Molecular changes in mesothelioma with an impact on prognosis and treatment

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Abstract (250 words):

Over recent decades, genetic and epigenetic abnormalities have been investigated in malignant pleural mesothelioma (MPM) by studying gene mutations, DNA methylation, gene and miRNA expression profiling. These researches have improved patients' outcomes by increasing our level of confidence in MPM diagnosis and prognosis.

Molecular changes in MPM consist in altered expression, activation or inactivation of critical genes in oncogenesis, especially tumor suppressor genes at the *INK4* and *NF2* loci. Deregulation of signaling pathways related to differentiation, survival, proliferation, apoptosis, cell cycle control, metabolism, migration and invasion have been demonstrated in complementary studies. Activation of membrane receptor tyrosine kinase has been frequently observed. More recent data have indicated the presence of alterations that could be targeted at a global level (methylation). Molecular analyses of series of MPM cases showed that defined alterations are generally present in MPM subsets, consistent with inter-individual variations of molecular alterations. This suggests that identification of patient subgroups will be essential in order to develop more specific therapies.

Some of the findings have already been used as the basis for several studies testing various targeted clinical approaches mainly on specific receptor tyrosine kinases, but mostly with limited success. Various experimental researches have been also developed, especially to abolish proliferation and trigger apoptosis in MPM cells. Further basic research studies are needed to predict a positive response in MPM, in order to avoid a rapidly unfavorable course and to prevent wasting resources with inappropriate treatments. Demonstration of multiple alterations present in MPM should encourage research into combined or more global therapies.

1. Introduction

Over recent decades, various studies have been conducted to define the molecular characteristics of malignant mesothelioma (MM) cells. Genome-wide array-based approaches have allowed progress in MM research by identifying changes at the genetic and epigenetic levels. Genetic and epigenetic abnormalities have been investigated by identification of gene mutations, copy number changes, DNA methylation, and gene and miRNA expression profiling. The development of biological resources, frozen tissue and serum banks, tissue arrays and virtual banks, has also provided efficient tools to characterize MM cells and identify various types of tissue and serum markers. Reviews on genomic abnormalities and signal transduction dysregulation have been previously published [1-3]. The goal of this paper is to summarize the molecular changes in MM, focusing on more recent advances in malignant pleural mesothelioma (MPM), and discussing the level of confidence and limitations of these results, their impact on prognosis and treatment and the future research that is required to fill the gaps and enhance the benefit of basic research to improve the patient's outcome.

2. Genomic and epigenetic changes in mesothelioma

Genomic and epigenetic changes of potential interest for MPM histology, diagnosis and prognosis are described in **Table 1**.

2.1. Chromosomal alterations

Genomic alterations in human MPM have been previously reported in numerous studies based on various methods: cytogenetic analysis of standard karyotype, classical comparative genomic hybridization (CGH), CGH array, single nucleotide polymorphism (SNP) array and representational oligonucleotide microarray analysis (ROMA). Cytogenetic studies first demonstrated that numerous chromosomal abnormalities are associated with MPM, including both various structural and numerical changes, and recurrent alterations [4-5]. These earlier studies have already been reviewed in detail [1-2]. **Table 2** shows the recurrent regions of chromosomal alterations reported in recent studies using high-throughput analyses. MPM cell cultures and primary tumors both share similar patterns of chromosomal alterations. However, the frequency of alterations in some particular chromosomal

regions is generally higher in cultured cells, likely due to the presence of normal cells in tumor samples as mentioned by several authors. Losses of chromosomal regions are always more common than gains. Frequent losses are localized on chromosomes 1p, 3p, 4q, 6q, 9p, 13q, 14q, and 22q and gains involve chromosomes 1q, 5p, 7p, 8q and 17q [6-12]. A recent large-scale analysis of gene mutations based on second-generation sequencing in one tumor specimen confirmed the presence of numerous DNA rearrangements in MPM [13].

Chromosomal alterations and clinicopathological features. Differences in genomic alterations have been described in MPM according to the histological subtype or the patient's asbestos exposure status. Although recurrent regions of chromosomal alterations are roughly similar between epithelioid and sarcomatoid MPM, significant differences in the frequency of genomic alterations have been observed, such as losses in chromosomal regions 3p14-p21, 8p12-pter, and 17p12–pter or gain in 7q [6]. Experimental studies have shown that asbestos fibers induce chromosomal abnormalities in normal human mesothelial cells [14-15]. Significant correlations have been described between high contents of asbestos fibers in lung tissue and partial or total losses of chromosomes 1, 4 and 9, and chromosomal rearrangements involving a breakpoint at 1p11-p22 [16-17]. More recently, comparison between recurrent altered regions in asbestos-exposed and non-exposed patients showed a significant difference in the 14q11.2-q21 region, which was also lost in fiber-induced murine mesothelioma [12].

Chromosomal alterations and diagnosis. None of the individual genomic aberrations observed are specific for MPM, as they are also found in other types of tumors. However, some of these genomic aberrations could be used to distinguish benign mesothelial proliferations from MPM. This is the case for the deletion involving the 9p21.3 locus, the site of the cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*) which is one of the most frequent alterations in MPM and is often homozygous. Detection of *CDKN2A* deletion by fluorescence in-situ hybridization (FISH) has therefore been evaluated for the diagnosis of MPM [18-20]. CGH analysis has also been used in an attempt to distinguish MPM from adenocarcinoma and large-cell anaplastic carcinoma of the lung. The frequency of several genomic alterations can be used to differentiate mesothelioma from lung carcinoma with a sensitivity and specificity of 89% and 63%, respectively [21]. It has also been suggested that CGH analysis could be useful to distinguish sarcomatoid MPM from other types of spindle cell tumors of the pleura [22].

Chromosomal alterations and patient outcome. Correlations between patient survival and chromosomal imbalance have also been studied. Chromosome copy number and alterations of the short arm of chromosome 7 have been reported to be inversely correlated with survival [16, 23]. Univariate and multivariate analyses in a larger number of MPM showed that homozygous *CDKN2A* deletion, detected by FISH analysis, is a significant independent adverse prognostic factor [24-25]. Classification of MPM patients into two groups defined by short-term (less than 12 months) and long-term recurrence after surgery also suggested an association between 9p21.3 deletion encompassing the *CDKN2A* locus and the short-term group [9]. In the same ROMA analysis, chromosomal instability corresponding to the number of genomic alterations was shown to be higher in MPM patients characterized by a shorter time to relapse [9]. In deciduoid MPM, a variant of epithelioid MPM, survival was also found to be longer in patients with a smaller number of losses [26]. Interestingly, a correlation was demonstrated between chromosomal instability and tumorigenicity of human mesothelioma xenografts in nude mice [12]. These data indicate a correlation between the number of genomic alterations and the aggressive behavior of MPM and further studies are needed to determine whether chromosomal instability can be used as a prognostic factor.

Data on chromosome imbalance could also be useful to design new treatment strategies: a relevant example targets the methylthioadenosine phosphorylase (*MTAP*) gene. Homozygous co-deletion of the *MTAP* gene and the *CDKN2A* gene has been observed in the majority of pleural mesotheliomas [24]. The *MTAP* gene is a key enzyme in the salvage pathway of AMP synthesis complementary of the *de novo* purine biosynthesis pathway. Inhibitors of *de novo* purine biosynthesis induced selective killing of *MTAP*-negative cells in culture [27]. One clinical trial on MPM using L-alanosine showed that this inhibitor was ineffective at the dose used [28]. Further studies are necessary to conclude on the value of this treatment strategy.

Genomic alteration studies have already contributed to our knowledge on the mechanisms of mesothelial carcinogenesis, especially by identifying or confirming the involvement of tumor suppressor genes (TSG) such as *CDKN2A* in MPM. They have also identified potential markers for diagnosis, prognosis and treatment. New genes of interest could be identified by using technologies providing more precise localization of altered chromosomal regions and, especially, by performing

integrated mining of genomic data linked with epigenetic, miRNA profiling, and transcriptomic data in the same cultured cells or primary tumors.

2.2. DNA methylation

Numerous genes have been shown to be downregulated in mesothelioma cells by epigenetic regulation such as DNA methylation of their transcriptional promoters. These changes dysregulate several signaling pathways, including the Wnt pathway, in which several negative regulators are silenced by hypermethylation [29-32]. The global epigenetic profile determined by high-throughput methylation analysis differs between MPM and normal pleura indicating that MPM, like other cancers, have aberrant CpG island methylation [33-34]. Gene profiles of hypermethylation also differ between MPM and other tumors [33-37]. These data support the hypothesis that a specific DNA methylation program is induced during mesothelial carcinogenesis.

DNA methylation and clinicopathological features. DNA methylation of gene loci in MPM is dependent on age, ethnic origin, histological subtype and asbestos exposure and could explain discrepancies between the frequencies of DNA methylation in published studies as well as the experimental method used to detect it. Age-dependent changes in DNA methylation have been reported in the literature [38]. An age-associated increase of DNA methylation has been reported in MPM patients [39]. The methylation status of the insulin growth factor binding protein *IGFBP2* and bone morphogenetic protein *GDF10* loci has also been shown to be significantly higher in MPM from Japanese patients than in USA patients [40-41].

The frequencies of DNA methylation of TRAIL receptors (*TNFRSF10C* and *TNFRSF10D*) and tumor suppressor *RASSF1* have been reported to be significantly higher in epithelioid MPM than in sarcomatoid MPM histological subtypes [35, 42]. These data were not confirmed in another study for *RASSF1*, but methylation of another gene, *MT2A*, encoding heavy metal binding protein was shown to differ between these two histological subtypes [43]. High-throughput methylation analysis showed that epithelioid and sarcomatoid mesotheliomas had differential methylation at 87 CpG loci [44].

A significant association between asbestos exposure and DNA methylation at the *MT1A*, and *MT2A* gene loci has also been described in MPM [43]. Methylation of TSG loci, *APC*, *CCND2*, *CDKN2A*, *CDKN2B*, *HPPBP1* and *RASSF1* was studied in comparison with asbestos exposure. Only DNA

methylation at the *RASSF1* locus was correlated with an increased number of asbestos bodies in the patient's lung. A trend towards an increasing number of methylated cell cycle control genes and increasing asbestos body counts was also observed [39]. Recently, high-throughput methylation analysis confirmed distinct methylation profiles between MPM from asbestos-exposed and non-exposed patients and a significant positive association between asbestos fiber burden and methylation status of *CDKN2A*, *CDKN2B*, *RASSF1* and *MT1A* in about one hundred other loci [33].

DNA methylation and diagnosis. DNA methylation could be useful for the diagnosis of MPM. Differences in the frequency of DNA methylation have been described for several genes between MPM and lung adenocarcinoma or non-malignant pulmonary tissues [35-36, 43]. High-throughput methylation analysis covering several thousand CpG islands confirmed the potential value of DNA methylation profile to distinguish MPM from these two other tissues. Accurate diagnosis could be based on the global methylation profile, but further studies on larger populations are needed before using a limited number of hypermethylated loci [33-34, 44]. It was recently suggested that DNA methylation at the three loci *TMEM30B*, *KAZALD1* and *MAPK13*, could be useful in the differential diagnosis of MPM [34].

DNA methylation and patient outcome. DNA methylation status of individual genes such as the transcriptional repressor *HIC1*, the pro-apoptotic protein *PYCARD*, the tumor suppressor *LZTS1* and the transporter *SLC6A20* has been associated with either a good or poor prognosis [43, 45]. High-throughput methylation analysis showed that patients with MPM with a low frequency of DNA methylation had a significantly longer survival [34]. Furthermore, classification based on the methylation profile of patients undergoing surgical resection before any other treatment identified subgroups characterized by different clinical outcomes [33]. These data highlight the potential prognostic value of DNA methylation analysis.

In view of the aberrant epigenetic events observed in MPM, the clinical value of histone deacetylase inhibitors (HDACi) has been studied in preclinical models using MPM cell lines and mouse xenograft models. Phase I and II clinical trials in patients with MPM have been conducted using several different HDACi, either alone or in combination with conventional chemotherapy. The encouraging results of

these early-phase trials led to a phase III, multicenter, randomized, placebo-controlled study of one of these HDACi in patients with advanced MPM [46].

Like chromosome imbalance studies, epigenetic analyses identified genes or pathways potentially involved in mesothelial carcinogenesis such as the Wnt pathway. At the present time, only the global methylation profile appears to be relevant for diagnosis or to evaluate the patient's survival, thereby limiting its clinical applications. Furthermore, epigenetic regulation mechanisms in MPM have been mainly studied in terms of DNA methylation, but insufficient data are available on regulation of histone modifications, despite their crucial role to maintain chromatin stability. Such data are necessary to support clinical trials based on HDACi.

2.3. miRNA expression

Micro-RNAs (miRNAs) are emerging as key players in the control of a multitude of biological processes and are aberrantly expressed in several tumors including MPM. MiRNA expression has been shown to differ between MPM tumors and normal pleura [47] and between MPM cell lines and immortalized mesothelial cells [48]. MPM histological subtypes also demonstrate a specific miRNA expression pattern [47-48]. Potential targets of these deregulated miRNAs include TSGs, oncogenes and genes involved in specific signaling pathways [47, 49]. However, a link between miRNA expression and mesothelial carcinogenesis has been demonstrated by experimental analysis for only miR-31 and miR-29c. MiR-31 is frequently lost in MPM due to its chromosomal location at 9p21.3, and miR-29c expression is higher in epithelial MPM of patients with a good prognosis (time to progression greater than one year). Overexpression induced by transfection of these two miRNAs decreased *in vitro* proliferation, migration, invasion, and colony formation of the same two MPM cell lines [50-51].

MiRNA and diagnosis. MiRNAs have been proposed as diagnostic tools. Downregulation of seven miRNAs (miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205 and miR-429) was shown to be characteristic of MPM regardless of their histological subtypes, and could be used to distinguish MPM from adenocarcinoma [49]. Another study demonstrated that the selection of three miRNAs (miR-193, miR-200c and miR-192) could distinguish MPM from various carcinomas invading the lung and pleura [52].

MiRNA and patient outcome. Some recent data suggest that miRNA expression could also be used as prognostic tools, as downregulation of both miR-17 and miR-30c in sarcomatoid MPM and upregulation of miR-29c in epithelioid MPM were significantly associated with better patient survival [48, 51].

MiRNA expression analysis is a promising tool to improve the accuracy of diagnosis and could be complementary to immunohistochemical markers. This analysis also opens up new perspectives for the prognostic assessment of MPM in the near future. However, a better knowledge of miRNA signatures of MPM is still necessary, as certain discrepancies have been observed between miRNA profiling studies. Functional studies in cultured cells and animal models are also needed to determine the precise contribution of miRNAs to mesothelial carcinogenesis and whether or not they can be used as potential targets for anticancer therapy.

3. Molecular changes in malignant mesothelioma

3.1. Gene mutations

Knowledge of gene mutations provides insight into specific mechanistic pathways that can be altered in MPM cells, opening the way for future targeted therapies. A number of genes are known to be recurrently mutated in MM.

TP53. The *TP53* gene, a TSG located at 17p13.1 that controls cell cycle and apoptosis, is mutated in many types of human cancers. Its mutation frequency is about 20% in human MPM, a fairly low rate in comparison with other human cancers [3]. Point mutations are the main types of alterations in MM. Six point mutations are indicated in the IARC p53 database, five missense mutations and one stop mutation (http://p53.free.fr/Database/p53_database.html). In a study conducted to determine the frequency of simian virus 40 (SV40) in Egyptian MM patients, altered p53 and pRb expressions were found in 57.5% and 52.5% of patients, respectively, with no p53 mutation [53]. These authors assessed the prognostic impact of altered expression of *RB1* and *TP53* gene status. Univariate analysis showed a significant correlation between overall survival and p53 overexpression ($P = 0.05$). Although debated, SV40 has been associated with MM, and is assumed to act as a cofactor of asbestos in carcinogenesis. In some MM, p53 protein function could be inactivated after binding to the Large T

(Tag) SV40 protein, but SV40Tag expression in MM remains controversial [54]. In a recent study, no expression of SV40-specific miRNA was detected in human malignant pleural mesothelioma (MM) samples [55].

No relationship has yet been established between *TP53* mutation and clinical impact. The uncertainties concerning p53 status in MPM appear to make it difficult to establish relationships between p53 status and prognosis and/or treatment.

Neurofibromatosis 2 (*NF2*). The *NF2* TSG, located on 22q12 was one of the first TSGs shown to be inactivated in MPM [56-57]. Early conventional cytogenetic studies reported a loss of chromosome 22 in human MM [58-59]. *NF2* inactivation is frequent, with rates ranging from 20 to 60% depending on the material used, tissue or cells, and the method (classical CGH, DNA sequencing…). Various types of lesions have been described, including small and large deletions, homozygous deletions, nonsense and missense mutations. The role of *NF2* in mesothelial carcinogenesis will be described in the paragraph on the hippo pathway.

INK4 locus. A second recurrent gene alteration occurring in human MM consists of inactivation of genes located at the *CDKN2A* locus. The *CDKN2A* locus encodes both $p16^{INK4A}$ and $p14^{ARF}$ which share common exons, but no common amino acid sequence. Alterations at this locus have been demonstrated by DNA sequencing, FISH and methylation as reported above. The most frequent alteration is homozygous deletion in about 70% of cases [3]. This alteration is related to asbestos exposure in lung cancer and is also observed in mesotheliomas induced by mineral fibers in mice [60-61]. The *CDKN2B* gene adjacent to *CDKN2A* is also frequently codeleted in MPM, but at a lower frequency [62].

FISH detection of *CDKN2A* deletion has been proposed to differentiate between reactive and malignant mesothelial cells on paraffin-embedded sections and effusion cytology [19-20, 63]. Several authors have reported that loss of the encoded protein $p16^{INK4A}$, as assessed by immunohistochemistry and FISH analyses confirmed by gene profiling microarray studies, is associated with lower survival [25, 64-67].

CTNNB1. The beta-catenin status in MPM cells was reported in one study of 2 primary tumors and 8 cell lines in which one homozygous deletion was found in one cell line [68]. A modification of the

subcellular localization of beta-catenin was reported in another study, consistent with activation of beta-catenin as transcriptional cofactor [69].

There is now a general consensus that several TSGs are frequently altered in MPM: *NF2*, *CDKN2A*, *CDKN2B*, and, less frequently, *TP53*. In contrast, no recurrent oncogene mutation has yet been identified in MPM.

3.2. Gene expression profiling

Data from array-based studies indicate deregulation of gene expression in MPM. These studies were conducted in order to improve histological classifications and prognosis (**Tables 3-5**). These data were recently reviewed by Gray *et al.* [70].

Comparison with normal cells. Early studies were carried out with MPM cell lines compared to normal pleural mesothelial cells (**Table 3**). Using a cDNA array including 588 genes : 26 genes that play a role in signaling pathways (*MAP3K14*/*NIK*, a serine/threonine protein-kinase that stimulates NF-kappaB activity; *JAG1*/*JAGGED1* a ligand of the notch1 receptor), cell cycle (cyclin D1, *CCND1;* cyclin D3, *CCND3;* CDK phosphatase, *CDC25B)*, cell growth (fibroblast growth factor 3 and 12, *FGF3* and *FGF12;* platelet-derived growth factor receptor B, *PDGFRB*) and DNA damage repair (*XRCC5*/*Ku80*) were overexpressed, and 13 genes encoding growth factors such as *FGF1* and *FGF7* (fibroblast growth factor 1 and 7), *CCND2* (a regulatory subunit of cyclin-dependent kinases, involved in cell cycle G1/S transition), *KDR/VEGFR2* (vascular endothelial growth factor receptor 2), *PDGFRA* (platelet-derived growth factor receptor), *RARß* (retinoic acid receptor ß2) and genes encoding proteins involved in cell adhesion, motility and invasion were underexpressed [71].

Differentially expressed genes were also related to tumor invasiveness and resistance to anticancer defenses [72]. In another study, in a series of 14 differentially expressed genes, 8 were upregulated: *CFB* (complement factor B), *FTL* (ferritin light polypeptide), *IGFBP7* (insulin-like growth factor binding protein 7), *RARRES1* (retinoic acid receptor responder 1), *RARRES2* (retinoic acid receptor responder 2), *RBP1* (retinol-binding protein 1), *SAT* (spermidine/spermine N1-acetyltransferase) and *TXN* (thioredoxin), while 6 were downregulated: *ALOX5AP* (arachidonate 5-lipoxygenase-activating protein), *CLNS1A* (chloride channel nucleotide-sensitive 1A), *EIF4A2* (eukaryotic translation

initiation factor 4A2), *ELK3* (ETS-domain protein, SRF accessory protein 2), *DF2/REQ* (apoptosis response zinc finger gene), and *SYPL* (synaptophysin-like protein) [73].

The expression of 588 cancer-related genes was screened in 16 MPM tumors using normal mesothelial cell lines and pleural mesothelium as references [74]. Eleven genes, *COL1A2*, *COL6A1* (collagen), *tPA*, *MMP9* (protease), *CDH3, L1CAM, ITGB4, PLXNA3/PLXN3, KRT14/K14* (cell adhesion or cell surface molecule), *SEMA3C* (semaphorin), and *CXCL10/INP10* (chemokine), were overexpressed in MPM [74].

Microarray expression data of 40 MM tumor specimens, 4 normal lung specimens and 5 normal pleura specimens were reported by Gordon *et al.* [75]. These authors identified genes that were significantly differentially expressed in tumors compared to normal samples. There were 328 overexpressed genes and 311 underexpressed genes in MM tumors. These authors proposed 3 novel candidate oncogenes *NME2* (nucleoside diphosphate kinase), *EID1/CRI1* (regulator of EP300 and RB1) and the *PDGFC* (platelet-derived growth factor), and one candidate tumor suppressor *GSN* (cytoskeleton regulator) in MPM [75].

In another study, MM tissue specimens from 16 patients were compared to 4 control pleural tissue samples using cDNA microarray filters with 4132 clones [76]. Interestingly, upregulation of many genes involved in the glycolysis pathway and the Krebs cycle was observed, in agreement with the ability of cancer cells to rely on aerobic glycolysis, the “Warburg effect”. Other upregulated genes were involved in mRNA translation and cytoskeletal reorganization pathways. These authors also identified gp96 (adenotin, GRP94, *HSP90B1*), LRP (lung-related resistance protein, *MVP*), galectin-3 binding protein (*LGALS3BP*) and Mr 67,000 laminin receptor (*RPSA*), but this last gene was not expressed on tumor cells, but on infiltrating vessels.

More recently, Crispi *et al*. [77] compared MPM tissues from 9 patients to normal pleural tissues from patients undergoing resection for a non neoplastic disease. Components of the condensin complex (e.g. *BRRN1*, *CNAP1*, *NCAPD3*) and members of the kinesin family (e.g. *KIF14*, *KIF23*, *KIFC1*) were upregulated. Other upregulated genes were related to cell proliferation and its control such as cyclin-dependent kinase *CDK1/CDC2*, cyclin genes *CCNA2*, *CCNB1*, *CCNB2* and *CCNL2*, the *DLG7*

component of the mitotic apparatus, the checkpoint kinase involved in response to DNA damage *CHEK1/CHK1,* and *BUB1* and *MAD2L1,* components of the spindle checkpoint.

Romagnoli *et al.* [78] used a quantitative polymerase chain reaction (PCR)-based, low-density array focusing on genes involved in cell cycle regulation. They studied 45 MPM tumor samples and normal tissue samples obtained by pleural wiping of surgical samples with no evidence of pleural disease. Several genes were differentially expressed: either downregulated in cancer cells (*UBE1L, CCND2*), or upregulated (*CHEK1*/*CHK1, CCNH, CCNB1, p18-CDKN2C, CDC2, FOXM1, CDC6*). Overexpression of the cell cycle regulator Chk1 was confirmed in an independent set of 87 MM by immunohistochemistry using tissue microarrays. In another study, gene expression studies confirmed by reverse transcriptase (RT)-PCR showed downregulation of the putative TSG *FUS1/TUSC2* and the cytokine *OSM* (oncostatin M) compared to normal samples (matched normal peritoneum specimens) [9, 79]. Downregulation of *FUS1/TUSC2* and *PL6/TMEM115* was also observed in comparison with matched normal pleura specimens [9].

Features of malignant mesothelioma cells related to MM histology. Several studies have provided data on MM classification (**Table 4**). A microarray transcriptional profiling study of 10 MPM cell lines and 4 MPM primary tumor specimens distinguished epithelial, sarcomatoid and biphasic MPM. Upregulated genes included *ST14*, a gene encoding matriptase, and a membrane serine protease degrading the ECM, overexpressed in epithelial MPM [80]. In the comparative study with normal cells quoted above, *SEMA3C*, *ITGB4*, *CDH3* and *COL6A1* were highly expressed in the epithelioid MPM subtype, *L1CAM*, *K14*, *INP10* were overexpressed in the mixed MPM subtype and *MMP9* and *PLXN3* were overexpressed in the sarcomatoid MPM subtype [74]. Statistically significant distinct gene expression patterns between epithelial and non-epithelial tumors were reported to be correlated with distinctive subclasses from hierarchical clustering in a series of 40 MPM [75]. In a series of 99 tumors, genes typical of epithelial differentiation, the cell-surface transmembrane proteins uroplakins 1B and 3B (*UPK1B* and *UPK3B*) and the protease kallikrein 11 (*KLK11*) were more highly expressed in epithelioid MM [25]. Romagnoli *et al.* [78] compared epithelioid and nonepithelioid MPM using a quantitative PCR-based low-density array. Two genes were overexpressed in epithelioid MPM, the transcription factor *TFDP2* and the protooncogene *ABL1*, whereas the transcription factor *TWIST1*

was overexpressed in the nonepithelioid group. In an attempt to classify genes according to their correlation with survival, more favorable genes were associated with epithelioid morphology and unfavorable genes were associated with sarcomatoid type or epithelioid MM with poor outcome [25].

Features of malignant mesothelioma cells related to the outcome of MM patients. Other authors have tried to improve prognostic assessment by using gene expression analyses (transcriptome and/or quantitative RT-PCR) of MM primary tumors or cell lines (**Table 5**). In a study investigating mesothelioma surgical specimens, calculation of three gene expression ratios, *KIAA0977*/*GDIA1*, *L6*/*CTHBP*, and *L6*/*GDIA1* was found to be a good predictor of surgical treatment-related outcome. Samples with geometric means greater than 1 and less than 1 were assigned to good-outcome and poor-outcome groups, respectively [81]. In a cohort of 39 patients undergoing surgery, these authors validated previous findings and identified new sets of gene expression ratios *CD9*/*KIAA1199*, *CD9*/*THBD*, *DLG5*/*KIAA1199*, and *DLG5*/*THBD* allowing classification of tumors according to patient outcome [82]. In a more recent study, Gordon *et al*. [83] investigated 120 consecutive patients with malignant pleural mesothelioma treated by surgery. None of the patients had received preoperative neoadjuvant chemotherapy or radiation therapy. By analyzing data for four genes, they defined three ratios of gene expression (*TM4SF1/PKM2*, *TM4SF1/ARHDDIA*, *COBLL1/ARHDDIA*), which, associated with other prognostic factors, were able to discriminate high-risk and low-risk patients.

A cohort of 1,153 samples from patients diagnosed with 11 distinct types of cancer including 17 patients with mesothelioma from the study by Gordon *et al.* [81] was investigated by microarray analysis, looking for molecular signatures based on the polycomb group *BMI-1*-associated gene expression pathway, a pathway essential for self-renewal of hematopoietic and neural stem cells [84]. Expression of the 11-gene signature was a powerful predictor of poor prognosis in cancer patients. These 11 genes were *Gbx2*, *KI67*, *CCNB1*, *BUB1*, *KNTC2*, *USP22*, *HCFC1*, *RNF2*, *ANK3*, *FGFR2*, and *CES1* [84].

Several studies have identified specific genes associated with patient outcome. In a study comparing MM samples from patients with short-term recurrence after surgery (STR) and patients with longer time to relapse (LTR), the cadherin *CDH2* was upregulated especially in the STR group. In contrast,

the chaperone protein *DNAJA1* showed reduced expression in the STR cohort. In addition, the authors noted no discrimination between epithelial and biphasic histological types [9].

Aurora kinases A and B (*AURKA* and *AURKB*) are serine/threonine kinases that play an important role in chromosome alignment, segregation and cytokinesis during mitosis. They were found to be overexpressed in a study of 99 MPM [25]. The expression of aurora kinases and genes participating in cell division and mitotic control was further investigated in 29 MPM [85]. Expressions of *AURKA* and *AURKB* and related genes were correlated, and overexpression of *AURKB* determined by immunohistochemistry was significantly correlated with poor outcome [85].

A correlation between metalloproteinase *MMP14* expression and overall survival was reported in one study of 9 patients with MPM treated by standard thoracotomy for therapeutic purposes compared to 4 normal pleural samples [77]. High *MMP14* expression was associated with lower survival. This gene has been proposed as a potential MPM biomarker. Upregulation of *MELK* (maternal embryonic leucine zipper kinase) was associated with poor survival, confirming previous findings by Lopez-Rios *et al.* [25], but *BTG2*, which plays a role in regulation of G1/S transition, was associated with different outcomes in these 2 studies. Other genes, *BIRC5* an inhibitor of apoptosis, *KIF4A* an ATP-dependent microtubule-based motor protein and *SEPT9*, a member of the septin family involved in cytokinesis and cell cycle control, were upregulated and associated with poor prognosis [77]. In this study, a favorable survival was associated with downregulation of transcription factor *WT1* in contrast with a previous study, which associated long-term survival with upregulation of *WT1* [25].

Microarray analysis discriminated between normal and MM samples in a comparative study of 8 normal peritoneum and 7 stage I MM, subsequently validated on a large set of matched normal/MM samples by RT-PCR. Intense overexpression of *HAPLN1* (hyaluronan and proteoglycan link protein 1), a protein of the extracellular matrix (ECM), was observed in MM samples. Immunostaining with anti-HAPLN1 antibodies demonstrated that all MPM types (epithelial, mixed, and sarcomatoid) as well as reactive mesothelium expressed this gene. Moreover, *HAPLN1* expression was negatively correlated with time to progression and survival [86]. Functional studies using transfection assays revealed that MM cells overexpressing full-length *HAPLN1* or its functional domains strongly supported the protumorigenic role of *HAPLN1*.

A meta-analysis was carried out on published data on microarray analysis of gene expression profiles in mesothelioma, glioma and prostate cancer [87]. Mesothelioma data were derived from the study by Gordon *et al.* [81]. MM cases consisted of eight good responders who survived more than 17 months, while 10 patients in the poor responder group survived less than 6 months. A list of genes generated according to patient outcome showed similarities between the three types of cancers [87]. Thirteen highly expressed genes and one gene expressed at low levels were identified as being equally related to poor survival in the 3 types of cancers. These genes encode proteins of the ECM and regulators of ECM assembly, and angiogenesis genes [87]. These results are consistent with a more aggressive state of malignant cells, and a more deleterious tumor microenvironment. These results may be of interest for combining tumor-specific and more global therapies.

An analysis of 6 MPM compared to normal visceral and parietal pleural tissues has focused on differential gene expression and identification of pathways that could be related to the drug and irradiation resistance of pleural MM [88]. Several genes encoding proteins known to control DNA replication, cell cycle regulation and DNA repair were identified as over- or underexpressed in MPM could account for MPM resistance mechanism to chemotherapies [88].

These studies show changes in the expression of genes involved in several regulatory pathways. Discrimination between epithelioid and non-epithelioid MPM was reported in several studies without apparent benefit for classification of MPM subtypes in comparison with classical histological analysis. Other studies developed a gene ratio approach to predict outcome in patients having undergone surgery. No extrapolation can be made to other therapeutic settings, such as chemotherapy, can be made at the present time. Several specific genes were identified as potential predictors of patient outcome. Although providing a number of candidate areas to kill cancer cells or abolish their growth, these results need to be confirmed on a larger number of cases before proceeding to clinical applications. An important issue is to determine the most pertinent individual approach in relation to the various biological features of MM cells.

3.3. Pathway regulation

Receptor tyrosine kinases. Membrane receptor tyrosine kinases (RTKs) drive downstream cell signaling to cell proliferation and cell cycle control, survival and differentiation [89]. Downstream

networks from RTKs can be activated by RTK mutation or sustained signaling by autocrine or paracrine mechanisms, providing a useful context to therapeutically counter the effects of RTK activation.

Epidermal growth factor receptor (*EGFR*) is generally not mutated in human MPM. However, in an immunohistochemical study, EGFR was expressed in 44% of MPM cases [90]. EGFR protein status was statistically significantly associated with a favorable prognosis, but was not an independent prognostic factor, when compared to clinicopathological status [90]. A tissue array study was performed on epithelioid tissue samples from 48 MPM cases for comparison between long-term survival and short-term survival, associated with the expression of other proteins involved in the corresponding pathway [91]. A relationship was found between EGFR expression and long-term survival, whereas PDGFR signaling was more strongly associated with short-term survival [91]. In contrast, no relationship was found between survival and EGFR protein or mRNA expression [92].

EGFR alteration cannot be considered to be critical in MPM at the present time, which could explain why, although high EGFR expression is found in MPM, EGFR inhibitors, gefitinib and erlotinib, did not induce any significant tumor response when applied in phase II studies in patients with MPM [93]. Response rates were situated between 0 and 4% and median overall survival was between 4.6 and 13.1 months in phase II trials including patients with either first-line chemotherapy failure or no previous treatment [94-96].

KIT/CD117 encodes a stem cell factor receptor. In MPM, *KIT* expression has mostly been studied by immunohistochemistry, showing a low percentage of positive tumors [97]. No expression was detected by RT-PCR in a study of 37 MPM [98]. *KIT* has not been shown to be characteristic of MPM at the present time.

Vascular endothelial growth factor receptors (VEGFRs). Several immunohistochemical studies demonstrated an enhanced expression of vascular endothelial growth factor (VEGF) in a large proportion of MPM in comparison with non neoplastic specimens [99]. Contradictory results were found regarding the correlation between VEGF expression and survival. VEGF was not identified as a prognostic factor in studies of 52 and 37 MPM specimens, respectively [100-101]. In contrast, in a study of 40 MPM tissues, VEGF showed significant correlation with short survival, and was an independent

prognostic factor [102]. MPM cells express both VEGF and VEGFRs (fms-related tyrosine kinases, *FLT1* and *FLT4*) and fetal liver kinase (*KDR/FLK1*) [103-106]. An autocrine role of VEGF has been suggested, using neutralizing antibodies against VEGF or the VEGFR, or antisense oligonucleotides against VEGF that significantly reduced MM cellular proliferation [105, 107]. VEGF expression can be regulated by lipoxygenases. Human MPM cells, but not normal mesothelial cells, express a catalytically active 5-LO (arachidonate 5-lipoxygenase). A 5-LO antisense oligonucleotide potently and time-dependently reduced VEGF mRNA and constitutive VEGF accumulation in the conditioned media of MPM cells [108]. These results indicate that VEGF may have multiple effects, as a key regulator of MM growth via activation of its tyrosine kinase receptors, and as promoter of tumor angiogenesis.

Despite unsuccessful early trials of anti-VEGF therapy, numerous clinical trials are testing the benefit of VEGF inhibitors in combination with chemotherapy [95, 109].

Platelet-derived growth factor receptors (PDGFRs). MM cell growth may be linked to autocrine or paracrine stimulation by platelet-derived growth factor (PDGF), and the regulation by PDGF appears to be complex in MM cells. Normal human mesothelial cells express low levels of PDGF-A mRNA chain and the PDGF-B mRNA was not detectable [110]. These cells express PDGFR-A mRNA and protein and had weak to undetectable levels of the PDGFR-B mRNA and protein [111]. In contrast, human MM cells express high level of PDGF-A and PDGF-B, as well as PDGFR-B [110-111]. However, expression of PDGFR-B is controversial and weak to undetectable levels were reported [110-113]. Nevertheless, an autocrine proliferation can be suggested in MM, as it may occur via binding of homodimer of PDGF-B chains [114]. PDGF has been suggested as a regulatory factor for proliferation of MM cells, either directly, or indirectly via the hyaluronan/CD44 pathway. Hyaluronan is an important constituent of the extracellular matrix. PDGF-BB-stimulated normal human mesothelial cells express both hyaluronan synthase and hyaluronan [115-116].

PDGF-A-stimulated autocrine loop does not seem to play a positive role in mesothelioma proliferation *in vitro*, but nude mice injected with MM cells that over-express PDGF-A showed increased tumor incidence and reduced latency period to tumor formation [117-118]. These data suggest that PDGF-A could contribute to tumor formation via a paracrine mechanism to generate favorable environmental conditions, e.g. by stimulating angiogenesis, for tumor proliferation [118].

Like EGFR targeted therapy, the PDGFRs inhibitor, imatinib mesylate, was ineffective in clinical trials [119-120].

Insulin growth factor receptors (IGFRs). Human MM cells express IGF and IGFR [121]. IGF-I appears to function as an autocrine growth stimulus in human mesothelial cells [122]. When activated, IGFR phosphorylates multiple classes of signal transduction adaptators, including insulin receptor substrates (IRS). IRS-1 was found to induce cell proliferation in response to IGF-1, whereas cell migration was induced by IRS-2 [123]. In addition, various members of the insulin-like growth factor binding protein (IGFBP) family have been investigated in MPM. IGFBPs form a complex with IGFR subunit and IGF, and have been shown to either inhibit or stimulate the growth promoting effect of IGF. IGFBPs can be either expressed or unexpressed in MM, modulating the aggressiveness of the MM phenotype [121, 124-125].

Hepatocyte growth factor receptor (*MET*) is a proto-oncogene. Mutation in the *MET* gene appears to be uncommon in MPM. No mutation was reported in a study of 20 cell lines [126], but 5 point mutations and one deletion were identified in a series of 43 primary tumors and 7 cell lines [127]. The encoded protein is involved in pathways regulating development, cell growth and survival, motility and invasion. It is expressed in most MPM and in reactive mesothelium but not in normal mesothelial cells [128-129]. Hepatocyte growth factor/Scattering factor (HGF/SF), the related Met ligand, is also expressed in some but not all MPM cells. *In vitro* stimulation of MPM cells by HGF/SF increased spreading, motility and/or invasiveness, but these effects were dependent on the cell line [127, 130-131]. Experimental studies with cultured MPM cells demonstrated that inhibition of MET by RNA interference or protein kinase inhibitor resulted in G1/S arrest and reduction of the activity of Akt and Erk1/2 signaling in some cell lines [127, 131]. However, no correlation was found between levels of MET and ERK1/2 phosphorylation [126]. In the light of these results showing a tumor-dependent activation of HGF/MET signaling, HGF/MET status could define various MPM subclasses.

The activation status of MET and other RTKs, EGFR family (Erb1, Erb2, Erb3), PDGF−A and PDGFR−B was investigated in 20 MPM cell lines and 23 primary specimens of MPM, and the effect of MET-specific inhibitors (MET-shRNA interference vector and RTK inhibitors) was investigated on

cell lines [126]. The results showed that inhibition of single RTK was not sufficient to obtain a tumor suppressor effect but that inhibition of multiple RTK should be considered [126].

MAPK. As several RTK receptors are tyrosine-phosphorylated in some MM, downstream activation of the MAPK (mitogen-activated protein kinase) proliferation-associated signaling pathway is likely. Several studies have investigated phosphorylation of proteins of the MAPK cascade, extracellular-regulated kinases (ERKs), Jun amino-terminal kinases/stress-activated kinases (JNKs/SAPKs), and p38 MAPK. Other studies have tried to modulate MAPK pathways in order to inhibit cell survival and induce apoptosis.

Phospho-ERK expression was studied by immunohistochemistry in 50 biopsy specimens including non-small-cell lung cancer and normal lung, and pleural tissue comprising 10 MPM (6 epithelioid, 1 sarcomatoid and 3 biphasic) [132]. MPM showed significant ERK phosphorylation compared to lung cancer and normal tissues [132]. Activation of ERK, JNK, and p38 MAPK was investigated in 28 MPM and 8 peritoneal MM (32 effusions and 4 biopsies) and 14 samples of reactive mesothelium by assessing the expression of phosphorylated proteins by immunohistochemistry and western blot. MAPK activation did not differentiate between benign and malignant mesothelial cells [133]. The authors argued against a major role for this pathway in the malignant transformation of mesothelial cells. They also noted that MAPK expression and phosphorylation were better predictive factors of outcome, in agreement with data obtained in ovarian cancer [133]. Arsenic trioxide (As_2O_3) is a chemical compound that has been reported to inhibit cell proliferation and induce apoptosis in tumor cells via the MAPK pathways. As_2O_3 inhibited proliferation and induced apoptosis in one mesothelioma cell line [134]. As_2O_3 did not alter phosphorylation of either Akt or Src, while ERK1/2 and JNK1/2, but not p38 MAPK, were markedly phosphorylated after As_2O_3 treatment, indicating the involvement of the JNK-dependent ERK-dependent pathway in the cell response [134]. However, p38 MAPK appears to be involved in the response to TGF-beta. In 6 human MM cell lines, migration and invasion linked to the production of metalloproteinases were stimulated by TGF-beta1 via phosphorylation of p38 MAP kinase. The authors suggested that this pathway could be targeted to reduce mesothelioma progression [113]. Ou *et al.* [135] determined the relative levels of tyrosine phosphorylation of 42 distinct RTKs in

mesothelioma cell lines established from surgical specimens and found coordinated activation of RTKs EGFR, ERBB3, AXL and MET. As MAPK can be activated by heat shock proteins (HSP), these authors studied the effect of HSP90 inhibition on ERK1/2 activation. HSP90 inhibition reduced TKs phosphorylation and induced apoptosis [135]. The effect of other HSPs was also investigated in the context of the possible use of hyperthermic chemotherapy [136]. HSP40 was upregulated in response to heat stress, associated with activation of the ERK1/2 and p38 pathways in a study of 3 MPM cell lines, suggesting that treatment could be more effective by blocking these pathways [136]. HSP90 overexpression has been reported in MPM [88], and *DNAJA1*, a member of the HSP40 family, showed decreased expression in MPM with short-term recurrence of the disease [9].

These results show that regulation of mesothelioma cells via MAPK pathways is complex. Targeting these pathways to abolish cell proliferation could be proposed, but the treatment strategy would be difficult to define at the present time. MAPK activation is important for cell survival and can also be linked to apoptosis events. More specific investigations taking into account specific tumor characteristics and microenvironment must be conducted in order to trigger cell growth inhibition and apoptosis.

PI3K/AKT. Constitutive activation of RTKs in MM results in downstream signaling cascades including phosphatidylinositol-3-kinase (PI3K-AKT), a cascade regulating cell growth processes, cell migration and apoptosis. Phosphorylation of AKT protein, the active form of the protein, has been demonstrated in MM cells. Immunohistochemical analysis revealed elevated levels of phospho-AKT in nearly two-thirds of human primary MPM. A strong association with elevated phospho-mTOR positivity in the same tumors confirmed activation of the Akt pathway [137]. Activation of AKT triggers anti-apoptotic mechanisms. However, while the PI3K-Akt signaling pathway was activated in adherent MPM cells, loss of anchorage resulted in inactivation of this pathway and failed to restore apoptosis [138]. Inactivation of PTEN (phosphatase and tensin homolog deleted from chromosome 10), a TSG and negative regulator of the PI3K-AKT pathway, could account for PI3K-AKT activation. PTEN homozygous deletion has been reported in a small subset of MPM cell lines [139-140]. A tissue microarray-based study carried out on 206 tumor tissues demonstrated that loss of PTEN expression was observed in 62% of cases [141]. In this study, PTEN expression was correlated with better survival

554 from data available in 129 patients. PTEN was an independent prognostic biomarker in mesothelioma
555 patients [141].

556 ***Wnt pathway.*** The Wnt signaling pathway regulates developmental processes, cell proliferation and
557 cell polarity. It is driven by membrane protein activation involving low-density lipoprotein receptor-
558 related protein (LRP) and Frizzled, and G-protein-coupled receptors. Activation of the Wnt signaling
559 pathway prevents beta-catenin phosphorylation and its subsequent ubiquitination and degradation.
560 Beta-catenin plays a central role in the Wnt pathway activity, as beta-catenin can act as a coactivator
561 of transcription, allowing the expression of a variety of genes exerting pleiotropic effects [142]. While no
562 recurrent mutation of beta-catenin has been described in MPM, the Wnt pathway could be altered as a
563 result of promoter hypermethylation of regulatory genes [29, 31-32]. Apart from this canonical Wnt/beta-
564 catenin pathway, a non-canonical beta-catenin-independent Wnt pathway can also transduce signals in
565 MPM cells. This was demonstrated in beta-catenin-deficient MPM cells, in which inhibition of Wnt
566 signaling produced growth reduction and apoptosis [30, 143].
567 Gene expression profiling of MM cell lines, primary MPM tumors and normal pleural tissue has been
568 studied by using a custom array designed to profile the expression of genes involved in the Wnt
569 signaling pathway and downstream to Wnt signaling [144]. In the sixteen matched samples (malignant
570 tissue and normal adjacent pleura) investigated, numerous Wnt genes (*WNT1*, *WNT2*, *WNT5*) and
571 Wnt-related genes (*MYC*, *CCND1*, *JUN*) were upregulated. *WNT2* was most frequently upregulated.
572 In contrast, *WNT8A* and some WNT antagonists (*DKK1*, *SFRP2* and *SFRP4*) were downregulated. A
573 role of *WNT2* in cell survival was demonstrated using anti-Wnt2 antibody and Wnt2 siRNA,
574 associated with inhibition of the downstream effectors of the Wnt pathway [144]. Wnt signaling
575 inhibition is dependent on several factors including the Dickkopf (*DKK*) gene family. One member,
576 *REIC/Dickkopf-3*, is downregulated in numerous human cancers [145]. In four human MM cell lines,
577 *REIC/Dickkopf-3* expression was lower than in normal tissue, and overexpression by transduction in
578 one cell line induced apoptosis via a JNK-dependent pathway [145]. Moreover, a preclinical study
579 consisting of orthotopic inoculation of *REIC/Dickkopf-3*-deficient luciferase-labeled MM cells
580 followed by intrapleural injection of recombinant *REIC/Dickkopf-3*-adenovirus resulted in a strong
581 antitumor effect [145]. These results suggest that deregulation of the Wnt signaling pathway can be

involved in mesothelial carcinogenesis, and that identification of key targets could be of interest to suppress tumor development.

Hippo pathway. Merlin, the protein encoded by NF2, regulates cell growth by signaling via the Hippo pathway to inhibit the function of the transcriptional coactivator and candidate oncogene *YAP1* via its phosphorylation. Overexpression of *YAP1* was found in one MM cell line [146]. Moreover, Yap1 protein physically and functionally interacted with merlin and, in *NF2*-transfected cells, merlin expression reduced the nuclear localization of Yap1, suggesting that merlin can inhibit Yap1 function by sequestration [146]. Inactivating homozygous deletions or mutations of *LATS2* were recently demonstrated by CGH and DNA sequencing analyses in about 22% of MPM including 20 cell lines and 25 primary tumors [147]. Disruption of *NF2* signaling plays a major role in the development of MPM in view of the high rate of mutations in this tumor. Despite a wild-type status of *NF2*, the merlin also appears to be present in an inactivated phosphorylated form in MPM cells [148]. Recent data suggest that the Hippo pathway involving the merlin could be targeted for treatment strategies. There is now a general consensus concerning inactivation of the Hippo pathway in MPM. To the best of our knowledge, *NF2* expression has not been associated with any specific MPM subtype or specific characteristics and has not been linked to prognosis. Investigation of merlin function in MPM could be useful to develop new therapies. Some examples have been published in the literature. Using *NF2*-negative MM cell lines transduced with a recombinant *NF2* Adenovirus (Ad*NF2*), cDNA microarray analyses revealed differences in gene expression profiles characterized by a decrease in cyclin D1 (*CCND1*) expression, a gene upregulated in MPM, in cells transduced with Ad*NF2* compared to those transduced with the control adenovirus. In parallel, CDK4, the catalytic partner of cyclin D1, was inactivated and pRb was dephosphorylated, in agreement with efficient control of the G1/S transition in NF2-expressing cells. G1 cell cycle arrest was confirmed by cell cycle analysis [149]. In this study, the authors found that the effect of *NF2* was related to repression of cyclin-D1 promoter activity via PAK1 inhibition [149]. *NF2* function could also be related to regulation of motility and invasiveness in MM cells, as demonstrated by downregulation of focal adhesion kinase (FAK), and inhibition of motility and invasiveness following *NF2*-transfection and overexpression of FAK in 2 *NF2*-deficient mesothelioma cell lines [150]. A relationship between NF2 expression and apoptosis in MM cells has

been reported in other studies. In a study on the role of integrin-specific signaling in the control of apoptosis factors, NF2 was demonstrated to have an inactivating role on integrin-dependent mTORC1 signaling [151]. In this study, eleven MM cell lines were analyzed, four not expressing merlin and 7 expressing merlin, for their activity in mTORC1, ERK, and AKT. While activation of ERK or AKT was not correlated with the loss of merlin or activation of mTORC1, inactivation of merlin promoted mTORC1 signaling independently of AKT or ERK [151].

Ubiquitin-proteasome. Differences of the expression of genes involved in the ubiquitin/proteasome pathway have been observed between MM and normal tissue or according to histological subtype. Several genes encoding proteasome complex subunits were upregulated in MPM tumors compared to normal parietal pleura [88]. Others proteins involved in the ubiquitin/proteasome pathway, such as the FAS-associated factor *FAF1* which inhibits protein degradation of ubiquitinylated proteins, were recurrently altered at the genomic level in MM of $p19^{ARF}$ (+/-) mice and were downregulated in human MM [152-153]. In peritoneal MM, several genes involved in the ubiquitin-proteasome pathway were upregulated in biphasic tumors compared to epithelioid tumors [154]. In pleural MM, subunits of the proteasome complex (*PSME3*, *PSMA3* and *PSMA4*) and ubiquitin-conjugating enzyme (*UBE2S*) were upregulated in the epithelioid phenotype variant compared to the sarcomatoid phenotype variant of the same MPM cell lines [155].

Several studies have analyzed the impact of proteasome inhibitors on MPM malignancy in preclinical models. Bortezomib (PS-341 or Velcade®), a specific inhibitor of 20S proteasome activity, induces *in vitro* apoptosis and *in vivo* tumor growth inhibition in mice of one MPM cell line [156]. Other proteasome inhibitors, PSI or MG-132, were also shown to induce apoptosis in some MPM cell lines [157-158]. Using MPM cell lines in monolayer culture, bortezomib was shown to increase the cytotoxicity of chemotherapeutic agents [159]. However, MM cell lines, when grown as multicellular spheroids, acquired resistance to apoptosis induced by a combination of the proteasome inhibitor MG-132 and other apoptotic stimuli [160]. Results of ongoing phase II clinical trials using bortezomib combined with cisplatin will indicate the efficacy of proteasome inhibitors in the management of MM (ClinicalTrials.gov Identifier: NCT00458913).

Cell cycle regulation.

Alteration of genes located at the *INK4* locus, encompassing *CDKN2A* and *CDKN2B*, is a feature of human MM. Inactivation of these genes allows uncontrolled cell proliferation. While some MM do not show mutation or methylation of these genes, another level of regulation could occur via deregulation of miRNA expression (see above, chapter 2.3). Several authors have developed experimental studies trying to restore cell cycle control in MM by adenovirus-mediated expression of $p16^{INK4A}$ and $p14^{ARF}$ in human MM cells, and found effects on both cell cycle progression and reduction of tumor growth in immunocompromised mice [161-163].

Cell cycle control can be affected in MM cells by the loss of other negative regulators, CDK (cyclin–dependent kinases) inhibitors or by the overexpression of CDKs and cyclins (CCNs), and regulators of the mitotic checkpoints [85, 88]. The expression profile of 60 genes involved in cell cycle has been investigated in forty-five MM tumor samples and normal pleural tissue [78]. Among genes overexpressed in MM, several were involved in cell cycle checkpoints such as CDK1/CDC2 (cyclin-dependent kinase 1), CDC6 (cell division cycle 6, a regulator of replication), CDKN2C (cyclin-dependent kinase inhibitor 2C, p18), CCNH (cyclin H), CCNB1 (cyclin B1, controling the cell cycle at the G2/M transition), CHEK1 (Chk1 is required for checkpoint-mediated cell cycle arrest in response to DNA damage) and FOXM1 (forkhead transcription factor, a regulator of gene expression in the G2 phase). In contrast, CCND2 (cyclin D2, a regulator of Cdk4 and Cdk6, controls the cell cycle at the G1/S transition) was underexpressed [78]. Aurora kinases are involved in microtubule formation and are important regulators of the mitotic spindle checkpoint system, controlling progression of mitosis until all chromosomes are properly aligned during metaphase. An overexpression of aurora kinases has been reported in different studies [25, 85]. Aurora B levels increase after gamma irradiation, and MM cells arrest at the G2/M checkpoint of the cell cycle to repair DNA damage before proceeding through mitosis [164]. Stathmin is also important for the evolution of mitosis as it is involved in the regulation of the microtubule dynamics, by inhibiting the formation of microtubules and/or promoting their depolymerization. Kim *et al*. [165] identified potential genes involved in pathogenesis of MPM. They investigated seven MM cell lines, fresh mesothelioma tissues and adjacent normal pleural tissues using cDNA microarray chips. Multiple genes were overexpressed in MM cell lines compared to the human mesothelial cell strain LP-9 derived from the ascitic fluid of

a patient with an ovarian carcinoma, and stathmin was one of the most strongly overexpressed genes [165-166]. Protein expression of stathmin was observed in MPM tissues but not in matched normal pleural samples [165].

Because of these different alterations, response to DNA damage can be impaired in MPM cells entailing chromosomal instability. Well-controlled cell cycle progression is necessary for cells to respond to both endogenous and exogenous DNA damage. Although MPM cell cycle may be arrested in response to DNA damaging agents, it may be assumed that MPM cells recover, likely due to their inability to trigger the apoptotic mechanism. Moreover, a heterogeneity exists between different tumors. After exposure to gamma-radiation, human MPM cells were arrested either in one or more phases of the cell cycle, demonstrating a heterogeneity in cell cycle control. G1 arrest was p21WAF1/CIP1- and p53-dependent [167]. As mentioned in chapter 3.1 p53 can be inactivated in MPM, and its inactivation will facilitate chromosomal instability, in relation to loss of cell cycle control, especially in response to DNA damage. Regulation of p53 function occurs via post-translational mechanisms and interaction with several protein. MDM4 was recently shown to control p53 function in a human MM cell line [168].

Overall, these studies demonstrate that cell cycle dysregulation occurs in all phases, at the level of checkpoint control and related factors, encouraging the search for stimulation of death pathways in MPM cells.

Apoptosis. Malignant MM responds poorly to standard therapy [169]. Mesothelioma tissue usually has a lower apoptotic index than other carcinomas [170], suggesting major defects in the apoptotic machinery. Apoptosis is mediated by two signaling pathways, the extrinsic and intrinsic pathways. The extrinsic pathway is initiated by death receptors, while the intrinsic pathway is triggered by internal apoptotic signals and involves the release of cytochrome c from the mitochondrial intermembrane space. These two pathways merge and share mechanisms of the caspase cascades [171]. In the extrinsic pathway, the death receptor agonist TRAIL can induce apoptosis with a high specificity toward tumor cells and is currently being tested in clinical trials in a variety of human cancers. In mesothelioma, TRAIL has been to shown to enhance the chemosensitivity of tumor cells to various therapeutic agents, such as doxorubicin, gemcitabine, cis-platinum or etoposide. However, most MM cells are resistant to

694 apoptosis induced by TRAIL alone [172]. This resistance can be explained notably by overexpression of
695 the caspase-8 inhibitor, *FLIP*/*CFLAR*, and by the methylation of TRAIL receptors in MM cells [173].
696 Several multimodal approaches have subsequently been applied to sensitize MM cells to TRAIL. Heat
697 stress, as well as subtoxic doses of alpha-tocopheryl succinate or anisomycin can sensitize MM cells
698 to TRAIL and induce apoptosis *in vitro*, via Bid-dependent mitochondrial amplification of the
699 apoptotic signal [174-176]. Inversely, the multikinase inhibitor sorafenib showed synergistic effects with
700 TRAIL in cells resistant to TRAIL, independently of caspase activation [177]. Interestingly, in contrast
701 with mesothelioma cell monolayers, tumor fragment spheroids exhibit higher resistance to apoptosis
702 and notably to TRAIL-combined treatments, and this resistance is mediated by the mTor/S6K pathway
703 [160, 178]. In the intrinsic pathway, the mitochondrial membrane potential and permeability are regulated
704 by the Bcl-2 family of proteins. Members of this family include both proapoptotic proteins such as
705 Bax, Bak, Bad, Bid or Bim, and antiapoptotic proteins, such as Bcl-2, Bcl-xL and Mcl-1. Bcl-2 is
706 rarely expressed in mesothelioma [170], while high levels of Bcl-xL are commonly observed [179]. Several
707 studies have shown that downregulation of Bcl-xL could decrease baseline tumor cell viability and
708 improve sensitivity to chemotherapeutic agents, both *in vitro* and *in vivo* [180-182]. Mcl-1 has also been
709 implicated in the apoptotic resistance of mesothelioma cells [158, 179]. Recently, Varin *et al.* showed that
710 Bcl-xL and Mcl-1 cooperated to protect mesothelioma cells from cell death and that their concomitant
711 targeting was sufficient to induce apoptosis [183]. Most members of the proapoptotic Bcl-2 family appear
712 to be expressed in mesothelioma with functional integrity, suggesting that the loss of their apoptosis-
713 inducing properties is due to sequestration by Bcl-xL or Mcl-1 [184]. In particular, functional inhibition
714 of Bim contributes to survival in the spheroid model of mesothelioma cells [138].
715 The inhibitor of apoptosis protein (IAP) survivin, encoded by the *BIRC5* gene, was highly expressed
716 in all MM primary tumors (12 samples) and cell lines (7/8) compared with normal pleura [185]. Survivin
717 expression in 34 MM tumors was confirmed by immunohistochemistry and linked to an apoptotic
718 defect [170]. Downregulation of survivin with anti-survivin oligonucleotides induced apoptosis when
719 tested in one cell line [185]. Inhibition of survivin expression has been shown to decrease tumor cell
720 growth and enhance drug response [186]. XIAP is also frequently expressed in malignant mesothelioma,
721 and is notably upregulated in mesothelioma effusions and peritoneal mesothelioma [187]. Moreover,

722 XIAP inhibition has been shown to increase the sensitivity of mesothelioma cells to TRAIL-induced
723 apoptosis [188]. Together, these results suggest that combined approaches, triggering the extrinsic and
724 intrinsic pathways or the caspase cascade, are promising for the treatment of mesothelioma.
725
726 ***Telomere.*** Human telomeres progressively shorten during cell division, and critical shortening is
727 believed to limit the cellular life span, and is involved in conferring growth-promoting properties to
728 tumor cells. Telomere lengthening is due to telomerase (*TERT*) activity, which was found in a large
729 proportion of the 22 primary pleural MM and the 4 MM cell lines in comparison with mesothelial cells
730 from normal pleura using the telomeric repeat amplification protocol (TRAP) [189]. These findings were
731 confirmed in a more recent study carried out in peritoneal MM and another mechanism, alternative
732 lengthening of telomeres, was also demonstrated to maintain telomere length [190]. Interestingly, in their
733 series of 44 MM peritoneal lesions from 38 patients, these authors found that telomerase activity was a
734 significant prognostic factor for 4-year relapse and disease-free survival. Telomerase activity was
735 reduced in MM cell lines in comparison with normal cells by inhibition of *MetAP2* (methionine
736 aminopeptidase) with angiostatic agents fumagillin and ovalicin. This enzyme is overexpressed, in
737 MM cells [191].
738
739
740 **3. Conclusions**
741 Molecular studies have identified somatic genetic and epigenetic alterations in MPM cells, associated
742 with altered expression, activation or inactivation of critical genes in oncogenesis. Deregulation of
743 signaling pathways related to differentiation, survival, proliferation, apoptosis, cell cycle control,
744 metabolism, migration and invasion has been demonstrated in complementary studies. These changes
745 were found by investigating individual gene status in genomic and transcriptomic studies, and were
746 supported by immunohistological studies. MPM cells show a large spectrum of abnormalities shared
747 with other malignancies, or more specific alterations such as those of the *NF2* gene. Comparative
748 studies of series of MPM have usually demonstrated that both alterations in a given gene and
749 combined genetic and epigenetic alterations are present in MPM subsets, consistent with inter-

individual variations of molecular alterations. There are therefore at least two levels of heterogeneity, at the genome level and at the gene level, suggesting that identification of patient subgroups would be essential in order to develop more specific therapies. Moreover, the tumor microenvironment, consisting of a large number of different cell types, adds another level of complexity to identify the best strategy to improve the outcome of this disease. This tumor heterogeneity could explain differences in patient survival and response to treatments.

This review provides insight into a limited number of genes known to be frequently altered in MPM, *INK4* locus and *NF2*, and a larger number of candidates that may play a role in MPM carcinogenesis, especially those involved in various signaling pathways. Further studies should define the clustering of these genes in specific MPM subsets. These findings have already been the basis for several studies testing various targeted therapeutic approaches on specific RTKs, but mostly with limited success. Demonstration of the multiple alterations present in the tumor should encourage research into combined or more global therapies. Other studies have emphasized deregulation of signaling pathways, but no pathway seems to be specific or a particularly relevant target, as certain discrepancies have been observed concerning the response of MPM cells to specific inhibitors, and key regulatory players in one pathway may interact with another pathway. Focusing on apoptosis is probably an interesting strategy to counteract or trigger the activity of several of these pathways. More recent data have indicated the presence of alterations that could be targeted at a global level (methylation). Studies are ongoing to take advantage of these abnormalities for MPM treatment. Prediction of a positive response in MPM would avoid a rapidly unfavorable course and avoid wasting time and resources with inappropriate treatments. The critical issue concerning targeted therapy is to focus on the most relevant target(s). Some molecules, pathways and/or epigenetic changes should be selected, provided they are key factors in MPM. This is not an easy task regarding the interplay between the various regulatory pathways, and the diversity of genomic alterations. Molecular studies must be developed to identify and classify genomic alterations in MPM cells and correlate these alterations with disease outcome in order to avoid random testing of therapies already used in other cancers, but with unknown relevance in MPM. In recent years, several studies have been designed to evaluate the predictive role of microarray data for MM outcome. Various authors have developed

predictors of survival, but in some studies the accuracy was lower than that of prognosis based on the usual methods comprising clinicopathological variables and morphology. Other authors have proposed innovative predictors based on gene expression ratios. These procedures are of great interest and deserve further validation.

Our improved understanding of MPM development and treatment is partly based on well designed preclinical studies. Numerous *in vitro* investigations are currently underway to suppress MPM cell growth and/or induce apoptosis by interacting with proteins regulating proliferation and survival, or by silence gene expression (RNA interference). These methods benefit from the data provided by molecular analyses providing preclinical proof of concept for the feasibility of such strategies. However, these studies were carried out in MPM specimens that do not necessarily present the same genomic status as the tumors of patients selected for the relevant therapy. In the context of preclinical investigations, animal models must be combined with studies prior to translation to humans. An important point to be emphasized here is the paramount importance of frozen and paraffin MPM tissue banks to allow better characterization and annotation of MPM, as well as panels for diagnostic certification. Databases and panels are already available, such as the Mesothelioma Virtual Bank (http://www.mesotissue.org) [192] or the International Mesothelioma Excellence Center (IM@EC).

Over recent years, considerable methodological progress has been made in the field of molecular approaches to study cancer biology and this progress has been applied to MPM. Improvements are still in progress. Other methodologies have not yet been applied to MPM, such as proteomics, cell imaging, integrative biology and will likely be useful in the future, in order to identify MPM biomarkers, exposure markers and MPM subgroups.

Various clinical studies have shown that future treatment strategies must not be based on monotherapy, but must comprise multi-site and multimodal treatment. As this disease is particularly aggressive, it requires a specific treatment strategy. Investigation of the tumor genome and related pathophysiological events has therefore become a key step to a better understanding and possible cure of this dreadful incurable cancer.

Table 1. Genomic and epigenetic changes of potential interest for MPM histology, diagnosis and prognosis.

Genes	Significance	Reference
Diagnosis		
Chromosomal alteration	Frequency different between MPM and lung carcinoma and others splindle tumors of the pleura	21-22
DNA methylation status of specific gene loci	Frequency different between MPM and lung adenocarcinoma and non-malignant pulmonary tissue	33-36, 43-44
MiRNA expression level	Difference between MPM and lung adenocarcinoma	49
MiRNA expression level	Difference between MPM and various carcinoma	52
Histology		
Chromosomal alteration	Frequency different between epithelioid and sarcomatoid MPM	6
DNA methylation status of specific gene loci	Frequency different between between epithelioid and sarcomatoid MPM	35, 42-44
Prognosis		
Chromosomes and chromosome 7p	Inverse correlation between copy number and survival	16, 23
CDKN2A locus (9p21.3) homozygous deletion	Correlation with shorter survival or shorter time to relapse	9, 25
Number of chromosomal alteration	Correlation with shorter time to relapse	9
Number of chromosomal region loss	Correlation with shorter survival in deciduoid MPM	26
DNA methylation status of *HIC1*, *PYCARD*, *LZTS1* and *SLC6A20* gene loci	Potential association with survival	43, 45
Occurence of DNA methylation	Correlation between low frequency and longer survival	34
DNA methylation profile	Prognostic prediction depending of specific profiles	33
MiR-17 and mIR-30c	Correlation between reduced expression and better survival in sarcomatoid MPM	48
MiR-29c	Correlation between increased expression and better survival in epithelioid MPM	51

Table 2. Recurrent regions of chromosomal alterations in MPM.

Alteration	CGH (90 tumors) [6]	CGH array (17 tumors) [7]	CGH array (26 tumors) [8]	ROMA (22 tumors) [9]	SNP array (23 tumors) [10]	SNP array (22 cultured cells) [11]	CGH array (33 cultured cells) [12]
Gain	1q23-q32 (16%)	1q (44%)			1q23 (35%) 1q32 (22%)		
		5p (44%)		5p14 (55%)	5p (22%)		5p15.3-p11 (51%)
	7p14–p15 (14%)	7p (44%)			7p14-p15 (22%)		7p22-p11.2 (37%)
	8q22–q23 (18%)	8q24 (56%)		8q23-q24 (36%)	8q22-q23 (20%) 8q24 (22%)		
	15q22–q25 (14%)				15q22-q25 (17%)		
			17q21.32-q25 (27%)	17q21-q23 (24%)		17q23.2 (55%)	
				18q12.1 (36%)			
		20p (33%)			20p (9%)		
							20q11.2-q13.1 (34%)
Loss		1p36.33 (11%) 1p36.1 (33%)		1p36.22-p36.23 (36%) 1p36.11-p36.12 (55%)	1p36.1 (30%) 1p36.33 (39%)	1p36.3-p36.2 (55%)	1p36.3-p35 (51%)
	1p21 (21%)	1p21.3 (56%)	1p31.1-p13.2 (42%)	1p13.2-p13.3 (36%)	1p21.3 (30%)	1p22.3-p22.1 (82%)	1p31-p12 (40%)
	3p21 (16%)	3p21.3 (33%)	3p22.1-p14.2 (42%)	3p21.31 (27%) 3p14.3-p14.2 (32%)	3p21.3 (44%)	3p22.1-p21.31 (77%)	3p23-p14 (63%)
	4q31–q32 (29%) 4p12–p13 (25%)	4q22 (56%) 4q34-q35.2 (33%)			4p12 (26%) 4q22 (30%) 4q31-q32 (35%)	Chr4 (53%)	Chr4 (54%)
	6q22 (16%)	6q25 (44%)	6q22.1 (58%)		6q22 (26%) 6q25 (39%)		6q14-q27 (57%)
							8p23-p12 (31%)
	9p21 (34%)	9p21.3 (100%)	9p21.3 (65%)	9p21.3 (32%) 9p21.1 (36%)	9p21 (39%)	9p21.3 (100%)	9p24-q21 (91%)
				9q34.11 (41%)			
	10p13–p15 (16%)	10p (44%)			10p13 (9%)		10p15-p12 (37%)
							10q23-q26 (37%)
						11q23.2-q23.3 (64%)	
							12p13 (54%)
	13q13–q14 (19%)	13q33.2 (44%)	13q11-q14.12 (35%)		13q13-14 (17%)	13q12.2-q13.2 (73%)	13q (60%)
	14q12–q24 (23%)				14q12-q24 (22%)		14q11.2-q21 (40%)
		14q32.13 (56%)	14q22.1-32 (38%)		14q32.13 (17%)	14q32.2 (73%)	14q24-q32 (40%)
						15q15.1 (55%)	15q13-q21 (40%)
	17p13–p12 (16%)			17p13.1 (46%)	17p12 (17%)		17p13-p11.2 (34%)
				17q21.31 (32%)			
		18q (33%)			18q (13%)	18q12.3 (59%)	18q12-q23 (46%)
				19p13.2 (55%)			19p13.1-p12 (31%)
				19q13.32 (55%)			19q13.2-q13.4 (31%)
	22q (32%)	22q (33%)	22q11-q12.3 (35%)	22q12.2 (74%)	22q (43%)	Chr22 (78%)	22q (80%)

Table 3. Genes of potential interest for MPM characterization

Genes	Reference
Overexpressed in comparison with normal cells	
MAP3K14/NIK, JAG1/JAGGED1, CCND1, CCND3, CDC25B, FGF3, FGF12, PDGFRB, XRCC5/Ku80	[71]
CFB, FTL, IGFBP7, RARRES1, RARRES2, RBP1, SAT, TXN	[72]
COL1A2, COL6A1, tPA, MMP9, CDH3, L1CAM, ITGB4, PLXNA3/PLXN3, KRT14/K14, SEMA3C, CXCL10/INP10	[74]
Genes involved in glycolysis	[76]
HSP90B1, LRP, LGALS3BP	[76]
Members of the condensin complex and of the kinesin family	[77]
CDK1/CDC2, CCNA2, CCNB1, CCNB2, CCNL2, DLG7, CHEK1/CHK1, BUB1, MAD2L1	[77]
CHEK1/CHK1, CCNH, CCNB1, p18-CDKN2C, CDC2, FOXM1, CDC6	[78]
Underexpressed in comparison with normal cells	
FGF1, FGF7, CCND2, KDR/VEGFR2, RARß	[71]
ALOX5AP, CLNS1A, EIF4A2, ELK3, DF2/REQ, SYPL	[72]
UBE1L, CCND2	[78]
FUS1/TUSC2, OSM, PL6/TMEM115	[9, 79]

817 **Table 4. Genes of potential interest for characterization of MPM subtypes**
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Genes	Significance	Reference
Histology		
ST14	Overexpressed in epithelial MM in comparison with sarcomatoid and biphasic MM	[80]
SEMA3C, ITGB4, CDH3, COL6A1	Overexpressed in epithelioid MM in comparison with normal cells	[74]
L1CAM, K14, INP10	Overexpressed in biphasic MM in comparison with normal cells	[74]
MMP9, PLXN3	Overexpressed in sarcomatoid MM in comparison with normal cells	[74]
UPK1B, UPK3B, KLK11	Overexpressed in epithelioid vs non epithlioid MM	[25]
TFDP2, ABL1	Overexpressed in epithelioid vs non epithlioid MM	[78]
TWIST11	Overexpressed in non epithelioid vs epithlioid MM	[78]

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Table 5. Genes of potential interest for MPM characterization and predictive prognostic value

Genes	Significance	Reference
Patients' outcome		
P16/CDKN2A	Gene loss or no protein expression associated with low survival	[25, 64-67]
KIAA0977/GDIA1, *L6/CTHBP*, *L6/GDIA1*	Gene ratios predict outcome	[81]
CD9/*KIAA1199*, *CD9*/*THBD*, *DLG5*/*KIAA1199*, *DLG5*/*THBD*	Gene ratios predict outcome	[82]
TM4SF1/PKM2 *TM4SF1/ARHDDIA* *COBLL1/ARHDDIA*	Gene ratio discriminate high risk and low risk patients	[83]
Gbx2, *KI67*, *CCNB1*, *BUB1*, *KNTC2*, *USP22*, *HCFC1*, *RNF2*, *ANK3*, *FGFR2*, *CES1*	Expression associated with poor prognostic	[84]
CDH2	Overexpressed in the short-term recurrence group	[9]
DNAJA1	Underexpressed in the short-term recurrence group	[9]
AURKA, *AURKB*	Expression associated with poor outcome	[85]
MELK	Upregulation associated with poor survival	[25]
BIRC5, *KIF4A*, *SEPT9*	Upregulation associated with poor prognosis	[77]
HAPLN1	Expression negatively correlated with survival	[86]
DNAJA1	Underexpressed in the short-term recurrence group	[9]
MMP14	High expression associated with lower survival	[77]
LELK1	Upregulation associated with poor survival survival	[25]
Thirteen genes involved in ECM, regulators of ECM assembly, angiogenesis	High expression associated with poor survival	[87]

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