

Diagnostic and prognostic biomarkers for malignant mesothelioma: an update

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Abstract: Malignant mesothelioma (MM) is an aggressive and lethal cancer, mostly related to inhalation of asbestos and erionite fibers. MM is associated with poor prognosis, because of its resistance to current therapies, even if higher survival occurs in patients diagnosed and treated when at stage I of the disease. However, these do not exceed 5% of the total number of cases, due to the inadequacy of the existing biomarkers for early and accurate diagnosis. Therefore, new effective biomarkers are needed for MM detection at earlier stages and to develop tailored therapies. Here we review the most promising biomarkers in MM to date: mesothelin, soluble mesothelin-related peptides (SMRPs), megakaryocyte potentiating factor (MPF), Osteopontin (OPN), Fibulin-3, high mobility group B1 (HMGB1), microRNAs (miRNAs), multiplex protein signatures. The validation of these biomarkers will allow their use, alone or in combination, for monitoring individuals from cohorts at risk of MM and attaining early detection of MM that is instrumental in improving patient survival.

Keywords: Asbestos; biomarkers; diagnosis; mesothelioma; prevention

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Introduction

Malignant mesothelioma (MM), is an aggressive cancer arising from the surfaces of the pleural and peritoneal cavities, and it is predominantly caused by exposure to asbestos or asbestos-like fibers (1). These carcinogenic fibers induce cell necrosis, with consequent release of high mobility group protein B1 (HMGB1) and activation of the Nalp3 inflammasome, leading to chronic inflammation, DNA damage and carcinogenesis (2-4). There are approximately 3,200 cases of MM per year in the United States and at least 34,000 cases worldwide in 2013 (1,5). Asbestos continues to be used worldwide, particularly in newly industrializing countries, such as China and India (6,7), therefore the incidence of MM is expected to increase

during the course of the next decades.

Exposure to asbestos fibers and to carcinogenic mineral fibers not commonly classified as asbestos is considered as the main cause of MM (1,4,5,8-10). However, additional factors including SV40 infection (11-13), exposure to radiation, especially high doses radiotherapy of lymphoma and other chest malignancies (1), may also cause mesothelioma, possibly in concert with asbestos (14,15).

Moreover, we demonstrated that germline heterozygous inactivating mutations of the *BRCA1* associated protein-1 (*BAP1*) gene cause the high penetrance hereditary BAP1 cancer syndrome (16,17). The individuals carrying the germline heterozygous *BAP1* mutations develop multiple cancers including mesothelioma, even if not occupationally exposed to asbestos (8), suggesting that they may have

acquired an increased susceptibility to very low levels of asbestos exposure, otherwise harmless to the general population. This hypothesis was supported by the findings in germline *BAP1* heterozygous mice, where minimal exposure to carcinogenic fibers highly increased the risk of mesothelioma (15).

MM is classified into three histological subtypes. The epithelioid subtype characterizes about 70% of all MMs and is less aggressive than the sarcomatoid type, which is highly resistant to chemotherapy and associated with the poorest survival. The biphasic subtype has intermediate characteristics and possibly corresponds to a transition between the other two histological subtypes (18). However, differential diagnosis of MM is challenging, because MM morphology is similar to other cancers. In the epithelioid MM subtype morphology can be confused with that of non-small cell lung carcinomas, renal cell carcinomas, and others (19). The morphology of biphasic MMs can be similar to the one of synovial sarcomas and other biphasic malignancies, whilst sarcomatoid mesothelioma is often morphologically indistinguishable from other spindle cell tumors, including carcinosarcoma. This diagnostic uncertainty is a serious and critical issue because patients with different cancers need different treatments and may have different prognosis. The accuracy of MM diagnosis has been improved by using a set of immunohistochemical (IHC) markers, including mesothelial markers (calretinin, the most sensitive, and WT-1, the most specific) and carcinoma-related markers (CEA, CD15, Ber-EP4, MOC-31, TTF-1) for differential diagnosis of carcinoma (19,20). It is by combining the results obtained with these markers together that a correct diagnosis can be obtained, because—as an example—calretinin and WT1 staining is not exclusively specific for MM, but can be found also in other malignancies. Additional IHC markers can be used to distinguish MM from other malignancies: PAX8 positivity in a pleural tumor allows identifying metastatic renal cell carcinoma, and positive staining for WT1, estrogen receptor (ER), and progesterone receptor (PR) in a peritoneal malignancy allows a differential diagnosis of ovarian carcinoma from MM. Notably, these tumors are morphologically indistinguishable and both stain positively for WT1. However, although these IHC diagnostic tools are of undeniable help to diagnose correctly most MMs, there are still about 10% of cases in which the diagnosis remains dubious because, for example, in both MM and lung carcinoma markers result as either positive or negative, or only a fraction of tumor cells are positive (19). Moreover,

in the absence of evident MM tumor cell invasion into the surrounding normal tissues, it is particularly challenging to distinguish atypical mesothelial hyperplasia from epithelioid type MM, as well as biphasic and sarcomatoid MM subtypes from organizing pleuritis (21,22). Misdiagnosis of MM is a general problem worldwide. In France, the accuracy of initial diagnosis of MM was reported only in 67% of cases (23). We recently reviewed 92 pathological diagnoses of MM from China and found that the diagnostic accuracy rate was about 56.6% (24). In our experience, there are about 1/10 cases of MMs misdiagnosed in the US. Most of the times, these misdiagnoses can be attributed to pathologists who have rarely seen this malignancy, who attempt diagnosis on very small biopsies and/or cytology specimens, or who make a diagnosis with an insufficient set of IHC markers. Misdiagnosis leads to delayed treatment, negatively impacting on patient survival. Therefore, more specific and sensitive diagnostic biomarkers for MM are urgently needed to increase the accuracy of diagnosis of this aggressive and rapidly progressing cancer.

MM develops with a latency of 20–60 years from asbestos exposure (1). Because the tumor is difficult to diagnose and the initial symptoms can be insidious, the disease is often diagnosed at an advanced stage, and this delay is associated with a median survival of about 12 months (5,18). Moreover, MM, in general, is poorly responsive to current therapies, but this is especially true in advanced stages of the disease. Monotherapy and combination therapy have been widely investigated for the treatment of MM however, all therapeutic strategies failed to significantly benefit patients so far (25–28). On the other hand, it has been shown that when patients are diagnosed and treated at an early stage (stage I) of MM, the overall median survival is significantly improved (29,30). Therefore, the relevance of early diagnosis of MM is very high for a better response to therapy. However, only 5% of MM patients are currently diagnosed at an early stage (5).

Monitoring cohorts at high risk of developing MM, because of documented exposure to asbestos or other mineral fibers, or because they carry germline *BAP1* mutations, can be a helpful strategy to make MM diagnoses at earlier stages, when the malignancy may be more susceptible to therapy. In this perspective, the availability of specific and sensitive biomarkers for asbestos exposure and/or MM would facilitate monitoring cohorts over the course of the years.

A biomarker generally refers to a measurable indicator of some biological state or condition, including a pathogenic

process. Different novel biomarkers have been utilized in pre-clinical studies and diagnosis to predict, detect and monitor cancers along several last decades.

For MM diagnosis biomarkers include metabolites, proteins, and microRNAs (miRNAs). An ideal biomarker should selectively detect MM patients from other malignancies or asbestos-exposed subjects from non-exposed individuals. The ideal sample types for detecting biomarkers are blood and pleural effusion (PE), which can be easily collected. This review focuses on some of the most frequently used and promising biomarkers for early detection and diagnosis of MM.

Soluble mesothelin-related peptides (SMRPs)

Mesothelin is a 71-kDa precursor protein, which is physiologically cleaved into two mature proteins: the 31-kDa NH₂-terminal megakaryocyte potentiating factor (MPF), secreted into the blood, and the 40-kDa COOH-terminal glycosylated phosphatidylinositol-linked glycoprotein, which is a plasma membrane-bound protein. After further processing, the SMRPs is released from the cell, and this is the most studied and so far the only Food and Drug Administration (FDA)-approved biomarker for MM (31,32). Mesothelin is expressed at low levels in normal mesothelial cells and is undetectable in most normal tissues. On the contrary, mesothelin is overexpressed in several human cancers, including MM (33,34), pancreatic adenocarcinoma (35), ovarian (36) and lung cancers (37). The level of serum SMRPs has been proposed for identifying MM patients among asbestos-unexposed/exposed individuals and individuals with benign pleural diseases, with a sensitivity of 60–90% and specificity of 80–85% (38–41). Furthermore, it has been proposed that serum SMRPs levels may also differentiate MM patients from patients with pleural metastases of different types of carcinomas (39,40,42). However, serum mesothelin levels are elevated also in patients with renal impairment, and therefore renal function has to be taken into account during the interpretation of this assay (43).

SMRPs in PEs may also be a useful diagnostic biomarker for MM. SMRPs levels were evaluated in MM PE by enzyme-linked immunosorbent assay (ELISA) for their diagnostic performance in differentiating MM from benign pathologies and non-MM pleural metastasis and proposed as a novel diagnostic tool for MM (44). Furthermore, SMRPs levels detected in pleural effusion (PE-SMRPs) were compared with those detected in serum (S-SMRPs) for

their performance in diagnosis of patients with MM, non-MM pleural metastases, and benign pleural diseases. The result suggested that PE-SMRPs had a higher diagnostic performance than S-SMRPs in identifying MM (45).

Two meta-analysis evaluated sensitivity, specificity, and accuracy of serum SMRPs in the diagnosis of MM. A systematic review and meta-analysis of 12 studies in 717 MM patients and 2,851 control subjects including healthy control and patients with non-MM diseases. This study revealed 64% sensitivity [95% confidence interval (CI) 61–68%] and 89% specificity (95% CI 88–90%) of serum SMRPs in the diagnosis of MM (46). The meta-analysis of Hollevoet *et al.* examined 16 studies in 4,491 controls and 1,026 MM patients (47). The results showed a sensitivity of 32% (95% CI 26–40%) and 95% specificity for mesothelin. These results indicate that, although SMRPs may help to identify MM, the sensitivity of the assay is inadequate, limiting its application in early diagnosis of MM (47).

MPF

MPF is a 31-kDa secreted cytokine, which derives from the cleavage of mesothelin, similarly to SMRPs (31). Like SMRPs, MPF was evaluated by ELISA in serum samples from MM patients and different control subjects. Higher serum MPF levels were detected in MM patients, compared to healthy subjects, individuals with benign asbestos-related diseases, or lung cancer patients (48,49).

A prospective multicenter study was performed to measure the diagnostic performance of MPF in MM (50). This study enrolled 507 participants, grouped into six cohorts: 101 healthy controls, 46 individuals with benign respiratory disease, 89 healthy asbestos-exposed individuals, 123 patients with benign asbestos-related diseases, 63 lung cancer and 85 MM patients. The result of this large study revealed that both serum SMRPs and MPF levels allow differentiating MM patients from the other cohorts ($P < 0.001$). Moreover, no significant difference was found between SMRPs and MPF (SMRPs = 0.871, MPF = 0.849; $P = 0.28$). A further study confirmed equivalent diagnostic performances of SMRPs and MPF in distinguishing MM from other diseases, using samples from PE and/or serum (31).

The influence of clinical covariates on SMRPs and MPF levels and their diagnostic value were examined by a multicenter study in a total of 594 participants, including 106 MM patients and 488 control subjects, which found that SMRPs and MPF levels were independently associated with

age, glomerular filtration rate (GFR), and body mass index (BMI) in control subjects, and with GFR and tumor stage in patients with MM. Moreover, the diagnostic performances of SMRPs and MPF were significantly affected by the distribution of these covariates, implying that age, GFR, and BMI should be routinely recorded when measuring these biomarkers. The definition of the approaches to account for these covariates requires further validation (51).

Osteopontin (OPN)

OPN is a secreted glycoprotein that plays critical roles in several biological processes, such as cell-matrix interaction, immunological regulation, tumor development, and cell migration (52-55). The circulating serum OPN levels are elevated in several cancers, including MM (56), colon (57), lung (58), and breast cancer (59). Therefore, serum OPN has been identified as a potential biomarker for early detection of MM (60,61). In particular, Pass *et al.* discovered the link between increased OPN levels and MM (60), by measuring OPN levels in tumor tissues and sera of 49 controls (with no documented exposure to asbestos), 69 asbestos-exposed individuals and 76 MM patients (60). This study showed that there was no significant difference in serum OPN levels between subjects with asbestos exposure and with no asbestos exposure. However, OPN levels in MM were significantly elevated compared to controls and asbestos-exposed individuals. The performance of serum OPN assay was good with 77.6% sensitivity and 85.5% specificity, pointing at OPN as a promising biomarker for the identification of MM patients (60).

A study comparing serum OPN levels from 96 patients with MM and 112 healthy asbestos-exposed subjects showed that assays based on serum OPN levels displayed good sensitivity and specificity to distinguish MM patients from asbestos-exposed individuals (62). However, the OPN assay was unable to distinguish MM from pleural metastases, carcinomas or benign pleural lesions associated with asbestos exposure (BPLAE or pleural plaques) (62). The diagnostic performance of OPN for MM was further investigated in several other studies (63-67) with conflicting results. Some of the discrepancies can be explained by the source of the sample used for the OPN assay. Indeed, two separate studies comparing serum and plasma OPN revealed that the diagnostic performance of the OPN assay was higher when performed in plasma than in serum (65,68), because of the lower stability of serum OPN caused by thrombin cleavage during the coagulation process, which

may yield unreliable results (62,69).

A more recent systematic review and meta-analysis evaluated the overall diagnostic accuracy of serum OPN measurement for diagnosing MM (64). The results of this study showed that pooled sensitivity was 57% (95% CI 52–61%), and specificity was 81% (95% CI 79–84%), the area under the curve (AUC) of the receiver operating characteristic (ROC) curve being 0.80. These data suggested that OPN was a helpful diagnostic biomarker for MM (64).

Fibulin-3

Human fibulin-3 is a secreted glycoprotein encoded by the epidermal growth factor (EGF)-containing fibulin-like extracellular matrix protein-1 (EFEMP-1) gene, involved in the regulation of MM cell proliferation and migration (70).

To assess fibulin-3 as a potential diagnostic marker for MM, its levels of expression were evaluated (71). In this study, plasma fibulin-3 levels, PE fibulin-3 levels, and tumor tissue fibulin-3 levels were all analyzed. The results showed that fibulin-3 preferentially stained tumor cells and that the ROC for fibulin-3 displayed a sensitivity of 96.7% and a specificity of 95.5%, findings suggesting that fibulin-3 was a valuable biomarker for MM. However, other research teams reported a much lower sensitivity of fibulin-3 for MM diagnosis (32,72).

A comprehensive meta-analysis was conducted on 7 studies in 468 MM cases to establish the clinical diagnostic value of fibulin-3 for MM (70). The pooled sensitivity and specificity of the fibulin-3 assay was 62% (95% CI 45–77%) and of 82% (95% CI 73–89%), respectively and MM cases were discriminated from controls with AUC of ROC of 0.81 (70). These data demonstrate the high diagnostic efficacy of fibulin-3, supporting previous evidence of the role of this protein as a promising diagnostic biomarker for MM (5,71,73).

High Mobility Group Box 1 protein (HMGB1)

HMGB1 is a damage-associated molecular pattern (DAMP) and mediates several biological processes such as transcription, cell proliferation, DNA repair and inflammation (74,75). HMGB1 has been extensively studied as nuclear protein, however, acetylation of HMGB1 prevents nuclear translocation, leading to its accumulation in the cytoplasm. Inflammatory cells, such as granulocytes and macrophages, can release acetylated HMGB1 from the

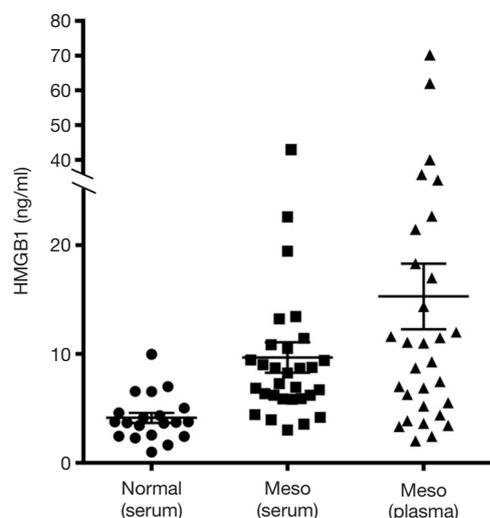


Figure 1 HMGB1 levels in sera and plasma of mesothelioma patients (30 tested) are significantly higher than the levels in healthy individuals (20 tested). Bars show mean of HMGB1 levels. HMGB1, High Mobility Group Box 1 protein; Meso, mesothelioma.

cytosol in the extracellular milieu, where it displays pro-inflammatory activity (76,77). HMGB1 is also passively released by cells undergoing programmed cell necrosis. We discovered that exposure of primary human mesothelial cells to asbestos fibers induces programmed necrosis and consequent release of HMGB1, which in turn triggers the process of cell transformation (3,4,78). Moreover, we demonstrated that MM cells become addicted to HMGB1 for growth and invasion. Accordingly, MM cells actively secrete HMGB1 in an autocrine fashion, as demonstrated by the interference of HMGB1 antagonists on MM growth *in vitro* and *in vivo* (78).

Based on these findings we hypothesized that the serum of individuals exposed to asbestos contain mainly non-acetylated HMGB1 (as expected by cells undergoing asbestos-induced programmed necrosis), while the serum of patients with MM contains mainly hyperacetylated HMGB1, as expected following active secretion of this cytokine by cancer cells. Consistently, we found that HMGB1 levels in serum and plasma were higher in MM patients compared to healthy individuals (Figure 1) (78) and these findings were confirmed by other studies reporting significantly higher serum HMGB1 levels in MM patients compared to individuals with benign asbestos-related diseases (79,80). A systematic review and meta-analysis

established HMGB1 as a prognostic marker for MM (79,81).

More recently, we discovered that hyperacetylated HMGB1 was significantly higher in MM patients compared to asbestos-exposed individuals and healthy controls and that HMGB1 levels do not appear to be influenced by tumor stage (82). The sensitivity and specificity of serum hyper-acetylated HMGB1 in discriminating MM patients and asbestos-exposed individuals and healthy controls was 100%, outperforming any other previously studied biomarkers. The combination of HMGB1 and fibulin-3 produced a better sensitivity and specificity in differentiating MM patients from patients with benign or malignant non-MM PE. These findings suggest that hyperacetylated HMGB1 is a valuable biomarker to identify MM patients among unexposed individuals or individuals occupationally exposed to asbestos (82). Validation studies are in progress.

microRNAs (miRNAs)

miRNAs are small non-coding RNA molecules of 18-22 nucleotides, which regulate gene expression at the post-transcriptional level by binding the 3'-untranslated regions of target miRNAs (83,84); 2,588 human miRNAs have been identified so far. These miRNAs are expected to target about 50% of human miRNAs (85), regulating many cellular activities, such as proliferation, differentiation, metabolism, apoptosis, senescence, angiogenesis, invasion (86). Deregulated miRNAs commonly occur in many cancers and different miRNAs have been proposed as promising diagnostic biomarkers in many cancers, including MM. In general miRNAs are excellent biomarkers because are stable and can be analyzed in routinely processed tissue samples (87), as well as in blood samples (88,89).

Several groups explored the miRNA expression profiles in MM tissues using microarrays (90-95). An initial analysis of miRNAs expression in MM (94) reported 12 miRNAs overexpressed and 9 miRNAs down-regulated in MM tissues compared with normal tissues. Interestingly, among over-expressed miRNAs, miR-30b*, miR-32*, miR-483-3p, miR-584, and miR-885-3p were predicted to regulate the tumor suppressor genes *CDKN2A* and *NF2*, while down-regulated miRNAs, miR-9, miR-7-1* and miR-203 were expected to target the oncogenes *HGF*, *PDGFA*, *EGF* and *JUN* (94). In a different study, the first miRNA signature of MM and mesothelial cells was obtained, revealing 10 miRNAs overexpressed and 19 miRNAs down-regulated in MM cells (93). The validation of these miRNAs by qRT-PCR in 24 MM specimens (epithelioid, biphasic, and

sarcomatoid) significantly linked several overexpressed miRNAs (miR-17-5p, miR-21, miR-29a, miR-30c, miR-30e-5p, miR-106a, and miR-143) to histopathological subtypes of MM. In addition, the expression of miR-17-5p and miR-30c was correlated with survival in sarcomatoid MM (93). Another study assigned to miR-29c* elevated expression a significantly higher survival of MM patients (96), and loss of miR-31 (linked with frequent homozygous loss of 9p21.3 chromosome in MM) was associated with tumor suppressor activity (97). Finally, a survey was performed on a panel of formalin-fixed paraffin embedded (FPPE) specimens of MM patients undergoing extrapleural pneumonectomy (EPP) or palliative surgery (P/D), to explore the association between miRNA expression and patient overall survival. The results generated the miR-Score, a signature of 6 miRNAs (miR-21-5p, miR-23a-3p, miR-30e-5p, miR-221-3p, miR-222-3p, and miR-31-5p), which allowed predicting long survival patients. The performance of the miR-Score was evaluated by ROC curve analysis that revealed 92.3% and 71.9% accuracy for patients undergoing EPP and P/D, respectively (98). These data suggested that deregulated miRNAs can be promising diagnostic biomarkers and prognostic factors for MM as well.

Also cell-free, circulating miRNAs have been suggested as biomarkers for MM (99-101). A plasma miRNA profiling was performed by comparing samples from MM patients and those from healthy controls. Three prognostic miRNA (miR-29c*, miR-92a, and the newly identified miR-625-3p) were validated as promising diagnostic markers for MM (92). Notably, miR29c* was already identified also as a prognostic marker in a previous study (96). Two distinct serum miRNA signatures were identified in MM patients with both diagnostic and prognostic significance (90).

Proteomics

The proteome is the complete set of proteins expressed by an organism or a system, at a certain time and under defined physiological or pathological conditions. A proteomics strategy is a high-throughput approach yielding a protein signature, which has been recently exploited for the effective screening of a high number biomarkers, significantly improving the diagnostic accuracy in different cancers (102), including MM (103,104).

The SOMAmer proteomic technology (105) has been used to screen serological diagnostic markers for MM in a multicenter case-control study including 117 MM patients and 142 control subjects with asbestos exposure (104). Over

1,000 proteins were screened, 64 candidate biomarkers were discovered, and a 13-marker random forest classifier was developed from the candidates, including inflammatory and proliferative proteins. This random forest model differentiated MM from controls with AUC of 0.99, both sensitivity and specificity of >90%, superior to the performance of mesothelin (AUC 0.82, sensitivity 66%, specificity 88%) (104). The potency of this proteomics approach, providing a multiplex biomarker signature, is likely a promising MM diagnostic tool (106).

A seven glycopeptide signature was identified by selected reaction monitoring (SRM) assay technology in MM cells and used to investigate surfaceome derived serum candidate biomarker panels for MM (107). The seven glycopeptide panel accurately discriminated MM from healthy controls and, in combination with mesothelin ELISA, significantly improved the diagnostic accuracy of mesothelin in differentiating MM from non-small-cell lung cancer (NSCLC) (107). Tissue-based proteomics studies in MM and benign biopsies (103) and in MM-derived exosomes (108) identified both already known biomarkers and novel potential candidates, whose biological significance need to be validated by further investigations.

Conclusions

Many million people have been exposed to asbestos in the US and worldwide, causing continuing increase of MM morbidity and mortality and a high number of individuals at risk of developing MM (82). MM is mostly diagnosed at an advanced stage when it is poorly responsive to the current therapies and it was demonstrated that early diagnosis significantly improves overall survival (5,29,30). However, the currently available tissue and serological diagnostic biomarkers are characterized by relatively poor sensitivity and specificity preventing the use of reliable tools both for identification of individuals exposed to asbestos and other carcinogenic fibers and for early detection in patients who are developing MM (109).

The only FDA-approved diagnostic biomarker for MM, serum SMRPs, has low sensitivity, and it was approved only to monitor tumor recurrence after therapy. Novel, and potentially more sensitive and specific MM biomarkers were evaluated, including MPF(31), OPN (60,64,66), fibulin-3 (70,72,73), HMGB1 (82), with promising results. In addition, a variety of deregulated miRNAs [reviewed in (109)], including the miR-score signature with prognostic significance (98), as well as different approaches based on

proteomics technology, have been proposed as suitable MM markers (100,109). It is conceivable that a combination of the most performing and valuable markers validated by the ongoing studies will allow more accurate MM diagnosis and earlier detection in the near future.

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Footnote

Conflicts of Interest: M.C. and H.Y. have pending patent applications on BAP1 and HMGB1; M.C. provides consultation for mesothelioma diagnosis.

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