HMGB1 and Its Hyperacetylated Isoform are Sensitive and Specific Serum Biomarkers to Detect Asbestos Exposure and to Identify Mesothelioma Patients

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Abstract

Purpose: To determine whether serum levels of high mobility group box protein 1 (HMGB1) could differentiate malignant mesothelioma patients, asbestos-exposed individuals, and unexposed controls.

Experimental Design: Hyperacetylated and nonacetylated HMGB1 (together referred to as total HMGB1) were blindly measured in blood collected from malignant mesothelioma patients (n = 22), individuals with verified chronic asbestos exposure (n = 20), patients with benign pleural effusions (n = 13) or malignant pleural effusions not due to malignant mesothelioma (n = 25), and healthy controls (n = 20). Blood levels of previously proposed malignant mesothelioma biomarkers fibulin-3, mesothelin, and osteopontin were also measured in nonhealthy individuals.

Results: HMGB1 serum levels reliably distinguished malignant mesothelioma patients, asbestos-exposed individuals, and unexposed controls. Total HMGB1 was significantly higher in malignant mesothelioma patients and asbestos-exposed individuals compared with healthy controls. Hyperacetylated HMGB1 was significantly higher in malignant mesothelioma patients compared with asbestos-exposed individuals and healthy controls, and did not vary with tumor stage. At the cut-off value of 2.00 ng/mL, the sensitivity and specificity of serum hyperacetylated HMGB1 in differentiating malignant mesothelioma patients from asbestos-exposed individuals and healthy controls was 100%, outperforming other previously proposed biomarkers. Combining HMGB1 and fibulin-3 provided increased sensitivity and specificity in differentiating malignant mesothelioma patients from patients with cytologically benign or malignant non-mesothelioma pleural effusion.

Conclusions: Our results are significant and clinically relevant as they provide the first biomarker of asbestos exposure and indicate that hyperacetylated HMGB1 is an accurate biomarker to differentiate malignant mesothelioma patients from individuals occupationally exposed to asbestos and unexposed controls. A trial to independently validate these findings will start soon. Clin Cancer Res; 22(12); 3087–96. ©2016 AACR.

Introduction

Malignant mesothelioma is an aggressive cancer associated with exposure to asbestos and other carcinogenic mineral fibers (1, 2). Asbestos is a generic name that identifies six naturally occurring silicate mineral fibers that were, and some of them still are, used commercially (3). In the United States, the use of asbestos increased dramatically during World War II and it peaked in the late 1970s, when over 700 tons of asbestos were imported. Asbestos use decreased considerably in the 1980s, following the report of the International Agency for Research on Cancer that identified asbestos as a group-1 human carcinogen (4). Nevertheless, the use of asbestos continues to increase exponentially in many developing countries, where an epidemic of malignant mesothelioma is expected in the coming decades (5). Malignant mesothelioma develops after a latency of 20 to 60 years from asbestos exposure (2). Accordingly, in the United States, the incidence of malignant mesothelioma has risen from less than 10 cases/year in the early 1950s to about 3,200 cases/year in 2004, and it has remained stable since then (1). In the United States, malignant mesothelioma continues to occur in former asbestos workers and in settings of prolonged indoor exposure to asbestos fibers (2). Moreover, with the increased development of rural areas, malignant mesothelioma has been associated with environmental exposure to asbestos (6) and to other carcinogenic mineral fibers, such as erionite, winchellite, richterite, and...
Translational Relevance

Over 20 million people in the United States and many more worldwide have been exposed to asbestos, the main cause of malignant mesothelioma. However, serological biomarkers to identify among potentially exposed cohorts those who have actually been exposed and thus are at risk for malignant mesothelioma, and among them those who are developing malignant mesothelioma, suffer from relatively poor sensitivity and specificity. We report that total serum HMGB1 is sensitive and specific in differentiating individuals professionally exposed to asbestos from unexposed healthy controls. Moreover, we discovered that a specific HMGB1 isoform, hyperacetylated HMGB1, accurately discriminates malignant mesothelioma patients from asbestos-exposed individuals with 100% specificity and sensitivity. Our findings open novel opportunities to identify asbestos-exposed individuals and among them those who have developed malignant mesothelioma.

HMGB1 localization and secretion are regulated by acetylation of the lysine residues in its two nuclear localization signals (NLS1/2). Nonacetylated HMGB1 is mostly localized in the nucleus, where it binds to chromatin and stabilizes nucleosomes. When HMGB1 is hyperacetylated, it is sequestered into the cytoplasm followed by active secretion into the extracellular space (25, 26).

Therefore, we hypothesized that (i) the HMGB1 secreted by malignant mesothelioma cells was hyperacetylated and that this isoform would be found in the sera of malignant mesothelioma patients; (ii) the HMGB1 released when HM die following asbestos exposure was the passively released nonacetylated HMGB1 isoform, and that this isoform would be present in the sera of asbestos-exposed individuals without malignant mesothelioma.

To test our hypothesis, we analyzed hyperacetylated and nonacetylated HMGB1 in the supernatant of malignant mesothelioma cell lines and primary HM cultures with or without exposure to asbestos, and in the serum samples from malignant mesothelioma patients and from individuals with a strong occupational history of asbestos exposure, and from unexposed healthy individuals as controls. We also assessed whether serum levels of HMGB1 and its hyperacetylated isoform would help discriminate malignant mesothelioma patients from patients with benign or malignant non-mesothelioma pleural effusions. Moreover, we compared the diagnostic accuracy of total and hyperacetylated HMGB1 to other proposed malignant mesothelioma biomarkers, including fibrin-3, mesothelin, and OPN.

Materials and Methods

In vitro studies

HM were established in tissue culture from pleural fluid of patients with congestive heart failure and characterized via immunohistochemistry as previously described (27). HM in tissue culture were treated with crocidolite asbestos fibers and tested between cell culture passages 2–5 (27). Malignant mesothelioma cell lines REN (28) and HP3 (29)—also referred to as Phi in our previous study (20)—were obtained respectively from Dr. Steven M. Albelda (University of Pennsylvania School of Medicine, Philadelphia, PA) and Dr. Harvey I. Pass within the last 3 years and have been previously characterized (20, 28, 29). Short-tandem repeat analysis was performed on these lines upon arrival. Reauthentication of the cell lines confirming cell identity was performed after completion of experiments in July 2015 by the company Genetica DNA Laboratories. Testing for Mycoplasma contamination was performed monthly using the LookOut PCR Detection Kit (Sigma-Aldrich). Cells were cultured for 48 hours in serum-free media. Supernatants were concentrated 50× before analysis.

Study population

Full description of participants’ demographic characteristics is included in Supplementary Table S1. Briefly, we tested the levels of HMGB1 isoforms and other malignant mesothelioma biomarkers respectively in serum and plasma from 60 patients who presented to the emergency room with pleural effusions: 13 of them were diagnosed with a benign pleural effusion; 22 of them were diagnosed with malignant mesothelioma; and 25 of them with a malignant non-mesothelioma pleural effusion (all diagnoses were confirmed by cytology and, for the malignant effusions by histopathology and immunohistochemistry). In addition, we studied serum and plasma from insulation workers from New York City, US, part of the “Selikoff cohort,” with a strong
history of occupational exposure to asbestos (ref. 30; n = 20); apparently healthy controls with no known history of asbestos exposure (n = 20) from Liverpool, UK. Malignant mesothelioma patients and patients with other effusions were recruited in a hospital setting in New York City, US, from 2005 through 2013. Patients were enrolled, after giving consent, for participation in the approved Institutional Review Boards and blood procurement protocols. The samples analyzed were from nonconsecutive randomly selected participants/patients, and collected prior to any treatment. The number of individuals recruited in each group (minimum of 20) ensured an 80% power to detect an effect size (Cohen’s d) of 1.0, with α = 0.05.

Mass spectrometry
HMGB1 isoforms present in our cell supernatants and in the human sera were blindly analyzed at the University of Liverpool by whole protein electro spray ionization-liquid chromatography-mass spectrometry (ESI-LC-MS). Posttranslational modifications were confirmed by tandem mass spectrometry (LC-MS/MS), as described (31–33). Briefly, prior to MS analysis, samples (50 μL of cell supernatants or 200 μL of human serum) were immunoprecipitated with 5 μg of rabbit anti-HMGB1 antibody (ab18256; Abcam), and then subjected to trypsin (Promega) or GluC (New England Biolabs) digestion according to the manufacturer’s instructions, and de-salted using ZipTip C18 pipette tips (EMD Millipore). Assay validation data showing the robustness of the protocol have already been extensively published elsewhere (32). As a further control, total HMGB1 was also measured on the human samples using a commercially available ELISA kit (IBL International). Plasma levels of fibulin-3 (USCN), mesothelin (R&D), and OPN (R&D) were measured in malignant mesothelioma patients and asbestos-exposed individuals with indicated commercially available ELISA kits following the manufactures’ instructions. To measure plasma mesothelin, we did not use the FDA-approved soluble mesothelin-related peptides Mesomark kit (Fujirebio Diagnostics Inc.) that is not available for research purposes in the United States, but a different commercially available ELISA kit (R&D). In our hands, testing a subset of samples from this cohort, there is a very good correlation between the two kits (R² = 0.75; P < 0.0001; Supplementary Figure S1A).

Statistical analysis
Using the Bartlett test, all the tested biomarkers presented significant heterogeneous variances between groups. Therefore, significance of two-group comparisons was calculated using the Mann–Whitney nonparametric test. P values of <0.05 were considered significant. Results were expressed as median, 1st quartile to 3rd quartile. Sensitivities, specificities, and ROC AUC were calculated to evaluate each biomarker (total and hyperacetylated HMGB1, fibulin-3, mesothelin, OPN) as well as biomarker composite scores. The classification variables that we considered were healthy controls, asbestos-exposed individuals, malignant mesothelioma patients, patients with benign pleural effusions, and patients with malignant pleural effusions due to other causes. The test variables were the different biomarkers and biomarker composites. The analyses were conducted using GraphPad Prism 6.00 (GraphPad Software). Biomarker composite scores were calculated by logistic regression from standardized biomarker values (17) to discriminate malignant mesothelioma patients from patients with pleural effusions due to other causes. Logistic regressions were run using Stata 12 (StataCorp LP). Whenever present, cut-off values corresponded to the Youden’s J index (i.e., highest sum of sensitivity and specificity minus one; ref. 34).

Results
Initially, we evaluated HMGB1 from concentrated supernatant of HM and malignant mesothelioma cells in tissue culture. Unexposed HM did not release detectable HMGB1 into the extracellular space (Fig. 1). When HM are exposed to 5 μg/cm² of crocidolite asbestos, approximately 60% to 70% of them undergo programmed necrosis within 48 hours (27). Accordingly, in the supernatant of HM exposed to asbestos (Asb-HM), we consistently detected high levels of nonacetylated HMGB1, as expected from cells undergoing necrosis (Fig. 1A and C). Instead, in the supernatant of malignant mesothelioma cells, we detected hyperacetylated HMGB1, consistent with active secretion, as well as nonacetylated HMGB1 (Fig. 1B and C), likely released by a fraction of malignant mesothelioma cells undergoing necrosis, as they are grown in serum-free tissue culture condition during the course of the experiment. Altogether, supernatants from malignant mesothelioma cells showed higher levels of total (nonacetylated + hyperacetylated) HMGB1 with a prevalence of the hyperacetylated isoform, compared with the supernatants from HM exposed to asbestos that contained prevalently the nonacetylated isoform (Fig. 1C). The presence of nonacetylated HMGB1 in asbestos-exposed HM was associated with significant cell death with loss of the classical HM cobblestone appearance (Fig. 1D). The differences in levels of nonacetylated and hyperacetylated HMGB1 observed in tissue cultures support our hypothesis and prompted us to study HMGB1 in human sera.

We compared the levels of total HMGB1 and its isoforms in the sera from (i) 20 unexposed healthy controls, (ii) 20 insulators workers (30) that included individuals with 10 or more years of occupational asbestos exposure, who did not have pleural effusion or evidence of any malignancy at the time of sera collection, and (iii) 22 malignant mesothelioma patients who had been diagnosed following the development of pleural effusion, a common presentation in malignant mesothelioma.

In the serum samples from unexposed healthy controls, total levels of HMGB1 detected by MS were very low (1.4, 0.8–2.2 ng/mL), consistent with previously published HMGB1 values in healthy volunteers (32). Total HMGB1 serum levels were significantly higher in asbestos-exposed individuals (10.2, 5.7–12.1 ng/mL) compared with the levels in unexposed controls (P < 0.001). Malignant mesothelioma patients had the highest levels of total HMGB1 (25.0, 15.7–36.6 ng/mL) when compared with either other group (P < 0.001; Fig. 2A). The total levels of HMGB1 measured with a commercially available ELISA kit and with our MS protocol were very similar (R² = 0.92; P < 0.0001; Supplementary Figure S1B), corroborating the reliability of our approach. The levels of hyperacetylated HMGB1 were very low in both the healthy controls (0.5, 0.3–0.7 ng/mL) and asbestos-exposed individuals (0.4, 0.3–0.6 ng/mL). Malignant mesothelioma patients showed significantly higher levels of hyperacetylated HMGB1 (17.4, 10.3–21.9 ng/mL) compared with either other group (P < 0.001; Fig. 2B). Overall, hyperacetylated HMGB1 comprised approximately 10% of the total HMGB1 in the sera of asbestos-exposed individuals, and approximately 67% of the total HMGB1 in the sera of malignant mesothelioma patients (Supplementary Figure S2).

Next, we evaluated the sensitivity and specificity of total and hyperacetylated HMGB1 as potential biomarkers to discriminate
malignant mesothelioma patients from asbestos-exposed individuals and healthy controls. Both, total and hyperacetylated HMGB1 showed exceptional accuracy in discriminating malignant mesothelioma patients from healthy controls with an AUC of 0.999 (95% confidence interval [CI], 0.994–1.000) and 1.000 (95% CI, 1.000–1.000), respectively. Comparing asbestos-exposed individuals with healthy controls, the AUCs of total and hyperacetylated HMGB1 were 0.964 (95% CI, 0.893–1.000) and 0.574 (95% CI, 0.392–0.756), respectively (Table 1 and Supplementary Figure S3). These data suggest that total HMGB1 is a reliable biomarker to discriminate individuals with asbestos exposure and/or malignant mesothelioma from healthy controls. Comparing malignant mesothelioma patients and asbestos-exposed individuals, the AUC of total HMGB1 was 0.830 (95% CI, 0.687–0.972; Fig. 2C); at specificity 100%, the sensitivity was 72.73% (for values > 15.75 ng/mL, which also corresponded to the cut-off value); at sensitivity 100%, the specificity was 5%. The AUC of hyperacetylated HMGB1, when comparing malignant mesothelioma patients with asbestos-exposed individuals, was 1.000 (95% CI, 1.000–1.000; Fig. 2D), with cut-off value of 2.00 ng/mL. These results point to hyperacetylated HMGB1 as a novel, sensitive, and specific biomarker to discriminate malignant mesothelioma patients from asbestos-exposed individuals. We did not detect any significant difference in total or hyperacetylated HMGB1 serum levels in malignant mesothelioma patients with stages I and II versus III and IV (Fig. 2E and F), suggesting that early lesions are also associated with increased HMGB1 levels and that hyperacetylated HMGB1 may be a valuable screening tool for early detection of malignant mesothelioma among asbestos-exposed cohorts.

Next, we sought to determine whether total and hyperacetylated HMGB1 could also help differentiating malignant mesothelioma patients from patients with pleural effusions due to other cause. Thirteen serum samples from patients with cytologically benign pleural effusions and 25 serum samples from patients with pleural effusions due to non–mesothelioma malignancy were available for these studies (Supplementary Table S1). We found that malignant mesothelioma patients had significantly higher levels of total HMGB1 compared with patients with cytologically benign pleural effusions (6.4, 4.7–9.7 ng/mL; P < 0.001) and malignant (non–mesothelioma) pleural effusions (6.7, 4.2–10.0 ng/mL; P < 0.001; Fig. 3A). Similarly, levels of hyperacetylated HMGB1 were significantly higher in the sera from malignant mesothelioma patients compared with sera from patients with benign pleural effusions (5.2, 3.7–7.8 ng/mL; P < 0.001) or malignant (non–mesothelioma) pleural effusions (5.7, 3.3–8.2 ng/mL; P < 0.001; Fig. 3B). Next, we evaluated the sensitivity and specificity of total and hyperacetylated HMGB1 to discriminate malignant mesothelioma patients from patients with pleural effusions due to other cause. The AUC of total HMGB1 was 0.860 (95% CI, 0.736–0.984; Fig. 3C); at specificity 100%, the sensitivity was 63.64%; at sensitivity 100%, the specificity was 10.53%. The AUC for hyperacetylated HMGB1 was 0.837 (95% CI, 0.709–0.966; Fig. 3D); at specificity 100%, the sensitivity was 59.09%; at sensitivity 100%, the specificity was
Abbreviation: MM, malignant mesothelioma.

MM vs. asbestos-exposed 0.830 (0.687–0.964) vs. healthy 0.964 (0.893–1.000).

Significant difference was found.

Figure 2. HMGB1 serum levels distinguished malignant mesothelioma (MM) patients, asbestos-exposed individuals, and unexposed controls. A and B, quantification of total and hyperacetylated HMGB1 in serum samples from 20 healthy volunteers (H), 20 individuals occupationally exposed to asbestos for at least 10 or more years (Ax), and 22 malignant mesothelioma patients (MM). A, total HMGB1 serum levels are higher in asbestos-exposed individuals compared with healthy unexposed controls, and highest in malignant mesothelioma patients: *** P < 0.001. B, malignant mesothelioma patients showed significantly higher serum levels of hyperacetylated HMGB1 compared with either other group: *** P < 0.001. Horizontal lines at 15.75 ng/mL (A) and 2.00 ng/mL (B) show cut-off values that discriminate malignant mesothelioma patients from asbestos-exposed individuals. C and D, ROC curves of total HMGB1 (C) and hyperacetylated HMGB1 (D) comparing malignant mesothelioma patients with asbestos-exposed individuals. E and F, levels of total and hyperacetylated HMGB1 in patients with early or late stage of malignant mesothelioma (stages I–II vs. stages III–IV). No statistical significant difference was found.

10.53%. Best cut-off values to discriminate malignant mesothelioma patients from benign or malignant non-mesothelioma pleural effusions were 11.35 ng/mL (sensitivity, 81.82%; specificity, 89.47%) and 9.70 ng/mL (sensitivity, 77.27%; specificity, 89.47%), respectively, for total and hyperacetylated HMGB1. Overall, levels of total and hyperacetylated HMGB1 were helpful to distinguish malignant mesothelioma patients from patients with pleural effusions due to other cause.

Table 1. AUC of total and hyperacetylated HMGB1 comparing healthy controls, asbestos-exposed individuals, and MM patients

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<td>MM vs. healthy</td>
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<td>Asbestos-exposed vs. healthy</td>
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<td>MM vs. asbestos-exposed</td>
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Abbreviation: MM, malignant mesothelioma.
for fibulin-3 with either total or hyperacetylated HMGB1 (Fig. 5 and Supplementary Table S4).

In summary, hyperacetylated HMGB1 showed the highest AUC in discriminating malignant mesothelioma patients from asbestos-exposed individuals. The combination of fibulin-3 and HMGB1 increased the power of discrimination of malignant mesothelioma patients from patients with pleural effusions due to other cause.

Discussion

We report that elevated serum levels of total HMGB1 differentiate asbestos-exposed individuals and malignant mesothelioma patients from healthy unexposed controls. Moreover, we discovered that hyperacetylated HMGB1 is a very sensitive and specific biomarker to discriminate malignant mesothelioma patients from asbestos-exposed individuals and from healthy unexposed controls. These results were supported by in vitro experiments in which we found that asbestos-induced necrosis of HM leads to the passive release of nonacetylated HMGB1, whereas the supernatant of malignant mesothelioma cells contains mostly hyperacetylated HMGB1, which is the isoform that is actively secreted. We propose that HMGB1 and its hyperacetylated isoform can be a valuable tool to identify among potentially exposed people, those individuals who have been exposed to asbestos, and to identify among them those who have developed malignant mesothelioma.

The extremely high specificity and sensitivity (100%) of hyperacetylated HMGB1 to identify malignant mesothelioma patients from asbestos-exposed individuals (Fig. 2D) exceeded our most optimistic expectations. In this setting, hyperacetylated HMGB1 outperformed other previously proposed malignant mesothelioma biomarkers, two of them, OPN and fibulin-3, identified by our coauthor (H.I. Pass; refs. 15, 16) who performed the comparative analyses shown in Fig. 4.

We previously proposed total HMGB1 as a possible biomarker for asbestos exposure and malignant mesothelioma (20, 21), but we did not investigate its possible value in differentiating malignant mesothelioma patients from asbestos-exposed individuals. Here, we confirmed that the total serum levels of HMGB1 are elevated in both asbestos-exposed individuals and malignant mesothelioma patients, and reliably distinguish these cohorts from unexposed healthy controls. We further discovered that malignant mesothelioma patients have significantly higher total serum HMGB1 levels compared with asbestos-exposed individuals ($P < 0.001$). Although total HMGB1 levels are sensitive and specific to discriminate asbestos-exposed individuals, the relatively low AUC of 0.830 when comparing malignant mesothelioma patients with asbestos-exposed individuals would limit its clinical usefulness in identifying malignant mesothelioma patients among large cohorts of asbestos-exposed individuals. Strikingly, hyperacetylated HMGB1 reliably distinguished malignant mesothelioma patients from individuals occupationally exposed to asbestos with 100% sensitivity and specificity. Identifying malignant mesothelioma patients among the high-risk asbestos-exposed cohorts is extremely difficult, due to lack of early phenotypical evidences of malignant mesothelioma. The specificity and sensitivity of hyperacetylated HMGB1 should significantly facilitate this task.
We also measured the levels of various biomarkers in patients with benign or malignant non–mesothelioma pleural effusions and compared them with malignant mesothelioma patients (Supplementary Table S3; Figs. 3 and 4). We found that total and hyperacetylated HMGB1 were second to fibulin-3 in distinguishing malignant mesothelioma patients from patients with pleural effusions due to other cause and performed better than mesothelin and OPN. However, the combination of fibulin-3 and HMGB1 resulted in even higher power of discrimination, suggesting a clinical advantage in measuring both proteins.

In our sample set, mesothelin and OPN performed better than in some previous studies (18, 19). Fibulin-3 data were consistent with the original report (16) and with additional studies (35, 36), and were better than those reported by other groups (37, 38).
Close follow-up of these high-risk individuals, and testing for exposed and are at risk of developing malignant mesothelioma. Total HMGB1 will prove helpful to identify among potentially cannot be removed, suggesting that serial longitudinal analysis of bio-persistence of the asbestos.

In contrast with all of the above, individuals heavily exposed to HMGB1 frequently increased in the synovial patients with rheumatoid arthritis may have serum levels of in some cases of chronic autoimmune disease (45). Specifically, in patients with sepsis who succumbed to the infection (44) and baseline within hours to 7 days (41). Of note, the serum samples from the exposed cohort tested here were from insulators with many years of exposure to high amounts of asbestos (30). Further studies are needed to analyze whether HMGB1 levels are increased also in a setting of exposure to lower amounts of asbestos. Also, it has been shown that total HMGB1 in serum and plasma are elevated in severe trauma–43). HMGB1 is also increased –43). HMGB1 levels in serum and plasma are elevated in severe trauma and patients with cytologically malignant (non-mesothelioma) pleural effusions due to other cause. A and B, combined values derived from logistic regression analysis of fibrin-3 and either total or hyperacetylated HMGB1 in malignant mesothelioma patients and patients with benign pleural effusions (Ben-PE), and patients with cytologically malignant (non-mesothelioma) pleural effusions (Mal-PE). †††, P < 0.001. Horizontal lines at scores of −0.48 (A) and −0.22 (B) show cut-off values that discriminate malignant mesothelioma patients from patients with pleural effusions due to other cause. C and D, ROC curves of fibrin-3 alone, HMGB1 (total or hyperacetylated) alone, and combination of the previous showing increased AUC.

Figure 5.
Combination of fibrin-3 and HMGB1 in distinguishing malignant mesothelioma (MM) patients from patients with pleural effusions due to other cause. A and B, combined values derived from logistic regression analysis of fibrin-3 and either total or hyperacetylated HMGB1 in malignant mesothelioma patients and patients with benign pleural effusions (Ben-PE), and patients with cytologically malignant (non-mesothelioma) pleural effusions (Mal-PE). †††, P < 0.001. Horizontal lines at scores of −0.48 (A) and −0.22 (B) show cut-off values that discriminate malignant mesothelioma patients from patients with pleural effusions due to other cause. C and D, ROC curves of fibrin-3 alone, HMGB1 (total or hyperacetylated) alone, and combination of the previous showing increased AUC.

Possible partial explanation to this observation is that most circulating biomarkers are very sensitive to variability introduced by samples’ preparation and handling, and choice of adequate control groups. The problem of reproducibility in biomarker studies is very important, and standardization of protocols to collect and store samples is a necessary step that needs to be taken (39).

Of note, the serum samples from the exposed cohort tested here were from insulators with many years of exposure to high amounts of asbestos (30). Further studies are needed to analyze whether HMGB1 levels are increased also in a setting of exposure to lower amounts of asbestos. Also, it has been shown that total HMGB1 in serum and plasma are elevated in severe trauma and septic shock (40). However, these are transient increases that occur only in severe circumstances that require hospitalization in intensive care. Under these conditions, HMGB1 levels return to baseline within hours to 7 days (41–43). HMGB1 is also increased in patients with sepsis who succumbed to the infection (44) and in some cases of chronic autoimmune disease (45). Specifically, patients with rheumatoid arthritis may have serum levels of HMGB1 above baseline (46, 47), although the levels are more frequently increased in the synovial fluid than in the serum (48). In contrast with all of the above, individuals heavily exposed to asbestos have sustained high HMGB1 serum levels—due to the bio-persistence of the asbestos fibers lodging in the tissues that cannot be removed, suggesting that serial longitudinal analysis of total HMGB1 will prove helpful to identify among potentially exposed cohorts those individuals who have actually been exposed and are at risk of developing malignant mesothelioma.

Close follow-up of these high-risk individuals, and testing for hyperacetylated HMGB1 in their serum, should help detect malignant mesothelioma at an earlier stage when it is more susceptible to therapy. Notably, the levels of total and hyperacetylated HMGB1 were not influenced by stage, suggesting that HMGB1 isoforms might be a promising early malignant mesothelioma detection biomarker.

In vitro, HMGB1 can be hyperacetylated and released by monocytes and macrophages (25). So far, among the nonmalignant conditions, hyperacetylated HMGB1 has only been detected in alcoholic liver disease (ALD; ref. 31), acute acetaminophen-induced liver failure (32), and severe macrophage activation syndrome (33). In the latter two conditions, however, the increase of serum HMGB1 is transient and limited to the acute crisis when patients are in intensive care. ALD might represent a possible confounding factor. However, a specific phosphorylation at serine 34 has been identified in circulating HMGB1 from ALD patients (31). Instead, HMGB1 phosphorylation was not detected in any of our samples (data not shown), using the same analytical methodology of detection, and by the same investigator who performed the ALD study (D.J. Antoine; ref. 31). Therefore, mass spectrometry, by revealing the presence of phosphorylated HMGB1 isoforms, can reliably distinguish the hyperacetylated HMGB1 isoform found in ALD patients from the hyperacetylated HMGB1 isoform found in malignant mesothelioma patients.

Potential technical limitations of our results need to be considered: our sample size was relatively small, which could lead to an undesired over-fitting of the data; our patients and controls were not strictly matched for age, sex, ethnicity, smoking status, or other demographic/epidemiologic factors, potentially introducing unwanted confounding factors. Before total and hyperacetylated HMGB1 can be introduced into the clinic as biomarkers of asbestos exposure and malignant mesothelioma detection, our findings need to be independently validated in a larger cohort. Prospective longitudinal validation studies with matched case–controls will start soon to validate the results reported here in a larger cohort. In these studies, we will also simultaneously investigate whether HMGB1 levels can be affected, similar to mesothelin, by clinical covariates such as kidney function or body mass index (49), and whether potential correlations exist between HMGB1 isoforms and other known markers of chronic inflammation, such as C-reactive protein or neutrophil-to-lymphocyte ratio. Moreover, further studies will be performed to investigate the potential role of specific HMGB1 isoforms as markers of prognosis or tumor recurrence after surgical cytoreduction, and as therapeutic targets. Specific reagents able to detect HMGB1 isoforms in a hospital setting will have to be developed. In this regard, we are currently trying to develop ELISA assays for specific HMGB1 isoforms. Nevertheless, the exceptional potential relevance of our findings to asbestos-exposed individuals and malignant mesothelioma patients warrants immediate attention.

Disclosure of Potential Conflicts of Interest
H. Yang reports receiving a commercial research grant from Shino-Test Corporation. D.J. Antoine is listed as a co-inventor on a granted patent application on the use of HMGB1 as a biomarker and methods to detect it by mass spectrometry that is owned by the University of Liverpool. H.I. Pass, M. Carbone, and H. Yang are listed as co-inventors on a provisional patent application on a Biomarker of Mesothelioma that is owned by the University of Hawaii and New York University. M. Carbone and H. Yang are listed as co-inventors on a provisional patent application on Methods and Kits for Analysis of HMGB1 Isotypes that is owned by the University of Hawaii. No potential conflicts of interest were disclosed by the other authors.
Authors’ Contributions
Conception and design: A. Napolitano, D. J. Antoine, H. I. Pass, M. Carbone, H. Yang
Development of methodology: D. J. Antoine, C. Canino, H. I. Pass, H. Yang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Napolitano, D. J. Antoine, L. Pellegrini, C. M. Goparaju, H. I. Pass, M. Carbone, H. Yang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Napolitano, D. J. Antoine, L. Pellegrini, F. Baumann, I. Pagano, S. Pastorino, H. I. Pass, M. Carbone, H. Yang
Writing, review, and/or revision of the manuscript: A. Napolitano, D. J. Antoine, L. Pellegrini, F. Baumann, I. Pagano, S. Pastorino, H. I. Pass, M. Carbone, H. Yang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Napolitano, I. Pagano, C. M. Goparaju, F. Baumann, I. Pagano, S. Pastorino, H. I. Pass; and by the Wellcome Trust and the Medical Research Council (to D. J. Antoine)

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References


Expression of Concern: HMGB1 and Its Hyperacetylated Isoform are Sensitive and Specific Serum Biomarkers to Detect Asbestos Exposure and to Identify Mesothelioma Patients

Andrea Napolitano, Daniel J. Antoine, Laura Pellegrini, Francine Baumann, Ian Pagano, Sandra Pastorino, Chandra M. Goparaju, Kirill Prokrym, Claudia Canino, Harvey I. Pass, Michele Carbone, and Haining Yang

The editors are publishing this note to alert readers to a concern about this article (1). An ongoing investigation at the University of Liverpool revealed concerns regarding the integrity of the mass spectrometry data contributed by one of the authors of this article. The authors are aware of these concerns and have requested that the journal alert its readers of these concerns. The journal will provide an update once a resolution has been reached.

Reference

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