gene therapy

Gene therapy has been growing considerably in the last decade especially in Mesothelioma due to the poor response to the traditional chemotherapy. It has been shown previously, that loss of the $p16^{INK4a}$/$p14^{ARF}$ is the most common event in MM, and thus therapies targeting the re-expression of these genes in mesothelioma cell lines have shown interesting results. It has been demonstrated, that the transduction of $p16^{INK4a}$ expressing adenovirus in mesothelioma cells resulted in cell cycle arrest, inhibition of pRb phosphorylation, diminished cell growth, and eventual death of the transduced cells [90]. Likewise, in a human mesothelioma xenografts study, the re-expression of the $p16^{INK4a}$ led to: an increase in survival [91]; an increase in p53 protein levels; a reduction on the phosphorylation of pRb, as well as G_1-phase cell cycle arrest and apoptotic cell death [12]. The re-expression of $p16^{INK4a}$/$p14^{ARF}$ using different vectors did not show better results in comparison to the single re-expression of the $p16^{INK4a}$ [92]. Taken altogether, gene therapy targeting both genes seems to have promising results. The clinical gene therapy trials for mesothelioma has been recently revised elsewhere [93].

2.2. NF2

The Neurofibromatosis type 2 (NF2) is a dominantly inherited tumor predisposition syndrome characterized by the development of bilateral vestibular schwannomas of the eighth cranial nerve, and by other brain
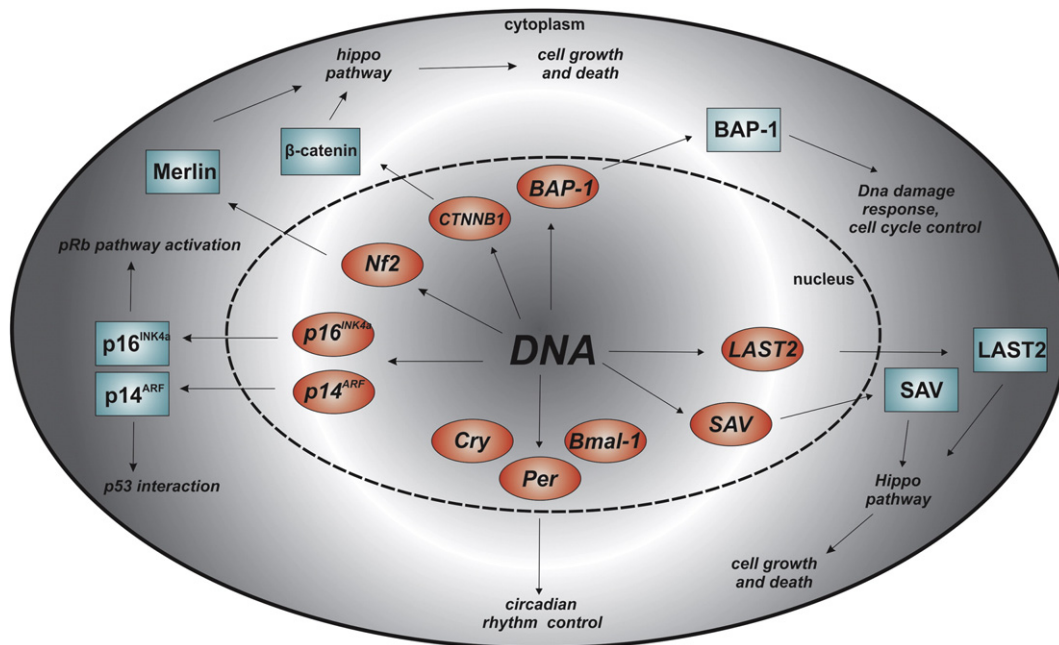
Fig. 3. The involvement of key genes and their respective proteins in the development of MM. The proteins p16[INK4a] and p14[ARF] are involved in pRb pathway activation and in p53 modulation, respectively. The *NF2* encodes the protein Merlin, *LAST*2 encodes the protein LAST2, β-catenin is encoded by *CTNNB1,* and *SAV* encodes the protein SAV, thus these proteins play an important role in the hippo pathway. *BAP-1* encodes the protein BAP-1 that plays an important role in DNA damage response and in cell cycle control. The genes *Cry*, *Per* and *Bmal*-1 are key players responsible for regulating the circadian rhythm control.

Table 1
Methods in each study vary with different sensitivity/specificity rates, and definitions of mutations. The methodologies described in the table are the main methods used, but more methods may have been applied. Fish = Fluorescence in situ hybridization; MM = Malignant Mesothelioma; HMM = Human Malignant mesothelioma; SSCP = single-strand conformation polymorphism.

Evaluation of $p16^{INK4a}/p14^{ARF}$ expression in MM		
Study	Methodology	Main results
$p16$ alterations and deletion mapping of 9p21–p22 in Malignant Mesothelioma [79]	Southern Blot and PCR. 40 cell lines and 23 primary tumors.	Homozygous deletion of $p16^{INK4a}$ in 34 (85%) cell lines and in 5 (22%) of primary tumors
$p16$ deletion in sarcomatoid tumors of the lung and pleura [249]	FISH Sarcomatoid malignant mesotheliomas samples	Deletion of 9p21 in 26 of 32 (81%) in malignant mesotheliomas
Codeletion of $p15$ and $p16$ in primary malignant mesothelioma [13]	FISH Primary mesotheliomas samples	Codeletion of $p15^{INK4b}$ and $p16^{INK4a}$ in 72% of mesotheliomas
Genomic profiling of malignant pleural mesothelioma with array-based comparative genomic hybridization shows frequent non-random chromosomal alteration regions including JUN amplification on 1p32 [247]	Genome-wide array-based CGH, RT-PCR 9 MPM cell lines and 17 MPM samples;	$p16^{INK4a}/p14^{ARF}$ deletion was found in 7 (41%) MPM samples and in 9 (100%) MPM cell lines.
Diagnostic importance of 9p21 homozygous deletion in malignant mesotheliomas [83]	FISH Pleural mesothelioma and Peritoneal mesothelioma samples	Homozygous deletion of the 9p21 in 35 of 52 cases (67%) of pleural mesothelioma and in 5 of 20 cases of peritoneal mesothelioma (25%)
Establishment and characterization of four malignant pleural mesothelioma cell lines from Japanese patients [248]	PCR, SSCP analysis and Western Blot 4 HMM cell lines	$p16^{INK4a}/p14^{ARF}$ in all four HMM cell lines
9p21 deletion in the diagnosis of malignant mesothelioma in serous effusions additional to immunocytochemistry, DNA–ICM, and AgNOR analysis [81]	FISH Malignant mesothelioma patient samples	9p21 homozygous deletion in 48.5%, heterozygous deletion in 36.4%
Promoter methylation of RASSF1A, RARβ2 and DAPK predict poor prognosis of patients with malignant mesothelioma [84]	Nested methylation-specific PCR DNA of mesothelioma patients	$p16^{INK4a}$ and $p14^{ARF}$ promoter region methylation in 28.2% and 44.2%, respectively
Morphology of 9p21 homozygous deletion-positive pleural mesothelioma cells analyzed using fluorescence in situ hybridization and virtual microscope system in effusion cytology [250]	FISH 15 epithelioid MPM	12 positive for a homozygous deletion and 3 positive for both homozygous and heterozygous deletions with a predominantly heterozygous pattern
9p21 deletion in the diagnosis of malignant mesothelioma, using fluorescence in situ hybridization analysis [82]	FISH Malignant mesothelioma patient samples	9p21 deletion in 35 of 40 (88%) cases with MM
Genomic gains and losses in malignant mesothelioma demonstrated by FISH analysis of paraffin-embedded tissues [249]	FISH Malignant mesothelioma patient samples	Loss $p16^{INK4a}/p14^{ARF}$ in epithelioid 23/30 (77%) and biphasic/sarcomatoid 12/12 (100%) mesotheliomas
Immunohistochemical analysis of the p16INK4 cyclin-dependent kinase inhibitor in malignant mesothelioma [32]	Immunohistochemical analysis and Immunoblot. Primary thoracic mesotheliomas and mesothelioma cell lines	Abnormal $p16^{INK4a}$ protein expression in 12 of 12 primary mesothelioma specimens and in 15 of 15 mesothelioma cell lines
Homozygous deletion of CDKN2A/ARF and codeletion of the methylthioadenosine phosphorylase gene in the majority of pleural mesotheliomas [247]	FISH Pleural mesothelioma samples	$p16^{INK4a}/p14^{ARF}$ homozygous deletion in 70 samples (74%). Homozygous loss of $p16^{INK4a}/p14^{ARF}$ in 49 of 71 epithelial (70%), 16 of 19 biphasic (89%) and 5 of 5 sarcomatous (100%) mesotheliomas.

tumors, including meningiomas and ependymomas [94]. This syndrome is caused by mutations and lack of expression of the tumor suppressor gene *NF2* (Fig. 3), which is located on chromosome 22q12, and encodes the 595 amino acid protein called Merlin (Moesin-ezrin-radixin-like protein) [95].

2.2.1. NF2 and MM

In 1995, two groups first demonstrated that this gene was mutated in approximately 40–50% of MM, and its inactivation was important in the tumorigenesis of MM [95,96]. Follow-up studies have confirmed the importance of this gene in the development of MM, and are

Table 2
Methods in each study vary with different sensitivity/specificity rates, and definitions of mutations. The methodologies described in the table are the main methods used, but more methods may have been applied. MM = Malignant Mesothelioma; HMM = Human malignant mesothelioma; SSCP = single-strand conformation polymorphism; Comparative genomic hybridization (CGH).

Evaluation of *NF2* expression in MM		
Study	Methodology	Main results
Frequent mutations of *NF2* and allelic loss from chromosome band 22q12 in malignant mesothelioma: Evidence for a two-hit mechanism of *NF2* inactivation [251]	Western blot and DNA sequence analyses 25 MM cell lines	14 of 25 (56%) showed no *NF2* expression; 18 of 25 (72%) showed losses at one or both loci tested.
Heterogeneity of mesothelioma cell lines as defined by altered genomic structure and expression of the *NF2* gene [252]	Northern blot, RT-PCR and PCR. 18 HMM cell lines	*NF2* alterations were identified at a genomic level in 7 (39%) cell lines and were associated with a marked decrease in the concentration of the *NF2* transcript.
Establishment and characterization of four malignant pleural mesothelioma cell lines from Japanese patients [248]	PCR, SSCP analysis and Western Blot 4 HMM cell lines	A point mutation of *NF2* was observed in 1 cell line.
Genomic profiling of malignant pleural mesothelioma with array-based comparative genomic hybridization shows frequent non-random chromosomal alteration regions including JUN amplification on 1p32 [247]	Genome-wide array-based CGH, RT-PCR 9 MPM cell lines and 17 MPM samples;	Small deletions resulting in frameshift mutation were found in 3 (18%) MPM samples.

summarized in Table 2. Furthermore, recent studies have strengthened this data. A study has found that 38% of MPM samples displayed NF2 mutation, and 29.4% displayed deletions, while no NF2 mutation was found in non-small cell lung cancer patients [97]. It has been shown that 38% of MM samples display chromosomal loss at 22q12 [82]; moreover, the NF2 was hypothesized to be an early molecular alteration due to loss of heterozygosity in a well-differentiated papillary mesothelioma of the peritoneum (WDPMP), a rare type of mesothelioma [98]. Not surprisingly, miRNA expression targeting NF2 has been reported in MM [99].

In order to better understand the role of NF2 in MM development, animal models have shed some light on the role of this gene. Altomare et al. [22] developed a NF2 $^{(+/-)}$ knockout mice and an environmental carcinogenesis model. The asbestos-exposed NF2 $^{(+/-)}$ mice exhibited an increase in tumor development, in comparison to the wild-type mice; in addition, it was observed biallelic inactivation in all nine asbestos-induced MM from NF2 $^{(+/-)}$ mice. Lastly, it was shown that tumors from NF2 $^{(+/-)}$ mice frequently showed homozygous deletion of the $p16^{INK4a}$, $p14^{ARF}$ and $p15^{INK4B}$, which is a similar feature of human MM. Following this line, Jongsma et al. [100], using a conditional knockout animal model, showed the importance of NF2, TP53 and $p16^{INK4a}$ and $p14^{ARF}$ on the development of MM.

Taken altogether, these animal studies resembling molecular features of human MM are important to better understand the roles of these genes, and also to design better treatment approaches targeting relevant pathways.

2.2.2. NF2 gene therapy

The overexpression of NF2 by viral vectors has been shown in several other cancers with interesting results on the cell cycle control and proliferation [101–104]. In MM, the re-expression of NF2 led to significant inhibition on cellular proliferation and invasiveness [105], cell proliferation inhibition, G_1 phase arrest, reduction on cyclin D1 expression, inhibition of CDK4 activity, and dephosphorylation of pRb [106].

2.3. BAP-1 and MM

Genetic susceptibility has been related to the development of MM. In a study in Turkish villages with high incidence of MM, it was shown that MM was genetically transmitted likely in an autosomal dominant way [107]. Another study has found that family members genetically predisposed to MM, when raised outside the villages did not seem to develop MM; in addition, when high-risk MM family members married into families with no history of MM, MM appeared in the descendants [108]; however, the effects of genetic factors on MM have been questioned [109].

On the other hand, very recently more solid evidence has been reported. The BAP1 (BRCA1-associated protein 1) is a tumor suppressor gene located on chromosome 3p21 and encodes the BAP1, which is a deubiquitinating enzyme that seems to regulate deubiquitination during DNA damage response, and the cell cycle (Fig. 3). In recent times, it has been shown BAP1 germline mutations in two families with high incidence of MM, and thus characterizing the existence of a BAP1-related cancer syndrome known by the presence of MM, uveal melanoma, [11,24,110], and possibly other types of cancer [111]. Furthermore, BAP1 somatic mutations have been identified in 23% of sporadic mesothelioma [112]. Interestingly, the lack of BAP1 activity has been shown to be more specifically involved in the pathogenesis of epithelioid MM rather than non-epithelioid MM [113]. A recent clinical study has shown that indeed 20% of MPM tumors harbor BAP1 somatic mutations; however, no difference in survival was found when comparing with BAP1 mutation status [114]. On the other hand, conflicting data has been reported, in which high levels of BAP1 (non-mutated form) expression was related to shorter overall survival in MPM tissue samples [115].

The role of BAP1 in the development of MM and other cancers, as well as its mutations have been recently addressed [116]. Furthermore, germline mutation on BAP1 confers increased susceptibility for the

development of MM and among other tumors [117], while somatic mutations have been shown to implicate transcriptional dysregulation in the pathogenesis of MPM. Taken altogether, genetics have been extensively implicated in the development and predisposition of MM, and in a near future the outcomes from these studies will help not only to better treat, but also to prevent more cases of MM, especially in those individuals more prone to its development.

2.4. LATS2

LATS (Large Tumor Suppressor) was first found as a tumor suppressor in Drosophila [118]. In humans the LATS1 and LATS2 have been identified, the latter one residing in a region (13q11-12), which frequently displays loss of heterozygosity in primary cancers [119,120]. The human LATS2 (Fig. 3) is a centrosomal protein, known to play an important role in the mitotic division [121], in mediating Hippo growth-inhibitory signaling [37], and to activate p53 [122]. The function of LATS in the cancer realm has been recently reviewed, and will not be further discussed here [123].

2.4.1. LATS2 and MM

Using CGH (Comparative genomic hybridization) analysis in 14 MM cell lines, three MM cell lines displayed homozygous deletion at 13q12.11, which was confirmed by PCR analysis; moreover, in 20 MM cell lines, 7 genetic mutations of LATS2 were found; in addition, 3 of 25 (12%) primary tumors displayed genetic alterations that led to inactivation. In the same study, the transduction of LATS2 in MM cells carrying mutations in this gene led to MM cell proliferation suppression. Therefore, LATS2 seems to play an important role in cell proliferation and survival; however further studies are needed in order to confirm whether this gene is important in the development of MM [25].

2.5. DNA methylation and MM

In MM, DNA methylation studies have brought interesting results. It has been shown that methylation profile can discriminate between normal pleura from mesothelioma [124]. Likewise, another study has found a unique methylation profile in MM, which eventually could be used as diagnostic markers [125]. The analysis of the methylation status of nine genes in serum DNA of mesothelioma patients demonstrated interesting results on patient survival [126]. Several other studies analyzing patient outcome, diagnosis, as well as epigenetic therapy, were extensively revised by other authors [15,127].

2.6. MicroRNA and MM

The miRNA expression is another important mechanism in the development of cancer due to its ability to control several biologic processes. Not surprisingly, it has been shown differences between the expression profile of miRNA in MM, in comparison to normal pleura; in addition, in each histopathological subtype of MM, specifically miRNA patterns have been found [99].

It has been shown that MM cell lines derived from patients with more aggressive cancer do not express mirR-31; moreover, the re-introduction of miR-31 led to suppression on cellular growth; thus showing a potential therapy for MM tumors that fail to express miR-31 [128]. Likewise, the miR-15/16 has been demonstrated to be downregulated in MPM cell lines in comparison to MET5-A. The restoration of miR-15/16 levels led to growth inhibition in MPM cell lines [129]. In addition, the relationship between miRNA expression, patient diagnosis and outcome has been extensively revised by other authors [15].

Therefore, miRNA have been proposed as diagnostic tool [130–132], as prognostic makers [163,164], and as treatment target option [129,133] for MM.

2.7. Other genes and MM

The salvador gene (*SAV*), component of Hippo cascade (Fig. 3), was first discovered in a *Drosophila* [134], and it has been suggested to be a tumor suppressor gene in some cancers [16,135]. Recently, it was shown that *SAV* had a homozygous deletion at chromosome 14q22 in one (5%) MM cell line; however the function of this gene has yet to be established in MM [25].

Furthermore, it has been found a homozygous deletion in the gene β-catenin (*CTNNB1*) (Fig. 3) in one (10%) MM cell line [136]; in addition, it was shown that *CTNNB1* is a positive growth-stimulating factor for many human cancers [137]; however its role in MM has not been fully addressed.

A recent study has suggested that the Hedgehog signaling pathway is active in MM cell lines [138]; in addition, it has been shown that 13 genes regulate this pathway in cancer; however only three of these genes (*PTCH1*, *SMO* and *SUFU*) were indeed mutated in 2 of 11 MM cell lines [138]. Regarding MM patient tumors, only one patient (7.15%) showed a mutation in *SMO* [139]. Recently, new genomes techniques have been applied showing new potential TSGs in MM [140]. Therefore, further studies are needed in order to confirm whether these genes are indeed important to MM tumorigenesis.

The circadian rhythms are generated by several genes and proteins, which ultimately regulate several biologic processes, such as: sleep regulation, body temperature, hormones, immune system response, and others [141]. It has been shown a correlation between a disruption of circadian rhythms and incidence of breast cancer [142]. Likewise, it has been shown that chronomodulated anticancer regimes in male patients display an increase on survival rates, in comparison to traditional chemotherapy [143]. In MM, more evidence has emerged showing that

the clock genes *PER* (period), *CRY* (cryptochrome) *BMAL1* (aryl hydrocarbon receptor nuclear translocator-like) (Fig. 3) were expressed in favor of tumor growth [144]. More recently, a therapeutic target approach inhibiting *BMAL1* expression has been proposed showing a reduction on tumor growth in MM cell lines expressing high levels of *BMAL1* [145].

3. Important pathways in MM

3.1. Receptors tyrosine kinases

The RTKs regulate cell cycle control and proliferation and are frequently activated in MM [146], leading to upregulation of RAS/RAF/MEK/MAPK and PI3K pathway (Fig. 4) [147].

The EGFR is often overexpressed in MM [148]. EGFR has been detected in 44% of MPM [28], and its expression has been correlated with long term survival [149]; however conflicting data has also been published [150]. Despite that MM displays high expression of EGFR, gefitinib [151] and Erlotinib [152], both EGFR inhibitors, were not active in MM in phase 2 clinical trials.

The VEGF and their specific transmembrane receptors (VEGFR-1 and VEGFR-2) are a potent inducers of angiogenesis, and are expressed in MM [30,153,154], being associated with a reduction on patient survival [30,155]; however, a study did not find a correlation between VEGF expression and an improvement on patient survival [156]. Likewise, it has been shown that SV40 induces VEGF expression in MM cell lines, which enhances their proliferation. VEGF may not only stimulate the tumor angiogenesis, but also tumor growth [157]. According to Lee et al. [27], prolonged survival rates in animals were observed in MPM cell lines treated with the antibody Bevacizumad in combination with
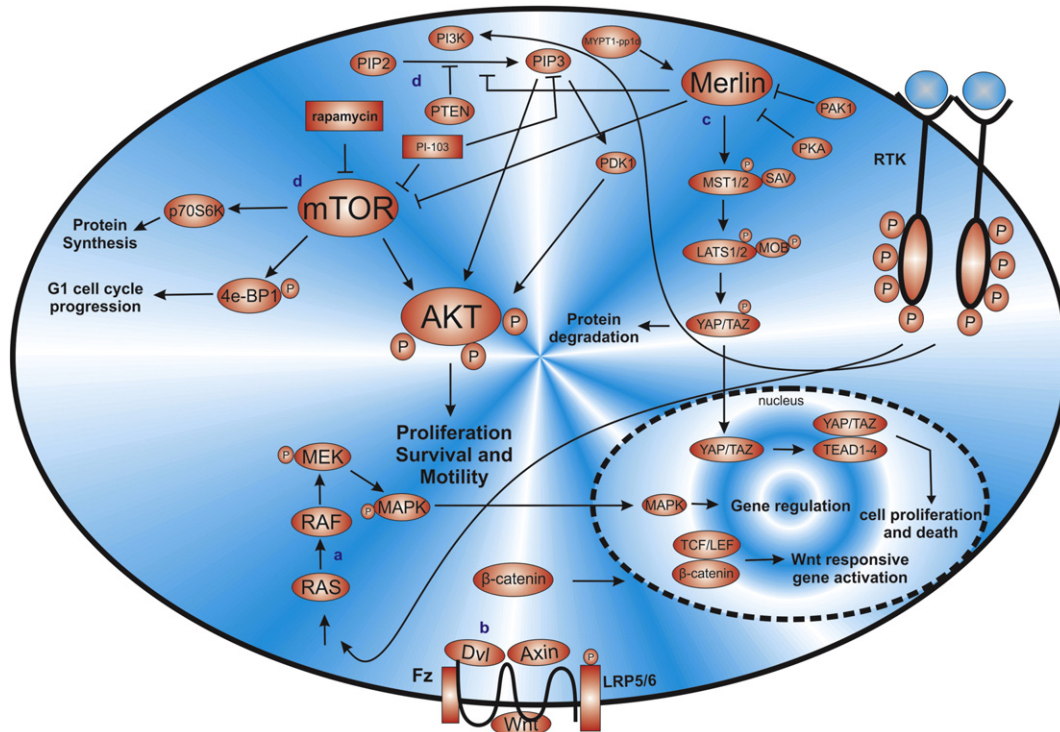
Fig. 4. The most common alterations in MM. a) the receptors tyrosine kinase (RTK) are frequently activated in MM and lead to an upregulation of RAS and PI3K pathway. RAS activates RAF, which phosphorylates MEK. The latter one phosphorylates MAPK that migrates to the nucleus and regulates gene expression. b) The Wnt pathway controls several cellular processes. Upon the presence of Wnt ligand, a complex involving Dvl, Axin, Fz and LRP5/6 leads to inhibition of β-catenin phosphorylation and degradation. Thus β-catenin migrates to the nucleus where complex with TCF/LEF leading to Wnt responsive genes activation. c) Merlin protein is encoded by *NF2* and it is negatively regulated by PAK1 and PKA, and positively regulated by MYPT1-PP1d. In addition, Merlin also acts as an inhibitor of PI3K and mTOR pathways. This protein plays an important role as an upstream regulator of the hippo pathway. Thus, upon upstream stimuli, MST 1/2, phosphorylates SAV1, LAST 1/2 and MOB1. The complex MST 1/2 and SAV1 directly phosphorylates LAST 1/2, which is required for LAST 1/2 activation. The complex LAST/2 and MOB1 directly interacts and phosphorylates YAP/TAZ. The latter one, when phosphorylated leads to protein degradation, while its dephosphorylated form enters into the nucleus mainly binding to the transcription factors TEAD1–4 to regulate genes involved in cell proliferation and cell death. d) PI3K/AKT/mTOR pathway is activated by the conversion of PIP2 into PIP3 by PI3K; however PTEN acts as an antagonist of this activation. PDK1 and mTOR phosphorylate AKT, which then exerts important roles on cell proliferation, survival and motility. mTOR leads to protein synthesis and G1 cell cycle progression and it is inhibited by rapamycin and by PI-103.

Pemetrexed. Regarding anti-VEGF therapy, erlotinib and bevacizumab in combination with chemotherapy were also evaluated in MM [158].

Insulin growth factor (IGF) and Insulin growth factor receptors (IGFR) are expressed by MM [159], and by normal mesothelial cell lines [27]; however, dysregulation of IGF pathway may lead to malignant transformation [27]. Stimulation by IGF-I resulted in enhanced activation of IGFR leading to cell proliferation [160]. A study has found that IGF-I was overexpressed in 11 MPM cell lines and in 4 primary tumors [161]; moreover, a recent study has demonstrated an interesting finding. The authors assayed the expression of IGF-I receptors (IGF-IR), and found considerable variability among several MM tumor samples and cell lines. Furthermore, in this same study, the IGF-I surface receptors were quantified by flow cytometry, confirming the previous results mentioned, and more remarkably, the anti-tumor efficacy with cixutumumab and inhibition of IGF-IR downstream signaling were highly correlated with IGF-IR sites per cell [162].

Lastly, Hepatocyte Growth Factor Receptor (MET) is a proto-oncogene that is commonly expressed in MM and it is not mutated [165,167]. Inhibition of MET has been proposed as therapeutic strategy option for MM [166,168]. Combined inhibition of MET and EGFR led to strong inhibition on cell proliferation and invasion of MPM cell lines [167].

3.2. Ras/Raf/MEK/MAPK pathway

The RAS/RAF/MEK/MAPK signaling pathway comprises cell surface receptors, transcription factors, which regulates gene expression. This pathway is one of the most dysregulated in cancer and regulates critical cellular function, such as: proliferation, growth and senescence. The RAS/RAF/MEK/MAPK pathway influences the regulation of apoptosis through interaction with BAD, Caspase 9 and BCL-2 [169].

RAS is a single GTPase molecule and it has tree isoforms (H-Ras, K-Ras and N-Ras). In its "off" state, RAS is bound to GDP; however, upon stimuli, RAS binds to GTP ("on" state) [170]. In its "on" state, RAS, combines with RAF and mobilizes the inactive protein from the cytoplasm recruiting the RAF kinases (ARAF, BRAF and CRAF) to the plasma membrane [171]. Onto the cell membrane, RAS activates the RAF, which then acts as a MAP kinase kinase kinase (MAPKKK) activating MEK1 and MEK2. The latter two, catalyze the activation and translocation into the nucleus of MAPK1 and MAPK2. Thus, once activated, MAPK1 and MAPK2 kinases phosphorylate several genes involved in several important cellular responses (Fig. 4) [171,172]. Also, it is known that RAS can activate other downstream pathways, such as: PI3K, RAC and RHO [171].

In cancer, point mutation of the *RAS* family gene comprises at about 30% of all human cancer [171]. The gene *BRAF* that encodes the BRAF is also frequently mutated in human cancer [173]. Therefore, this pathway is indeed important to the development of cancer, and several inhibitors have been tested or are under clinical trial, which were extensively revised by Santarpia et al. [171].

3.2.1. Ras/Raf/MEK/MAPK pathway in MM

It has been shown in MM that the expression of phosphorylated MAPK1/2 was increased in comparison to normal lung tissue [174]; however, it has also been shown that MAPK activation was not able to differentiate between benign and MM cells [175].

In an animal study, it was shown prolonged MAPK1/2 activation after exposure to asbestos, in comparison to animals exposed to similar and nonpathogenic particles [176]. Interestingly, MAPK1/2 phosphorylation also occurs in distal bronchioles in a murine model of fibrogenesis [177]. Recently, an in vitro study showed that MAPK2 is critical to transformation and homeostasis of epitheliod MM; thus showing different roles between MAPK1 and MAPK2 in epitheliod MM, at least in vitro studies [178].

3.3. PI3K/AKT/mTOR pathway

The phosphatidylinositol 3-kinase (PI3K) pathway regulates several cellular processes, such as survival, metabolism, proliferation, vesicle trafficking, apoptosis, growth and cell migration, and among other functions in specific cellular contexts [179].

Phosphoinositide 3-kinase is part of a family of intracellular lipid kinases that phosphorylate the 3′-hydroxyl group of phosphoinositides and phosphatidylinositols. One of the products of this reaction is the PIP3 (phosphatidylinositol-3,4,5-triphosphate), which is a second messenger lipid [180] essential for the translocation of AKT to the plasma membrane, where it is phosphorylated, and activated by phosphoinositide-dependent kinase 1 (PDK1). The phosphorylated AKT (p-AKT) conveys downstream signals promoting cellular proliferation and survival over apoptosis (Fig. 4) [22].

According to the structure, substrate specificity and lipid products, the PI3Ks are subdivided into 3 categories (classes I, II and III). Class I is subdivided into IA and IB. The first class is activated by tyrosine kinase receptors, G-protein coupled receptors and oncogenes, and it has been widely implicated in cancer. This class is composed of heterodimers, comprising regulatory p85 and catalytic p110 subunits. The class IB members consist of a p101-regulatory subunit and are activated by G protein-coupled receptors [180]. The classes II and III use phosphatidylinositol (PI) as a substrate to generate PIP3 [181].

The *PIK3CA* gene encodes the catalytic subunit p110α, which has been shown to increase the activity of this pathway [180]. This subunit phosphorylates the phosphatidylinositol biphosphate (PIP2) into PIP3 [182]. This second messenger is essential to translocate the serine/threonine kinase AKT (also known as PKB) onto the plasma membrane [26,183]. AKT activation regulates along with PI3K, a large number of cellular processes, including cell proliferation, survival and motility [184]. When PIP3 binds to AKT, the recruitment occurs onto the membrane, which is followed by phosphorylation by the mammalian target of rapamycin (mTOR) [185].

The mTOR pathway has an important role in the energy balance of mammalian cell growth and size, and therefore, it is a relevant therapeutic target for dysregulated cellular growth, including cancer [186]. Activation of mTOR leads to the phosphorylation of eukaryotic initiation factor 4E (eIF4E)-binding protein-1 (4E-BP1), thereby dissociating 4E-BP1 from the mRNA cap binding protein eIF4E to promote protein synthesis [187]. Also, mTOR regulates the activity of the ribosomal protein S6 kinase (p70S6K), that is required for cell growth and G_1 cell cycle progression [188].

3.3.1. PI3K/AKT/mTOR pathway and MM

Alterations on the PI3K/AKT/mTOR are associated with more aggressive diseases, such as cancer. This pathway is a major survival pathway in several tumors, and it has been shown to be active in mesothelioma cell lines [189]. When the signaling cascade is dysregulated, there is a disrupted translation of mRNAs that are involved in cellular processes, such as cell cycle progression, growth stimulation, cell survival, invasion and interaction with extracellular matrix [190], and apoptotic resistance [191].

AKT has a central role in a signaling pathway of which many of its components have been linked to tumorigenesis. AKT can be activated by a variety of mechanisms: loss or downregulation of *PTEN* [192], activation of PI3K in autocrine or paracrine stimulation of the RTK [192,193]; mutation of the PI3K catalytic or regulatory subunits [194], and/or Ras activation [195].

There are 3 isoforms of AKT: AKT-1, 2 and 3. Gene amplification of *AKT-1* and *AKT-2* are infrequent, but it has been reported in some types of cancers [196–198]. In MM, there is an increased activity of this kinase, which can be a pharmacological target used in order to increase the effectiveness of chemotherapy [22]. The lack of *PTEN* expression leads to elevated levels of p-AKT [199].

mTOR mediates survival in MM, thus Wilson et al. [23] reported that rapamycin (an mTOR inhibitor) was able to reduce the apoptosis resistance in more than 50% of MM tumors samples. Kim et al. [200] demonstrated a reduction on cell resistance to apoptosis due to inhibition of mTOR, by rapamycin.

One of the components of this pathway is the lipid phosphatase protein, PTEN, that dephosphorylates PIP3 into PIP2, thus acting as a central negative regulator of PI3K [181], by inhibiting AKT activation. PTEN also regulates chemotaxis and cell motility, both mechanisms that promote tumor invasion [84,85]. Few MM studies have shown *PTEN* homozygous deletion [22,26] or alteration on the expression [201,202], and their relationship with the suppression of cellular growth by AKT blockade. Therefore, these findings suggest that the protein phosphatase activity of PTEN may contribute to its tumor suppressor function in a subset of MMs.

The activated p110α catalytic subunit, which is encoded by the *PIK3CA* is a frequently mutated gene in cancer. Mutations in the p110α have been identified in about 12% of all human cancers [203]. Thus, there are three "hot spots" mutations identified on *PIK3CA*: E542K, E545K (helical domain) and H1047R (kinase domain) [204]. Several studies have linked the role of PTEN in negatively regulating the expression of AKT, and today, it is well accepted that the PI3K-AKT pathway is overexpressed in MM [22,26,201]. Furthermore, it has been shown that the overexpression of *PTEN* by transfecting cells with an adenoviral vector increased apoptosis in mesothelioma cell lines, due to AKT hypophosphorylation [201].

A study has found a reduction on mRNA levels of *PIK3CA* in patients with malignant peritoneal mesothelioma, which was correlated with an improvement on survival rate [205]. In another study, Suzuki et al. [26] demonstrated a dysregulation of PI3K-AKT pathway in MM cell lines. Furthermore, it has been shown AKT activation, and low or no expression of PTEN in MM cells lines [200].

As described above, several survival or anti-apoptotic mechanisms have been identified in MM, and they have been shown to influence survival and resistance to chemotherapy; however, it is not fully understood the role of mutations and mechanisms on the activity of AKT and PI3K, and their relationship with the development of MM.

3.3.2. Therapeutic targets

MM is a very aggressive tumor, but advances in the treatment of this disease have emerged. The tumor is highly resistant to chemotherapy, mainly due to resistance to apoptosis [206]. MM remains an unusual tumor, and thus it is difficult to study it in large clinical trials. Some animal and cellular models have been proposed for the study of this cancer.

PI3K/AKT/mTOR pathway is involved in MM carcinogenesis, and the elucidation of the downstream targets that dictate cellular response to this signaling pathway may have important implications for the development of MM treatment therapies. The treatment with PI3K inhibitors has been shown to inhibit the growth of many types of cancer cells by inducing cell cycle arrest and apoptosis [207,208].

Rapamycin treatment is able to inhibit activity of both mTOR and AKT, suggesting that it may be an effective therapeutic blockade of PI3K signaling [181]. It has been found that inhibitors of the PI3K and mTOR pathways were successful in enhancing apoptosis in human mesothelioma spheroids [200,209].

There is also a proposal to inhibit several targets of the PI3K pathway: Knight and Shokat [210] have developed a dual inhibitor of p110α and mTOR called PI-103. It has been effective in blocking proliferation of Glioma and other tumor cells in vitro. Furthermore, inhibition of other targets, including AKT and PDK1 has also been investigated [211]. Regarding inhibiting AKT activity, in addition to Rapamycin, the triciribine has been used to inhibit the phosphorylation of all 3 AKT isoforms, and tumor growth of cells overexpressing AKT in mouse xenograft models [212]. Several inhibitors of PI3K/AKTmTOR have been developed. However, these inhibitors may have limited effectiveness in human cancers.

3.4. The BCL-family and MM

In general, the majority of cancers, including MM [206], show resistance to apoptosis [213]. The BCL-2 family of proteins is important in controlling this process. This family is subdivided into pro- and anti-apoptotic members. All these proteins have at least one BCL-2 Homology (BH) domain. The BCL-2, BCL-XL, BCL-W and MCL-1 are among the anti-apoptotic members that inhibit cells from undergoing programmed death. On the other hand, the pro-apoptotic proteins members are BAD, BID and BIM [214], which induce permeability of the mitochondrial membrane, resulting in caspase activation [20]. There are also the multi-domain pro-apoptotic proteins, such as BAX and BAK, which contain BH1–BH3 domains [20].

MM cell lines rarely express BCL-2, but often express BCL-XL [35,36], and a normal contingent of pro-apoptotic *BCL-2* family genes [35,215]. Due to great resistance to apoptosis, low BCL-2/BAX ratio has been reported in mesothelioma cells, which implicates into a mechanism other than BCL-2 in regulating apoptosis [4,215].

Few studies have shown modulation of BAX, BAD, BIM, BAK and MCL-1 in MM. Yuan et al. [225] observed that MCL-1 was the major survival player in regulating MG132-induced apoptosis in MPM cell lines. Moreover, BAX and BAK require a subset of pro-apoptotic BCL-2 family proteins for activation, two of them (BID and BIM), induce oligo-merization and activation of BAX. It has been reported in MM, the loss of expression of BID (37%) and BIM (18%) [20]. Lastly, in a study using immunohistochemical analysis, it was found that 100% of a series of 35 mesothelioma samples expressed BAX [35].

3.4.1. Therapeutic targets

Few studies have shown therapeutic strategies that target activation or blockade of the BCL-2 family members. Following this line, Zhang et al. [216] and Raisova et al. [217] have shown that the ratio BAX/BCL-2 or BAX/BCL-XL in tumor cells is an important determinant of susceptibility to apoptosis, with more sensitive cells having higher BAX/BCL-2 or BAX/BCL-XL ratio.

The BCL-XL/BCL-2 inhibitors have been developed to disrupt the balance between pro-apoptotic and anti-apoptotic stimuli [218]. Several small-molecular ligands for BCL-2 and/or BCL-XL have also been identified [219].

Moreover, a study has demonstrated that pharmacological inhibition of BCL-XL expression by exposure to a histone deacetylase inhibitor, sodium butyrate (NaB), led to apoptotic cell death in MM. A recombinant adenoviral vector expressing pro-apoptotic *Bax*, has been shown to be effective in inducing apoptosis in human MM [220]. An antisense oligonucleotide therapy directed at *Bcl-xl* mRNA was used in vitro to downregulate the *BCL-XL* expression, and resulted in apoptosis and viability decrease in human mesothelioma cell lines [221]. Another therapy that has shown good outcomes was the combination of antisense oligonucleotide in combination with cisplatin, which reduced growth of established tumor xenografts in mice [222].

Furthermore, Varin et al. [223] showed that the simultaneous inhibition of BCL-XL and MCL-1 by small interfering RNA (siRNA) was able to induce massive cell death in the absence of chemotherapy, and was enough to avoid treatment resistance in MSTO-211H, a MM cell line; thus showing a strong molecular basis for the clinical evaluation of therapies targeting both BCL-XL and MCL-1, alone or in combination with traditional chemotherapy in the treatment of MM.

The JY-1-106 protein induces cancer cell death regardless of MCL-1 expression levels through the intrinsic apoptosis pathway, sensitizes tumor cells to chemotherapeutic agents and to metabolic stress; in addition, it induces apoptosis by disrupting BCL-XL and MCL-1 protein–protein interactions with BAK [224]. Another study has demonstrated that 2-methoxy antimycin A3 is able to induce apoptotic cell death without altering BCL-2 family protein expression [218].

Given the findings, we believe that more studies are needed to elucidate and/or discover possible therapeutic strategies to inhibit or activate the BCL-2 family members in MM, mainly focusing on the mechanisms that induce apoptosis.

3.5. The Merlin protein

The *NF2* encodes the protein Merlin. There are at least 10 known isoforms of Merlin protein, and the isoforms I and II are the most common. Merlin structural conformation is important to its activation. The "close" conformation is required for tumor suppressor activity, while the "open" conformation leads to loss of the tumor suppressor activity. Moreover, Merlin is phosphorylated by p21-activated kinase 1 (PAK1) or cAMP-dependent kinase A (PKA) on serine 518, and thus leading to its inactive form; on the other hand, the myosin phosphatase (MYPT1-PP1d) dephosphorylates Merlin (Fig. 4), which leads to its activation [226,227].

Furthermore, it has been shown that Merlin regulates cytoskeleton remodeling, cell motility, cell proliferation, morphology and motility [228]. Merlin also negatively regulates several signaling pathways, such as PI3K [229], mTOR [230], (EGFR) [231], and cyclin D1 expression [106]. In MM, Merlin phosphorylation has been reported, resulting in its inactivation [232]. Lastly, Merlin plays a crucial role as an upstream regulator of the hippo pathway [25].

3.5.1. The hippo pathway

Initially first identified in *Drosophila*, the hippo pathway controls cell proliferation, growth, differentiation and death [37]. The core components of this pathway are MST1/2, SAV, LATS 1/2, MOB and YAP/TAZ. A brief summary of the activation of this pathway is further described. Upon upstream stimuli, MST 1/2, phosphorylates SAV1, LATS 1/2 and MOB1. The complex MST 1/2 and SAV1 directly phosphorylates LATS 1/2, which is required for LATS 1/2 activation. The complex LATS/2 and MOB1 directly interacts and phosphorylates YAP/TAZ. The latter one, when phosphorylated leads to protein degradation, while its dephosphorylated form enters into the nucleus mainly binding to the transcription factors TEAD1–4 to regulate genes involved in cell proliferation and cell death (Fig. 4) [37,228].

3.5.2. Merlin-hippo pathway and MM

The hippo pathway has been implicated in the development of MM. Loss of LATS2 has been reported in MM (see LATS2 and MM section). *YAP*, an oncogene candidate, had its expression detected in more than 70% of MM tissues [25]. The *SAV*, which is a component of the hippo pathway, has been found altered in one MM cell line [see Other genes and MM section]. Furthermore, Merlin has been shown to inhibit the YAP nuclear function, the main downstream effector of Hippo pathway, through phosphorylation at serine 127 [233]. Another study has found that *YAP*-knockdown inhibits cell motility, proliferation, invasion and anchorage-independent growth in MM cell lines; in addition, it was found in MM cell lines with constitutive *YAP*-activation, that *YAP* activation promotes cell cycle progression, which results in more aggressive MM cells [38]. Lastly, the hippo pathway has been shown to interact with TGF-β pathway in regulating the connective tissue growth factor, which is associated with abundant extracellular matrix formation in MM tissues [39].

Taken altogether, the Merlin-hippo pathway plays an important role in driving MM tumorigenesis; however further studies are needed in order to better understand the role of LATS and *SAV* as tumor suppressor genes in regulating this pathway in MM, as well as to better understand the downstream consequences evoked by mutations on these genes.

3.6. Wnt pathway

Wnt signaling pathway plays a fundamental role in the determination of cell fate, proliferation, polarity, and cell death during embryonic development. This pathway was discovered more than 30 years ago [40], and it has been implicated in the development of human diseases [reviewed in 234], and in cancer [reviewed in 235].

Activation of Wnt signaling that alters transcription is called canonical, on the other hand, non-transcriptional Wnt signaling is called noncanonical. β-catenin is the essential downstream transcriptional effector of this pathway. Thus, in the absence of Wnt ligand, cytoplasmic β-catenin is degraded by the action of the Axin complex, which is composed of the scaffolding protein Axin, the tumor suppressor adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). The latter two phosphorylate β-catenin leading to proteosomal degradation, which inhibits β-catenin transcriptional activity (Fig. 4) [40].

On the other hand, upon the presence of Wnt ligand, Wnt/β-catenin canonical pathway is activated due to a complex formation between seven-pass transmembrane Frizzled (Fz) receptor and low-density lipoprotein receptor-related protein 5 or 6 (LRP6/LRP5). This complex together with the scaffolding protein Dishevelled (Dvl), leads to LRP6 phosphorylation, activation and recruitment of Axin complex to the receptors, which leads to inhibition of β-catenin phosphorylation and degradation by Axin complex (Fig. 4). Thus β-catenin migrates to the nucleus, where it forms a complex with TCF/LEF leading to Wnt-responsive gene activation [236].

3.6.1. Wnt pathway and MM

The Wnt signaling pathway has been reported in MM, through Dvl overexpression [41]. It has been shown that secreted frizzled-related proteins (sFRPs) antagonize, and act as a negative regulator of Wnt signaling pathway; in addition, it has been shown downregulation of *sFRPs* in approximately 85% of primary MM tumors due to promoter methylation. The re-expression of *sFRP* in MPM cell lines lacking *sFRPs* expression resulted in apoptosis and growth suppression [237].

The protein inhibitory factor-1 (WIF-1) antagonizes Wnt signaling, and it has been shown to be downregulated in MM cell lines, and in primary tumors due to promoter hypermethylation [238]. Promoter methylation of *WIF-1* was observed in 73.9% of mesothelioma tissues and in all 8 MM cell lines. *SFRP*1, 2 and 4 promoter methylation was observed in 21 of 37 (56.8%), 26 of 42 (61.9%) and 17 of 36 (47.2%) MM tissues, respectively [239]. Furthermore, nuclear accumulation of β-catenin has been reported in MM [240]; however conflicting data has also been published regarding nuclear accumulation of β-catenin [239,241].

A recent study has shown altered expression levels of a number of Wnt/Fzd signaling molecules in MM, and it has been proposed that the modulation of Wnt signaling in MM may sensitize it to cytotoxic drugs [242]. Likewise, it has been shown a lower overall survival rate of patients expressing tumors with high levels of Wnt2B, in comparison to tumors expressing low expression levels of Wnt2B [243]. Inhibition of Wnt2 by siRNA or by monoclonal antibody induced cell death in MPM cell lines [244], another study has found similar findings [245]. Lastly, β-catenin staining has been proposed as a marker in the diagnosis of mesothelial lesions [246].

Therefore, Wnt pathway has an important role on the development of MM and it is a good candidate for new therapeutic approaches targeting Wnt pathway inhibition.

4. Conclusions

As we have seen, once the diagnosis is made, most of the MM patients are left with a short life expectancy of less than a year; in addition, this cancer can take up to 40 years to manifest. Thus, this discrepancy between the start of the carcinogenic process and the diagnosis could be used in favor of the patient, since unlike other cancers, MM takes a long time to manifest. Moreover, the risk factors are of great importance in the development of this cancer, such as erionite, asbestos and SV40; however, more studies are needed in order to better comprehend how these players influence the development of MM, as well as the levels of exposure that are indeed dangerous for the population. Fortunately, the majority of the population is not exposed to such risk factors, something that extensively reduces the likelihood of developing MM.

However, early diagnostics, especially in the population at risk, such as people directly or indirectly exposed to asbestos, erionite or SV40, should be undertaken periodically. Yet, as we have seen, a lot of research is ongoing in order to create good and trustworthy diagnostic markers, which would eventually help many people. This fact in association with an ever-increasing knowledge on the biochemical and genomic pathways to drive the tumorigenesis of MM, gives us valuable clues, which would ultimately result in the production of more specific molecules and drugs, which would be used to fight against the process of malignant transformation, establishment and metastasis. Taken altogether, our work shows that several genes and pathways are involved in the development of MM; however, more studies are needed in order to better understand, prevent, diagnose, and treat this very aggressive cancer.

Acknowledgements

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