Chapter 12
Biomarkers of Response to Asbestos Exposure

Clementina Mesaros, Liwei Weng, and Ian A. Blair

Abstract Asbestos-related diseases (ARDs) resulting from exposure to asbestos include lung cancer and malignant mesothelioma (MM). This has significant health and economic implications that have been well documented. The 20–40-year latency periods of ARDs and their low incidence rates in the general population make preventative strategies and early treatment extremely challenging. The availability of well-validated diagnostic biomarkers of asbestos exposure would greatly facilitate both prevention and early treatment strategies. In this chapter, we have summarized the state of knowledge on biomarkers of response to asbestos exposure and highlighted recent advances, including the discovery of new specific biomarker based on the posttranslational modifications of the high mobility group box 1 (HMGB1) protein. Asbestos is inhaled and trapped primarily in lung tissue and so can only be detected in bronchoalveolar lavage fluid. This makes direct exposure assessments very difficult. In contrast, biomarkers of response, which reflect a change in biological function in response to asbestos exposure, have proved to be more useful. MM is the major biological response to asbestos that can be readily monitored, and numerous studies have used this disease as confirmation of a prior asbestos exposure. There is some new evidence that an increase in serum nonacetylated HMGB1 can serve as a biological response biomarker of asbestos exposure; whereas acetylated serum HMGB1 is associated with progression to MM. Finally, we discuss the potential merit of combined use of a multiplexed serum lipid biomarker panel with serum protein biomarkers.

Keywords Malignant mesothelioma • Diagnostic • Mesothelin • Osteopontin • Fibulin-3 • High mobility group box 1 • Acetylation • Slow off-rate modified aptamers • Mass spectrometry
12.1 Biomarkers of Malignant Mesothelioma (MM) and Other Asbestos-Related Diseases (ARDs)

Studies with animal models (Guo et al. 2014; Xu et al. 2014; Kalra et al. 2015) together with genetic (Testa et al. 2011; Carbone et al. 2013; Cheung et al. 2013; Carbone et al. 2015; Ohar et al. 2016) and epidemiological research (Becklake 1976; Lemen et al. 1980; Britton 2002; Stayner et al. 2013; Prazakova et al. 2014) have shown unequivocally that asbestos exposure can lead to ARDs such as MM and lung cancer as well as pulmonary fibrosis. MM is a heterogeneous, aggressive cancer that is mainly observed on the serosal surfaces of the pleura and to a lesser extent in the peritoneum as well as much less commonly in the lining of the testes and pericardium (Montjoy et al. 2009). Surgery can result in long-term benefit for early stage MMs, where there is a better chance that most or all of the cancer can be removed (Lang-Lazdunski 2014). Unfortunately, treatment of later stages of MM is not curative, and so the focus is more on palliative care (Sterman and Albelda 2005). The National Institute for Occupational Safety and Health analysis of the annual cause of death records for 1999–2005 (the most recent years available) revealed that there were 18,068 deaths from MM in the USA, with the number of reported MM deaths increasing from 2482 in 1999 to 2704 in 2005 (Bang et al. 2009). However, the annual death rate was stable at 14.1/million in 1999 and 14.0/million in 2005. Asbestos is still mined in Brazil, Canada, China, Kazakhstan, Russia, and Zimbabwe even though its use is restricted in many Western countries (Frank and Joshi 2014). Therefore, prolonged latency periods from exposure to diagnosis coupled with the ongoing mining and use of asbestos have led to the increasing prevalence of this deadly disease worldwide (Linton et al. 2012; Stayner et al. 2013). Although the occupational exposure to asbestos has declined in the USA through restrictions on its use, there are risks for nonoccupational environmental exposures at old mining sites such as Libby, Montana (Peipins et al. 2003), and old asbestos industrial manufacturing sites such as the BoRit superfund site in Ambler, PA (http://www.boritcag.org).

Early detection of ARDs, when surgical removal of tumors is more successful, provides an attractive therapeutic approach. However, most clinical cases result from exposures that occurred decades earlier (Selikoff et al. 1980; Reid et al. 2014). This means that the discovery and validation of biomarkers of response to asbestos exposure would greatly facilitate the detection of asbestos-exposed individuals prior to the onset of ARDs (Mesaros et al. 2015). This would allow the exposed individuals to be more closely monitored for any sign of disease and allow treatment before progression to inoperable cancer occurs. In addition, biological response biomarker assays conducted in populations living proximal to a site contaminated with asbestos (such as the BoRit site in Ambler) would allow the efficacy of site remediation to be assessed.
12.2 Asbestos Exposure and Biomarkers of Oxidative Stress

There is significant experimental evidence to suggest that oxidative stress is involved in the cellular response to asbestos exposure (Fung et al. 1997; Swain et al. 2004; Schurkes et al. 2004) and in the etiology of asbestos-induced pulmonary fibrosis (Cheresh et al. 2015; Marczynski et al. 2000b). Isoprostanes (Milne et al. 2005) and 8-oxo-2′-deoxyguanosine (dGuo) (Mesaros et al. 2012) are widely used as biomarkers of oxidative stress, although isoprostanes are the only rigorously validated biomarkers of oxidative stress (Kadiiska et al. 2005). Several studies have explored the use of isoprostanes (Pelclova et al. 2008) or 8-oxo-dGuo (Marczynski et al. 2000a; Valavanidis et al. 2009; Hanaoka et al. 1993; Yoshida et al. 2001; Pilger and Rudiger 2006; Chew and Toyokuni 2015) as biomarkers of asbestos exposure. Regrettably, neither of these biomarkers can distinguish asbestos exposure from other causes of oxidative stress such as cigarette smoking (Navarro-Compan et al. 2013; Mesaros et al. 2012; Ellegaard and Poulsen 2016) and atherosclerosis (Victor et al. 2009; Serban and Dragan 2014; Schulze and Lee, 2005; Armstrong et al. 2011; Peluso et al. 2012).

Inhaled asbestos fibers, which are typically longer (typically >5 μm) than they are wide (typically <2 μm diameter) (Boulanger et al. 2014), infiltrate the lung, reach the pleural surface, and are engulfed by phagocytic cells – primarily macrophages (Pooley 1972). It has been suggested that macrophages exposed to asbestos undergo frustrated phagocytosis of the elongated fibers. This process then causes chronic production of reactive oxygen species (ROS), activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, and cytokine release (Ramos-Nino et al. 2006). DNA damage caused by this ROS production (and subsequent mutagenesis) is one mechanism that could be involved in MM carcinogenesis (Mossman et al. 2013). This could occur through the direct action of ROS on DNA or through ROS-derived lipid peroxide-mediated DNA damage (Blair 2008).

Asbestos is also known to activate the nucleotide-binding oligomerization domain (NOD)-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome, which promotes the release of interleukin (IL)-1β and IL-18 (Hillegass et al. 2013). Only a small proportion of patients who have been exposed to asbestos develop MM. Consequently, the disease is thought to result from an interaction of genetic and environmental factors. For example, cyclin-dependent kinase inhibitor 4a (p16INK4a) and ARF tumor suppressor (p14ARF) genes are frequently inactivated somatically in MMs, and approximately 50% of MM tumors exhibit inactivation of the neurofibromin type 2 gene (NF2) due to a combination of somatic nonsense or missense mutations and the loss of the remaining wild-type allele (Cheng et al. 1994; Bianchi et al. 1995; Altimare et al. 2011; Sekido 2013; de Assis et al. 2014). In addition, mice with germline heterozygous inactivating mutations of these genes develop MM at an accelerated rate compared to genetically normal littermates when exposed to asbestos through intraperitoneal injections. Moreover,
germline and sporadic mutations of the tumor suppressor, breast cancer susceptibility (BRCA)-1 associated protein-1 (BAP1) gene, results in a predisposition for MM (Testa et al. 2011; Carbone et al. 2013; Cheung et al. 2013).

12.3 Biomarkers of Response to Asbestos Exposure

Asbestos fibers are not normally present in urine or plasma but they can appear in bronchoalveolar lavage (BAL) fluid. Quantification of asbestos fibers in BAL provides a qualitative/categorical approach to exposure assessment but has not been useful as a predictive biomarker (Sartorelli et al. 2007). This means that it is necessary to analyze biomarkers of response to asbestos fibers rather than directly quantifying the numbers of fibers. Before being implemented as a diagnostics test in clinical settings, biological response biomarkers must first be fully characterized and validated in large sample sets. Candidate biomarkers of exposure to asbestos often lack diagnostic utility because of poor sensitivity and/or inadequate specificity through confounding exposures or individual variability. Additional problems can arise from bioanalytical issues such as pre-analytic stability, inconsistent sample preparation, inconsistent sample processing, or inadequate technology. This means that robust and reproducible bioanalytical assay methodology is required for accurate and reproducible biomarker analysis. Rigorously validated assays for response biomarkers of exposure to asbestos could serve a critical role in early detection of ARDs and in monitoring novel approaches to treatment.

The major route of exposure to asbestos is thought to be through inhalation although there is now some evidence that asbestos can be transported in water (Wu et al. 2015), so it could potentially also be absorbed orally. Inhaled fibers are transported throughout the respiratory system, penetrate pleural cells in the lining of the lung, and induce oxidative stress as noted above. Proteins involved in the immune response, cell proliferation, and the generation of fibrotic tissue are showing promise as potential biomarkers of asbestos exposure. Other factors likely to impact risk of ARDs include common environmental variables, germline genetic factors (Testa et al. 2011), and differential expression of genes that interact with such variables (Hillegass et al. 2010).

We have reviewed the current status of biomarkers of response of human populations exposed to asbestos. The review is focused primarily on MM biomarkers as this is a disease that arises primarily from asbestos exposure. Validation of the more promising MM biomarkers in larger population studies will facilitate early detection of the disease, improve preventative measures, and help assess the efficacy of novel therapies.

12.4 Mesothelin as a Biomarker of MM

Mesothelin is a 40-kDa glycoprotein, which results from proteolytic cleavage of a 69-kDa mesothelin precursor, and is overexpressed in several types of cancer including MM. The soluble form, soluble mesothelin-related protein (SMRP),
sometimes known as soluble mesothelin-related peptide, has emerged as a potential biomarker in serum or urine for the early detection of MM (Creaney et al. 2010a; Creaney et al. 2010b). A number of enzyme-linked immunosorbent assay (ELISA) kits have been developed to analyze SMRP including the MESOMARK serum assay, which is a US Food and Drug Administration (FDA)-approved biomarker for use as an aid in the monitoring of patients with epithelioid and biphasic MM (Table 12.1) (Beyer et al. 2007). A meta-analysis of 30 studies revealed a mean sensitivity of 66% and specificity 97% for SMRP as a serum biomarker of MM when compared with healthy controls (Fig. 12.1) (Cui et al. 2014).

A study conducted in Australia with a sandwich ELISA using two monoclonal antibodies showed increased levels of SMRP in MM patients when compared with healthy controls (Robinson et al. 2003). However, the small sample size did not permit adequate statistical power. A study on the occupational exposure to asbestos in the Czech Republic showed that subjects who had been exposed to asbestos with benign lung disease had higher serum levels of SMRP than normal subjects. Individuals with benign disease had lower serum SMRP levels than subjects with MM (Jakubec et al. 2015). A Turkish biomarker study analyzed serum SMRP in 24 patients with MM from naturally occurring asbestos, 279 subjects with pleural plaques, 123 healthy exposed, and 120 control subjects. This study revealed that serum SMRP had a sensitivity of 63% and specificity of only 74% for detecting the MM patients in this population (Bayram et al. 2014).

Several other studies have found higher levels of serum SMRP in asbestos-exposed individuals than unexposed controls (Pass et al. 2008; Rodriguez Portal et al.

### Table 12.1 Response biomarkers of asbestos exposure

<table>
<thead>
<tr>
<th>Asbestos biomarker</th>
<th>Abbrev</th>
<th>Description</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Soluble mesothelin-related peptide or soluble mesothelin-related protein</td>
<td>SMRP</td>
<td>Mesothelin and SMRP are 40-kDa glycoproteins from proteolytic cleavage of the 69-kDa mesothelin precursor protein</td>
<td>Robinson et al. (2003)</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>None</td>
<td>A 32-kDa integrin-binding protein involved in tumorigenesis, progression, and metastasis</td>
<td>Pass et al. (2005)</td>
</tr>
<tr>
<td>Fibulin-3</td>
<td>None</td>
<td>Fibulin-3 is a 57-kDa protein that belongs to a family of extracellular proteins expressed in the basement membranes of blood vessels</td>
<td>Pass et al. (2012)</td>
</tr>
<tr>
<td>Nonacetylated high mobility group box 1</td>
<td>HMGB1</td>
<td>HMGB1 is a 30-kDa chromatin protein. The unmodified protein has a nuclear location</td>
<td>Tabata et al. (2013a)</td>
</tr>
<tr>
<td>Acetylated high mobility group box 1</td>
<td>Acetylated HMGB1</td>
<td>Lysine hyperacetylation within two nuclear localization signals (Fig. 12.4) causes translocation of HMGB1 into the cytosol</td>
<td>Napolitano et al. (2016)</td>
</tr>
<tr>
<td>Proteomic biomarkers</td>
<td>None</td>
<td>A panel of 13 high abundance serum proteins identified by Slow Off-rate Modified Aptamer (SOMAmer) technology</td>
<td>Ostroff et al. (2012)</td>
</tr>
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One of these studies (Marini et al. 2011) also examined the correlation between mesothelin (SMRP) levels and frequency of micronuclei in blood. A statistically significant positive correlation of the SMRP levels with the frequency of mononuclei in the mononucleated lymphocytes was observed.

Another Australian study measured serum SMRP in a cohort of 514 asbestos-exposed subjects in which the severity of ARDs was assessed separately (Park et al. 2012). The serum SMRP level in the population with compensable ARDs was positively associated with disability assessment. Conversely, the mean SMRP level in healthy asbestos-exposed subjects was significantly lower than those with pleural plaques and in subjects with ARDs who received compensation. The authors concluded that serum SMRP levels correlated with the severity of compensable ARDs and that serum SMRP could potentially be applied to monitor the progress of ARDs.

Fig. 12.1 Forest plots of estimates of sensitivity and specificity for soluble mesothelin family proteins in serum for diagnosing malignant pleural mesothelioma. The point estimates of sensitivity and specificity from each study are shown as solid circles. Error bars are 95% CIs. Numbers indicate the reference numbers of studies cited in the original reference list. Modified with permission from Cui et al. (2014)
One known potential problem apart from the variable sensitivity and specificity of serum SMRP assays (de Assis et al. 2014) is the increased expression of SMRP in ovarian cancer (Wu et al. 2014). Additional confounding issues include the effects of sample storage, body mass index, glomerular filtration rate, age, and smoking status (Park et al. 2010).

12.5 Osteopontin as a Biomarker of MM

Osteopontin is a 32-kDa protein that is encoded by the \textit{SPP1} gene (secreted phosphoprotein 1) in humans (Table 12.1). It is an integrin-binding glycoprotein involved in tumorigenesis, progression, and metastasis that is overexpressed in lung cancer, MM, and several other types of cancer (Denhardt and Chambers 1994). High levels of osteopontin are correlated with tumor progression and metastasis. In a rat model, osteopontin was upregulated in asbestos-induced tumors (Sandhu et al. 2000). There is a report that osteopontin levels in plasma or serum were able to differentiate between healthy subjects exposed to asbestos and MM patients (Grigoriu et al. 2007). A number of other human population studies have reported the serum levels of osteopontin in asbestos-exposed subjects. In one study, osteopontin levels were reported for 69 asbestos-exposed subjects, 45 healthy controls, and 76 patients with surgically staged MM (Pass et al. 2005). Serum osteopontin levels were significantly higher in the group with pleural MM than in the group with exposure to asbestos but without MM. An analysis of serum osteopontin levels comparing the receiver operating characteristic (ROC) curve in the group exposed to asbestos with that of the group with MM had a sensitivity of 77.6% and a specificity of 85.5% (Pass et al. 2005). Another study, which was conducted in Turkey, had 120 healthy controls and 123 subjects exposed to naturally occurring asbestos (Bayram et al. 2014). The difference in the levels of osteopontin was significant ($p < 0.05$) in controls versus asbestos-exposed individuals but was not useful for predicting malignant transformation.

A large study analyzed SMRP and osteopontin levels in asbestos-exposed workers ($n = 1894$) together with a smaller number of unexposed controls ($n = 102$) (Felten et al. 2014). The levels of osteopontin were not significantly different between the two groups. This study also found no correlation between osteopontin levels with the length of the asbestos exposure. A systematic review and meta-analysis of six studies was conducted in order to evaluate the diagnostic accuracy of circulating osteopontin for MM (Hu et al. 2014). The overall mean diagnostic sensitivity and specificity were 65% and 81%, respectively (Fig. 12.2). The area under the ROC curve (AUC) was 0.83, and the diagnostic accuracy of both serum and plasma osteopontin was comparable. The authors concluded that osteopontin was an effective marker for MM diagnosis but that more studies with larger sample sizes and better designs were needed in order to rigorously assess its diagnostic power.
Fibulin-3 is a 57-kDa protein, which belongs to a family of extracellular proteins expressed on the basement membranes of blood vessels. It has emerged as a potential plasma protein from the studies of Pass et al. as a biomarker of asbestos exposure with the capability of distinguishing between exposed and disease states within multiple cohorts (Table 12.1) (Pass et al. 2012). Unusually, in matched samples, fibulin-3 levels were lower in serum than in plasma probably due to the presence of thrombin cleavage sites within fibulin-3. It was also found that fibulin-3 was not able to distinguish between patients with MM and asbestosis because serum levels were elevated in both groups (Corradi et al. 2013). Plasma fibulin-3 was significantly elevated in MM patients from a Sydney patient cohort, but not a Vienna patient cohort (Fig. 12.3) (Kirschner et al. 2015).

12.6 Fibulin-3 as a Biomarker of MM

Fig. 12.2 Forest plots to estimate sensitivity and specificity of osteopontin from six studies. Each solid circle represents an eligible study. The size of the solid circle reflects the sample size of each eligible study. Error bars represent 95% CI. Solid diamond symbols represent mean pooled sensitivity and specificity. Reprinted with permission from Hu et al. (2014)
In addition, the diagnostic accuracy was low, which raised questions as to whether fibulin-3 actually has diagnostic value (Kirschner et al. 2015). However, in another recent study, patients with fibulin-3 levels >34.25 ng/mL before treatment had more than four times higher probability for developing progressive disease within 18 months than patients with levels ≤34.25 ng/mL (Kovac et al. 2015). In addition, patients with fibulin-3 levels >34.25 ng/mL after treatment had increased odds for progressive disease within 18 months even if they had a complete response or stable disease. The authors concluded that a combination of serum SMRP and plasma fibulin-3 levels might be helpful in detecting the progression of MM. Clearly, additional validation studies will be required to fully elucidate the utility of fibulin-3 as a dependable biomarker of asbestos exposure in human populations. It would be interesting to assess multiplexed assays with other protein biomarkers for MM (Kovac et al. 2015; Creaney et al. 2015). In fact, a recent study has demonstrated that compared to an asbestos exposure group of 48 subjects, a group of 42 MM patients had significantly higher mean epidermal growth factor (EGFR), thioredoxin-1 (TRX), SMRP, and fibulin-3 levels (Demir et al. 2016).

Fig. 12.3  Fibulin-3 in patient plasma. Plasma fibulin-3 protein levels in the Sydney (a) and Vienna cohorts (b). Mean levels in both cohorts were below those previously reported. Mean ± s.d. are represented by the lines in the scatter plots, and the cutoffs applied in the original study (Pass et al. 2012) are indicated by dotted lines. The diagnostic accuracy of plasma fibulin-3 was low in both investigated cohorts: (c) Sydney cohort AUC = 0.63 (95% CI, 0.50–0.76) and (d) Vienna cohort AUC = 0.56 (95% CI, 0.41–0.71). Reprinted with permission from Kirschner et al. (2015)
12.7 High Mobility Group Box (HMGB) 1 Protein as a Biomarker of MM and Asbestos Exposure

HMGB1 is a 30-kDa DNA-binding nonhistone protein that is present in nucleus (Lotze and Tracey 2005). It consists of 215 amino acid residues that are organized into three domains, which include two tandem HMG box domains (A box and B box) that are arranged in an L-shaped configuration, and a C-terminal tail of 30 amino acid residues (Fig. 12.4). The nuclear localization of HMGB1 is due to the presence of two lysine-rich nuclear localization sequences (NLSs), spanning amino acids 28–44 (NLS1) and 179–185 (NLS2) (Fig. 12.4) (Lotze and Tracey 2005). HMGB1 is released from macrophages and monocytes by endogenous pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin [IL]-1β, and interferon [IFN]-γ (Wang et al. 2004). Because its N-terminus lacks a signal sequence, HMGB1 cannot

Fig. 12.4 HMGB1 structure. A linear diagram of high-mobility group box 1 protein (HMGB1) is shown, including the residues that constitute the A-box (pink), B-box (purple), and acidic tail (green). The proximal A-box and B-box of HMGB1 both contain putative nuclear-emigration signals, as identified by binding to the nuclear exportin chromosome-region maintenance 1. HMGB1 also contains 43 lysine residues, some of which are frequently acetylated in lipopolysaccharide-activated macrophages (shown in bold). These lysine residues are found within two nuclear-localization signals (indicated by dashed boxes): NLS1, which spans amino acids 28–44; and NLS2, which spans amino acids 179–185. Reprinted with permission from Lotze and Tracey (2005)
be released via the classical endoplasmic reticulum-Golgi secretory pathway. Instead, activated macrophages/monocytes acetylate the HMGB1 at three lysine residues in the NLS1 region and five lysine residues in the NLS2 region (Fig. 12.4) (Lotze and Tracey 2005), which leads to translocation into the cytoplasm and release into the extracellular milieu (Bonaldi et al. 2003; Chen et al. 2005).

The HMGB1 protein, which has a regulatory role in inflammatory immune responses, has received substantial attention as a potential biomarker of MM (Qi et al. 2013) (Table 12.1). Gene expression profiling of mesothelial cells exposed to asbestos has shown upregulation of many genes targeted by HMGB1 (Qi et al. 2013). Furthermore, exposing mice to asbestos resulted in increased serum levels of HMGB1 for 10 or more weeks after crocidolite exposure, but returned to background levels within 8 weeks after chrysotile exposure. Continuous administration of chrysotile was required for sustained high serum levels of HMGB1 (Qi et al. 2013). One study found elevated serum levels of HMGB1, in asbestos-exposed individuals when compared with both smoking and nonsmoking controls (Yang et al. 2010), indicating that serum HMGB1 could be exploited for assessing asbestos exposure in human populations. In agreement with this finding, elevated serum levels of HMGB1 have been found in MM patients (Jube et al. 2012; Tabata et al. 2013b; Yamada et al. 2011).

More recently, it was found that serum HMGB1 is extensively acetylated in the serum of MM patients (Napolitano et al. 2016). The quantification of HMGB1 has generally been conducted using immunoassay-based methodology such as ELISAs, which cannot readily distinguish the nonacetylated and acetylated forms (Zhou et al. 2016; Yamada et al. 2003; Zangar et al. 2006; Barnay-Verdier et al. 2011; Bergmann et al. 2016). Development of high-specificity liquid chromatography-mass spectrometry (LC-MS)-based methods by Antoine and his colleagues (Antoine et al. 2012; Ge et al. 2014; Napolitano et al. 2016) made it possible to distinguish the HMGB1 hyperacetylated forms from the unmodified form normally found in the nucleus (Lotze and Tracey 2005). The MS-based methods are based upon the use of electrospray ionization (ESI) analysis of intact HMGB1 protein followed by spectral deconvolution (Fig. 12.5) (Napolitano et al. 2016) or by the use of Glu-C protease digestion of the HMGB1 followed by LC-ESI/tandem mass spectrometry (MS/MS) analysis of the hyperacetylated NLS2-derived decapeptide K^{180}SKKKKEEEE^{189}, which contains five acetylated lysine residues (Fig. 12.6) (Ge et al. 2014). LC-MS analysis of synthetic acetylated peptide from the NLS2 region of HMGB1–K{(Ac)}SK{(Ac)}K{(Ac)}K{(Ac)}K{(Ac)}EEEE – revealed that it could be rapidly adsorbed on plastic surfaces (Antoine et al. 2012). As a result, glass vials were required throughout the assay procedure. To minimize losses during LC-MS analysis, a desalted tryptic digest of human serum albumin was used as a proteinaceous carrier for the Glu-C-derived peptides.

Hyperacetylated and nonacetylated HMGB1 (together referred to as total HMGB1) were analyzed blindly in blood collected from MM patients (n = 22), individuals with verified chronic asbestos exposure (n = 20), patients with benign pleural effusions (n = 13), malignant pleural effusions not due to MM (n = 25), and healthy control subjects (n = 20) (Napolitano et al. 2016). Blood levels of previously
proposed biomarkers (fibulin-3, SMRP, and osteopontin) were also analyzed in the non-healthy individuals. HMGB1 serum levels reliably distinguished MM patients, asbestos-exposed individuals, and unexposed controls (Napolitano et al. 2016). Total HMGB1 was significantly higher in MM patients and asbestos-exposed individuals compared with healthy controls. Hyperacetylated HMGB1 was significantly higher in MM patients compared with asbestos-exposed individuals and healthy controls and did not vary with tumor stage. At the cutoff value of 2.00 ng/mL, the sensitivity and specificity of serum hyperacetylated HMGB1 in differentiating MM patients from asbestos-exposed individuals and healthy controls was 100%, outperforming other previously proposed biomarkers. Furthermore, by combining HMGB1

Fig. 12.5 Asbestos-exposed human mesothelial (HM) and malignant mesothelioma (MM) cells release different HMGB1 isoforms. (a) Representative spectrum of whole protein ESI/MS analysis of HMGB1 in crocidolite asbestos-exposed HM, where only nonacetylated HMGB1 was detected. (b) Representative spectrum of whole protein ESI/MS spectrum of HMGB1 in MM cells where both hyperacetylated and nonacetylated HMGB1 were detected. Reprinted with permission from Napolitano et al. (2016)

Fig. 12.6 Diagnostic LC–MS/MS spectrum of Glu-C-derived peptide confirming the identification of hyper-acetylated HMGB1 derived from inflammatory cells present in patient sera during acetaminophen hepatotoxicity. Amino acids, b and y ions and peptide sequences are indicated on each spectrum. Acetylated lysine residues within HMGB1 are represented by K(Ac). Reprinted with permission from Antoine et al. (2012)
and fibulin-3, increased sensitivity and specificity was obtained for differentiating MM patients from patients with cytologically benign or malignant non-mesothelioma pleural effusion. If confirmed by other groups, these results are clearly highly significant and clinically relevant because they provide the first biomarker of asbestos exposure and indicate that hyperacetylated HMGB1 is an accurate biomarker to differentiate MM patients from individuals occupationally exposed to asbestos and unexposed controls. More extensive studies will reveal whether these exciting new findings offer an approach to distinguish subjects exposed to asbestos from those who have progressed to MM.

### 12.8 Proteomic Biomarkers of MM

A targeted proteomics approach using Slow Off-rate Modified Aptamer (SOMAmer) technology (Ostroff et al. 2012) was employed for the discovery, verification, and validation of MM biomarkers. SOMAmers have slow specific off-rates for dissociation of targeted analytes, which results in highly selective protein detection. This makes it possible to simultaneously quantify over 1000 proteins in unfractionated biologic samples (Vaught et al. 2010). The biomarker study by Ostroff and colleagues used serum from 117 MM cases and 142 asbestos-exposed control individuals. An initial set of 64 candidate high abundance protein biomarkers was discovered. A training set identified a panel of 13-protein biomarkers for the validation studies (Table 12.1). In a paired sample analysis, the sensitivity (91%) and specificity (94%) of the 13-protein panel and the AUC of the ROC curve of 0.99 were far superior to those observed for serum SMRP (Fig. 12.7). The 13-protein panel consists of both inflammatory and proliferative proteins, which are involved in biological processes that are strongly associated with asbestos-induced malignancy. Further validation studies will be required to determine whether this panel will be useful for screening and diagnosis of high-risk individuals.

### 12.9 Conclusions and Future Perspectives

There is a compelling need to rigorously validate the serum and plasma proteins that are upregulated in MM as a panel of useful biological response biomarkers of asbestos exposure (Table 12.1). In spite of the ban on mining it in the USA, asbestos is still being mined in other parts of the world where there are poor controls on potential exposure of workers to asbestos (Linton et al. 2012). Furthermore, the 20–40-year latency period before ARDs are detected means that they will continue to be a public health problem in the USA for many years (Carbone et al. 2012). A reliable biomarker panel capable of assessing whether a particular individual is at risk for ARDs would be a useful clinical tool in screening, diagnosis, and prevention. Perhaps more importantly, such a diagnostic biomarker panel could help alleviate
concerns about possible environmental exposure as well as help to ensure that effective removal of asbestos from the environment has been implemented. In the past, response biomarker studies have focused on small molecule biomarkers of oxidative stress, signaling factors for cell-mediated and humoral immune responses, and growth factors generated in response to inhalation of asbestos fibers. These studies were able to elucidate many of the important factors involved with the pathogenesis of ARDs as well as guide efforts for developing effective asbestos exposure biomarkers. This has led to the potentially exciting observation that acetylated HMGB1 may be a highly specific biomarker of MM and that elevated nonacetylated HMGB1 might be a useful response biomarker of asbestos exposure.

The discovery of acetylated HMGB1 in MM patients has highlighted the potential utility of LC-MS-based methodology, which is more specific than ELISA-based procedures as also evidenced in our recent studies of apolipoprotein A-I (Wang et al. 2015). Furthermore, LC-MS methodology can be readily adapted to multiplexing, so that multiple candidate proteins can be quantified with high sensitivity and specificity in a single analysis (Mesaros and Blair 2016). Another approach that could be applied to the discovery of biomarkers of response resulting from asbestos exposure.

**Fig. 12.7** ROC curves comparing 13-protein MM biomarker panel to mesothelin (SMRP). Performance of the 13-protein random forest (RF) classifier panel (red) compared to a commercial mesothelin (SMRP) assay (blue) on the same cohort of 32 MM cases and 34 asbestos-exposed controls. ROC curves are plotted with corresponding AUC values and 95% confidence intervals. Reprinted with permission from Ostroff et al. (2012)
exposure involves the implementation of untargeted serum lipidomics using ultrahigh-performance LC coupled with high-resolution MS (Snyder et al. 2015). This could lead to the discovery of a panel of serum lipid biomarkers of response to asbestos exposure that could be validated in more extensive biomarker studies. A serum lipid biomarker panel when combined with serum protein biomarkers would provide an extremely rigorous approach to monitoring populations at risk for environmental asbestos exposure such as near the BoRit site in Pennsylvania and the disused mine in Libby, Montana. Finally, highly specific and sensitive LC-MS assays of rigorously validated MM biomarkers would be extremely effective in assessing the impact of novel approaches to the treatment of MM such as the immune checkpoint inhibitors (Lievense et al. 2014; Ceresoli et al. 2016).

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References

Carbone M, Flores EG, Emi M et al (2015) Combined genetic and genealogic studies uncover a large BAP1 cancer syndrome kindred tracing back nine generations to a common ancestor from the 1700s. PLoS Genet 11:e1005633


Marini V, Michelazzi L, Cioe A et al (2011) Exposure to asbestos: correlation between blood levels of mesothelin and frequency of micronuclei in peripheral blood lymphocytes. Mutat Res 721:114–117


