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The undersigned, appointed by the

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"Epigenetic profiles, asbestos burden, and survival in pleural mesothelioma"

presented by Brock Christensen

candidate for the degree of Doctor of Philosophy and hereby
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Epigenetic profiles, asbestos burden, and survival in pleural mesothelioma

A thesis presented

by

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Epigenetic profiles, asbestos burden, and survival in pleural mesothelioma

Abstract

This thesis aimed to examine the relationships among aberrant epigenetic events, exposure to the carcinogenic mineral fiber asbestos, and patient outcomes in malignant pleural mesothelioma. Pleural mesothelioma is a rapidly fatal asbestos-associated malignancy with a median survival time of less than one year following diagnosis. Asbestos is the single most important contributor to the pathogenesis of pleural mesothelioma with approximately 80% of patients reporting a known exposure. First, the relationship between asbestos burden and survival in mesothelioma was examined using quantitative asbestos fiber burden measures in an effort to advance the understanding of the contribution of asbestos burden to disease prognosis. We found that lung tissue asbestos burden was a significant predictor of mesothelioma prognosis, and our data suggest that patient survival may be modified by susceptibility to this carcinogen. Next, an investigation of the relationship between epigenetic inactivation of six cell cycle control pathway genes and asbestos burden revealed that increasing asbestos burden was associated with an increased number of aberrant epigenetic silencing events, suggesting a novel tumorigenic mechanism of action of asbestos. Lastly, we aimed to clarify the relations among gene-locus specific methylation and tumor status,

asbestos burden, and disease survival with a comprehensive examination of aberrant epigenetic events at 1505 CpG loci associated with silencing of 803 cancer-related genes in pleural mesotheliomas and normal pleura. Classifying mesotheliomas and normal pleural samples based upon CpG methylation profile, we found that methylation profile classes differentiated tumor from normal pleura ($P < 0.0001$). In addition, examining tumors demonstrated that methylation profile class membership significantly predicts lung tissue asbestos burden ($P < 0.03$). Finally, we also found that both methylation class membership ($P < 0.01$), and asbestos burden (HR = 1.4, 95% CI, 1.1 – 1.8) were significant predictors of patient suvival in this disease. This work has demonstrated a role for asbestos as a significant contributor to aberrant epigenetic events in mesothelioma. Furthermore, this work has important clinical implications as we have shown that methylation profiles can differentiate diseased pleura from normal pleura, and that asbestos burden and methylation class membership are independent predictors of mesothelioma patient survival.

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Chapter 1

Introduction

Thesis overview

Malignant pleural mesothelioma is a rapidly fatal asbestos-associated tumor of the parietal pleura. Although asbestos has been established as risk factor for this disease in the modern medical literature for almost a half-century, its pathogenic mechanisms remain incompletely characterized. The aims of this work were to enhance the understanding of the contributions of asbestos exposure and epigenetic alterations to pleural mesothelioma, as well as to evaluate their potential impact on patient survival. These research goals were approached using an incident case series of patients enrolled through the International Mesothelioma Program at Brigham and Women's Hospital in Boston Massachusetts, and had the advantage of a quantitative measure of asbestos burden for the majority of study subjects.

To begin, Chapter two reports on the contribution of patient asbestos fiber burden to pleural mesothelioma prognosis. Next, using a pathway-based, candidate-gene approach to study epigenetic inactivation of tumor suppressor genes, cell cycle control related genes were investigated for the potential correlation between methylation silencing of these genes and patient asbestos burden. In the final chapter of this thesis, additional cases as well as non-tumor pleura samples were added to the study base, and the earlier candidate-gene work was expanded into a high-throughput study of epigenetic alterations in cancer-related genes. In this manner, epigenetic alteration profiles based on hundreds of CpG methylation measurements in each sample were generated. These epigenetic profiles were then used to explore the ability of aberrant methylation events to distinguish diseased pleura from non-diseased pleura. Finally, the relationships between profiles of epigenetic alteration and asbestos burden, as well as between these alterations and patient survival were investigated.

Mesothelioma presentation, diagnosis, and treatment

Mesothelioma is an asbestos-related tumor that arises from the mesothelial membranes surrounding the lungs, heart, and abdominal cavity. The parietal and visceral mesothelial membranes surrounding the lungs are collectively known as the pleura. Malignant pleural mesothelioma (MPM) affects the parietal pleura and is the most common form of mesothelioma, accounting for approximately 85% of cases (Robinson and Lake 2005). Patients suffering from MPM can present with any number of general symptoms; dull chest pain, dyspnea, cough, and / or weight loss and diagnosis can be difficult, especially in non-endemic regions (Antman 1980; Antman, Hassan et al. 2005; Scagliotti and Novello 2005). Although routine chest radiography will often reveal pleural effusion and occasional pleural masses evident of disease, histologic diagnosis is required to verify suspected MPM (differentiating MPM from lung adenocarcinoma, among other entities can be difficult) and to identify the tumor's histology which affects prognosis and, in some cases, treatment decisions (Boutin, Schlesser et al. 1998; Robinson and Lake 2005). Upon MPM diagnosis, aggressive therapy including surgery and chemotherapy has become the standard among eligible patients (Sugarbaker, Heher et al. 1991). Radical surgical approaches in treatment of this disease can include pleurectomy and decortication (P/D), and extrapleural pneumonectomy (EPP), and these are the most widely used treatments for MPM.

Mesothelioma incidence, latency, and survival

Approximately 1 in 100,000 people are diagnosed with MPM in The United States annually, incidence has been rising for thirty years (Antman, Hassan et al. 2005), and is not expected to peak until 2020 (Singhal and Kaiser 2002; Kukreja, Jaklitsch et al. 2004).

Startlingly, rates of mesothelioma in Europe are between 10 and 15 cases per 100,000, several times that of the United States, and are not expected to peak until 2020 (Peto, Decarli et al. 1999; La Vecchia, Decarli et al. 2000). The single most important risk factor for MPM, asbestos exposure, is known (primarily as a result of epidemiologic studies employing patient self-reports of occupational and environmental history) to occur in 70-80% or more of MPM patients (Tammilehto, Maasilta et al. 1992). Although asbestos use has been declining in the United States and Europe for decades, the 20-50 year latency of MPM is responsible for the continued worldwide increasing disease incidence. In contrast to the long latency period following asbestos exposure, MPM is a rapidly fatal cancer with a median survival time of less than one year upon diagnosis (Robinson and Lake 2005). Further influencing survival is disease histology; patients with biphasic and sarcomatoid tumors have reduced survival compared to patients with epithelioid tumors (Flores, Pass et al. 2008). Importantly, since asbestos exposure is often occupationally related, and men are more often employed in these positions, disease occurs three to five times more often in men than women (McDonald and McDonald 1980). The burden of both exposure and disease is heavier among men. Further, men are significantly more likely to have reduced survival compared to women with MPM (Spirtas, Connelly et al. 1988). Hence, while asbestos exposure burden could influence disease outcome, the relationship between asbestos exposure patterns and prognosis has been very poorly studied and is incompletely understood.

Forms of asbestos

Asbestos is a group of crystalline-hydrated silicate minerals that occur in a naturally fibrous form. Derived from the Greek word for inextinguishable, asbestos was used centuries

ago as a textile for clothing, and in oil lamp wicks (Cugell and Kamp 2004). There are two main groups of asbestos fibers; serpentine asbestos, also known as chrysotile, is comprised of shorter, curved fibers; and amphiboles, which are long and straight and have several forms such as crocidolite, amosite, anthophylite, tremolite, and actinolite. Although those who have studied mesothelioma have formed a general consensus that amphibole fibers are more pathogenic than serpentine fibers, evidence indicates that all types of asbestos fibers are carcinogenic (Godleski 2004). Inhalation of asbestos fibers leads to deposition at alveolar duct bifurcations and eventual migration to the pleural membranes (Brody, Hill et al. 1981; Viallat, Raybuad et al. 1986). Unfortunately, the process of fiber translocation is not well understood, but redistribution through airspaces, tissue spaces, or travel via lymphatics are among popular hypotheses (Nishimura and Broaddus 1998).

Occupational and incidental asbestos exposure

The original identification of asbestos-related disease leading to death was made over one hundred years ago by Dr. Montague Murray in a case of asbestosis (Tweedale and Hansen 1998). It was not until 1931 that the first case of MPM was reported in the literature, and not until a study of South-African miners in 1960 that the link between asbestos and MPM was confirmed (Klemperer P 1931; Wagner, Sleggs et al. 1960). Historically, most asbestos exposure is occupationally related and affects individuals who mine, manufacture, or apply asbestos containing products, including shipbuilders, construction workers, and auto mechanics (McDonald and McDonald 1980). In addition, paraoccupational exposure of family members of asbestos workers may lead to higher burdens of asbestos exposure compared to the general population, and potentially increased risk of developing MPM.

The devastating prognosis of pleural mesothelioma is accompanied by an incredible economic burden for both medical care and the litigation aimed at establishing liability for the consequences of asbestos exposure, estimated to be in excess of $265 billion over the next four decades in the United States (Bhagavatula, Moody et al. 2001). While asbestos use has been declining since the 1970s in the U.S. and in Europe due to some regulatory action controlling asbestos use, vast quantities of asbestos continue to be mined and exported throughout the world, and use is heavy in developing nations such as China, India, and Central America (Joshi and Gupta 2004; Kazan-Allen 2005). Although asbestos-containing insulation products were banned in the United States in 1972, other asbestos-containing products are still imported and are among multiple remaining asbestos exposure hazards, many being old products that remain in place. Removal of asbestos-containing insulation put in place prior to the 1972 ban can be costly to remove, and is often left in place unless extensive renovations or planed demolitions are being undertaken. Therefore, there remain significant sources of asbestos in the environment, and thus substantial risk for exposures, such as that observed in the dust from the World Trade Center towers collapse in New York City (Landrigan, Lioy et al. 2004).

Environmental asbestos exposure

In addition to incidental and occupational exposures to asbestos, there are also environmental exposures and other pathogenic mineral fibers. A common type of environmental exposure occurs when dust from surface soils containing mineral fibers are inhaled. Certain geographic locales, such as Afghanistan, Australia, Finland, Greece and Turkey are known to harbor naturally occurring surface amphibole fibers that can generate

significant exposures for residents (Noro 1968; Constantopoulos, Theodoracopoulos et al. 1991; Voisin, Marin et al. 1994; Dumortier, Gocmen et al. 2001). Examples of environmental exposures to asbestos that may not be readily apparent include talc products or talc mine tailings. Veins of mineral asbestos can occur coincident with talc deposits and depending on the geographic origin of the talc, asbestos may comprise a significant percentage of talc (Scancarello, Romeo et al. 1996). Another recent example of asbestos fiber contaminated mining deposits is the vermiculite mine in Libby Montana. In Libby, exposure to amphibole fiber contaminated vermiculite deposits has been associated with an increased risk for asbestos associated diseases (Sullivan 2007). Further, taconite mine tailings that were dumped into Lake Superior near Duluth Minnesota have resulted in the exposure of Duluth residents to high levels of amphibole asbestos fibers in their drinking water, and have put these individuals at a significantly increased risk of peritoneal mesothelioma (Sigurdson 1983). This situation has resulted in public controversy that continues and efforts are underway in 2008 by the state of Minnesota legislature to begin another study of the association of taconite mining with increasing incidence of MPM in the state (Safety 2007).

Pathogenic mechanisms of asbestos

The carcinogenic mechanisms of action of asbestos remain incompletely characterized. However, *in vitro* work has demonstrated the cytotoxic and clastogenic effects of asbestos fibers (Kelsey, Yano et al. 1986; Goodglick and Kane 1990; Jaurand 1997). In addition, attempted phagocytosis of fibers by macrophages and oxido-reduction reactions on fiber surfaces are known to generate reactive oxygen species (ROS) which are capable of inducing DNA damage (Wang, Jaurand et al. 1987). In mesothelial cell cultures, asbestos

fibers cause cell cycle arrest, and can activate *TP53* as well as upregulate proto-oncogenes *c-Fos* and *c-Jun*, and transcription factor NFκB (Levresse, Renier et al. 1997). Importantly, animal models of mesothelioma have been very helpful in advancing the understanding of the pathogenic mechanisms of asbestos, revealing that heterozygosity at particular genes such as *NF2* and the *CDKN2* locus can accelerate the induction of disease in the context of asbestos exposure (Kane 2006). Asbestos fibers have also been shown to damage chromosomes and alter mitosis by interfering with chromosome segregation (Yegles, Saint-Etienne et al. 1993). Specific regions of chromosome deletion often described in MPM include 1p22, 1p36, 3p21, 6q, 9p21, 15q11.1-15, and 22q (Lu, Jhanwar et al. 1994; Huncharek 1995; Xio, Li et al. 1995; Bell, Jhanwar et al. 1997; Bjorkqvist, Tammilehto et al. 1997; Balsara, Bell et al. 1999; Murthy and Testa 1999; Whitaker 2000). Finally, there is rapidly emerging literature suggesting that asbestos may participate in the induction of epigenetic tumor suppressor gene silencing via promoter CpG hypermethylation in MPM (Ohta, Shridhar et al. 1999; Murthy, Shen et al. 2000; Toyooka, Pass et al. 2001; Hirao, Bueno et al. 2002; Toyooka, Carbone et al. 2002; Wong, Zhou et al. 2002; Lee, He et al. 2004; Shivapurkar, Toyooka et al. 2004; He, Lee et al. 2005; Shigematsu, Suzuki et al. 2005; Suzuki, Toyooka et al. 2005; Tsou, Shen et al. 2005).

Other exposures

Although some mesothelioma patients may not have a *known* exposure to asbestos, they may not be truly unexposed, or may have developed the disease due to a different exposure, such as ionizing radiation, given some reports of sporadic MPM following radiotherapy (Cavazza, Travis et al. 1996). There has also been intense debate surrounding

the potential role of simian virus 40 (SV40) infection in MPM pathogenesis. The early region of the virus encodes large T antigen (Tag) which is known to bind and inactivate p53 and Rb. SV40 DNA, RNA, or protein have been found in human mesotheliomas in multiple labs all over the world and more is known about the pathogenic role of SV40 in mesothelioma than any other tumor type (Gazdar, Butel et al. 2002; Pass, Bocchetta et al. 2004). Further, in a mouse model of mesothelioma, SV40 oncoprotiens have been shown to increase asbestos-induced double-strand DNA breaks, and prevent mesothelial cell senescence (Pietruska and Kane 2007). However, others maintain that SV40 is not a significant contributing factor to mesothelioma pathogenesis as multiple studies have detected SV40 as often in normal lung samples and negative controls as in mesotheliomas (Shah 2004). Continuing research into the molecular-genetic consequences of asbestos exposure is imperative in order to improve our understanding of its mechanism of action in inducing MPM.

Genetic susceptibility

The rare incidence of mesothelioma, despite widespread exposures to asbestos, particularly environmental exposures in some parts of the globe, suggests that genetic susceptibility may contribute to MPM risk. Interestingly, despite widespread exposure to the asbestiform mineral fiber erionite in certain Turkish villages, close examination of MPM incidence revealed that risk appeared to be inherited (Carbone, Kratzke et al. 2002). Also, previous studies have shown that parental history of MPM is a possible contributor to an individual's risk of developing mesothelioma (Heineman, Bernstein et al. 1996; Huncharek, Kelsey et al. 1996). In fact, a wealth of evidence supporting a potential genetic susceptibility component to this disease has been collected (Li, Lokich et al. 1978; Risberg, Nickels et al.

1980; Martensson, Larsson et al. 1984; Lynch, Katz et al. 1985; Hammar, Bockus et al. 1989; Otte, Sigsgaard et al. 1990; Precerutti, Mayorga et al. 1990; Dawson, Gibbs et al. 1992; Ascoli, Scalzo et al. 1998). A more recent genetic epidemiologic study of MPM in Turkey, residents of homes where interiors were painted with a whitewash containing zeolite had endemic mesothelioma (Roushdy-Hammady, Siegel et al. 2001). These authors assembled and analyzed three-generation pedigrees that strongly suggest vertical transmission of mesothelioma in a setting where exposure to erionite is ubiquitous. These results are consistent with a report of familial mesothelioma following residential asbestos exposure (Li, Lokich et al. 1978), and with the reports of malignant mesothelioma in two pairs of siblings reported by Martensson et al in 1984 (Martensson, Larsson et al. 1984).

Epigenetics

Epigenetics is the study of heritable changes in gene function that cannot be explained by changes in DNA sequence (Russo 1996). While every cell in an individual human has the same genetic sequence, epigenetic marks are critical regulators of development and cellular fate determination. At the chromatin level, post-translational modification of specific histone lysine residues is critical to the control of gene transcription and is considered a mode of epigenetic regulation. Lysine residues of histone proteins are subject to methylation, and specifically, methylation of histone K9, and lack of K4 methylation promote a heterochromatic conformation of highly condensed nucleosomes (Nguyen, Weisenberger et al. 2002). Similar to, and associated with histone deacetylation, histone lysine methylation results in compacted DNA that prevents access of transcription factors and the transcription initiation complex to DNA, stably silencing gene expression.

Associated with these repressive epigenetic marks and higher-order nucleosome structure is DNA cytosine methylation, the most commonly studied mechanism of epigenetic silencing.

DNA methylation

Cytosine methylation occurs in the context of a CpG dinucleotide, and concentrations of CpGs known as CpG islands when sufficiently methylated, are associated with transcriptional gene silencing tantamount to "one hit" as part of Knudson's two hit hypothesis of carcinogenesis (Knudson 2000). Promoter CpG hypermethylation induced gene silencing is a functional inactivation akin to a protein truncating mutation, a mutation that introduces a stop codon, or gene deletion. Although CpG dinucleotides are underrepresented in the human genome, CpG islands often occur in gene promoter regions, (Bird 2002) and about half of human genes have a promoter CpG island. It is thought that under normal conditions these CpG islands are largely maintained in an unmethylated state permissive to transcription (Jones and Baylin 2002). However, the catalytic addition of a methyl group to cytosine by *de novo* DNA methylatransferase enzymes DNMT3a and DNMT3b causes methylcytosine binding proteins to be recruited and form complexes with histone deacetylases resulting in hypoacetylated histones and highly compacted nucleosomes (Jones and Baylin 2002). The secondary structure of DNA is then inaccessible to transcription activation complexes and the gene downstream of the hypermethylated CpG island is transcriptionally silenced.

DNA methylation in human cancer

DNA methylation associated gene silencing is a well recognized mechanism of epigenetic gene silencing that often occurs at tumor suppressor gene (TSG) loci in human cancer. Hundreds of reports of methylation induced silencing at TSGs in virtually all types of human cancer have been published (Jones and Baylin 2002; Baylin 2006). In contrast to hypermethylated promoter regions, the original link between methylation and cancer was a report of global hypomethylation in cancer cells compared to normal (Feinberg and Vogelstein 1983). Global hypomethylation is the result of loss of methylation of retrotransposons such as long interspersed nuclear elements (LINES) and short interspersed nuclear elements (SINES) such as Alu repeats. Loss of methylation at these regions, which are largely methylated in normal cells, contributes to increased genomic instability (Yoder, Walsh et al. 1997) and may activate transcription of oncogenic genes and non-coding RNAs. As mentioned previously, instances of local promoter hypermethylation induced gene silencing are important contributors to tumorigenesis, though specifically how genes are targeted for *de novo* methylation is not known.

Methylation and exposures

Interestingly, although monozygotic twins are genetically identical, they can have phenotypic differences that may be explained by differences in epigenetic profiles (Fraga, Ballestar et al. 2005). In fact, older monozygotic twins have been shown to exhibit overall content and distribution variations in methylated cytosine greater than the variations seen among younger twin pairs (Fraga, Ballestar et al. 2005). Thus, differences in environment may influence the disparate methylation profiles in twin pairs (and all humans) as varying

exposures are accumulated throughout life. Therefore, in addition to being heritable, epigenetic alterations are almost certainly influenced by an individual's environment.

In fact, exposures to carcinogens have been linked to TSG methylation silencing events in several types of human cancer. Hypermethylation of *RASSF1* in lung cancer is associated with a history of starting to smoke at a younger age (Marsit, Kim et al. 2005), while hypermethylation of *CDKN2A*, encoding p16INK4a has been associated with increasing dose of tobacco smoke (Kim DH, et al Cancer Res 2001 & Toyooka S et al Int J Cancer 2003). In bladder cancer cigarette smoking is significantly associated with methylation induced inactivation of *CDKN2A* (Marsit, Karagas et al. 2006). Additionally, arsenic exposure is another carcinogen that has been associated with TSG methylation in bladder cancer, at *RASSF1* and *PRSS3* (Marsit, Karagas et al. 2006). Alcohol consumption, HPV16 infection and smoking status have been associated with methylation inactivation of the *SFRP* family of Wnt antagonists in head and neck cancer (Marsit, McClean et al. 2006). A final example from bladder cancer that models the association between promoter methylation measurements at 16 TSGs and exposure variables revealed that smoking was significantly associated with an increased propensity to methylate at these loci (Marsit, Houseman et al. 2007). This same report also reported a significantly increased risk of death among bladder cancer patients with an increased propensity to methylate at these 16 TSG loci, indicating the potential contribution of exposure-related methylation events to patient outcome (Marsit, Houseman et al. 2007).

Methylation in MPM

Although more extensively studied in other exposure related solid tumors, rapidly emerging literature implicates methylation induced silencing of TSGs as an important contributor to MPM. In 2001, Tokooya *et al.* reported on differential methylation profiles of seven TSGs in malignant mesothelioma versus lung adenocarcinoma, in part, attempting to establish markers of diagnosis for distinguishing these tumors (Toyooka, Pass et al. 2001). These authors assessed 66 mesotheliomas and 40 lung adenocarcinomas for methylation at *RASSF1A, RARB, CDH13, GSTP1, MGMT, CDNK2A,* and *APC* and found significantly lower levels of methylation in mesotheliomas at all TSG loci except *RASSF1A* and *GSTP1* (Toyooka, Pass et al. 2001). More recently, the same group reported a prevalence of methylation of *SCGB3A1* in mesothelioma of 40% (Shigematsu, Suzuki et al. 2005). In addition, other authors have shown that methylation of *PYCARD* or *HIC1*, predicts poor survival in a case series of 50 MPM patients (Suzuki, Toyooka et al. 2005). A study of the methylation induced silencing of the Secreted Frizzled Related Proteins (SFRP) family in MPM, has demonstrated that these genes are frequently methylated in this disease (Lee, He et al. 2004; He, Lee et al. 2005). Finally, in an investigation of TSG promoter methylation in a case series of 52 MPMs, Tsou *et al.* reported independent associations between methylation at *MT1A* and *MT2A* and patient-reported exposure to asbestos, making an important link between asbestos exposure and epigenetic gene inactivation in MPM (Tsou, Galler et al. 2007).

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Chapter 2

Asbestos burden predicts survival in pleural mesothelioma

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Asbestos burden predicts survival in pleural mesothelioma

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Abstract

Malignant pleural mesothelioma (MPM) is a rapidly fatal asbestos-associated malignancy with a median survival time of less than one year following diagnosis. Treatment strategy is determined in part using known prognostic factors. The relationship between asbestos exposure and survival outcome in MPM was examined in an effort to advance the understanding of the contribution of asbestos exposure to MPM prognosis. We studied incident cases of MPM patients enrolled through the International Mesothelioma Program at Brigham and Women's Hospital in Boston using survival follow-up, self-reported asbestos exposure (n = 128), and a subset of cases (n = 80) with quantitative asbestos fiber burden measures. Consistent with the established literature, we found independent, significant associations between male gender and reduced survival ($P < 0.04$), as well as between non-epithelioid tumor histology and reduced survival ($P < 0.02$). While self-reported exposure to asbestos was not predictive of survival among our cases, stratifying quantitative asbestos fiber burden into groups of low (0 – 99 asbestos bodies), moderate (100 – 1099), and high fiber burden (>1099), suggested a survival duration association among these groups ($P = 0.06$). When adjusting for covariates in a Cox model, patients with low asbestos burden had a 3-fold elevated risk of death compared to patients with moderate fiber burden (95% CI, 0.95 – 9.5, $P = 0.06$), and patients with high asbestos burden had 4.8-fold elevated risk of death (95% CI, 1.5 – 15.0, $P < 0.01$) versus those with moderate exposure. Our data suggest that patient survival is associated with asbestos fiber burden in MPM and is perhaps modified by susceptibility.

Introduction

Malignant pleural mesothelioma (MPM) is a rapidly fatal malignancy with a median survival time of less than one year. The single most important risk factor for MPM is exposure to asbestos, which occurs in 70 – 80% or more of these patients (Tammilehto, Maasilta et al. 1992; Robinson and Lake 2005). Over 3,000 deaths can be attributed to MPM each year in the United States, and worldwide its incidence is on the rise (Pelin, Hirvonen et al. 1994; Price 1997; Morinaga, Kishimoto et al. 2001; Roushdy-Hammady, Siegel et al. 2001; Price and Ware 2004). As a result of the profound disease risk associated with exposure to asbestos and the occupational setting where exposure often occurs, litigation related to asbestos disease–estimated at $265 billion over the next 40 years–has become a tremendous economic burden (Bhagavatula, Moody et al. 2001). Although MPM incidence trends may plateau and begin to decline in the coming years in the U.S. (Price 1997; Price and Ware 2004), asbestos-containing products are still imported into the U.S. Moreover, asbestos use in other nations remains widespread and significant (Robinson and Lake 2005).

Following diagnosis of MPM, the options for treatment are in part dictated by known prognostic factors. Notable predictors of reduced survival in this disease are male gender and non-epithelioid histologies (Zellos and Christiani 2004). Recently, it was reported that a history of asbestos exposure is associated with reduced survival (Flores, Zakowski et al. 2007). In an effort to confirm and extend this observation, we used both self-reported (n = 128) and quantitative asbestos burden measures (n = 80) in a subset of cases to examine the relationship between asbestos exposure and MPM treatment outcome.

Materials and Methods

Study population and exposure data

Lung tissue and tumor tissue were obtained following surgical resection of pleural mesothelioma from incident cases seen at the International Mesothelioma Program at Brigham and Women's Hospital from 2000 – 2006. Quantification of asbestos bodies in samples of lung tissue from multiple sites in the resected lung (De Vuyst, Karjalainen et al. 1998) was carried out as previously described (Churg and Warnock 1977). All patients provided informed consent under the approval of the appropriate Institutional Review Boards. Clinical information was obtained from medical record review. Pathological diagnosis and date of diagnosis was obtained from the medical record of the initial diagnosis, either at Brigham or the primary referring clinic, after having been confirmed by a pathologist's review (JGG). Each patient was assessed for history of exposure to asbestos by a trained industrial hygienist as well as additional demographic and environmental data by obtaining their medical and occupational history with an in-person questionnaire or interview. Patients were followed up for survival using the national death index to determine date of death. Surviving patients were censored based on their last known clinic visit.

Statistical analysis

Univariate tests for association between asbestos exposure, asbestos body burden, patient demographic, and tumor characteristic data were carried out with the appropriate statistical tests using statistical analysis software (SAS). Similarly, tests for association between these variables and survival were carried out with Log-rank tests on Kaplan-Meier survival probability plot strata. Also, a Cox proportional hazards model was used to adjust for co-variates when examining overall patient survival.

Results

Tumor and lung tissue from patients was obtained during surgical resection, and although surgically treated patients tend to be slightly younger and have more epithelioid disease versus the total MPM patient population, this cohort is highly similar to other surgically treated cohorts (Pass, Wali et al. 2008). Survival data were available on all 128 cases, and of these, 83 cases had available asbestos body burden data. Among the cases with available asbestos body counts, three had extremely high counts–14,870, 19,681, and 303,852–compared to the median count of 158. In an effort to avoid an analysis anchored by extreme values, we did not include data from the three patients whose lungs had these asbestos burden values. In Table 2.1, exposure, demographic, and tumor histology data are presented for both all 128 cases and the subset of 80 cases with asbestos burden data. Cases with asbestos burden data did not differ significantly by these variables from cases without fiber burden data.

Table 2.1. Mesothelioma patient demographics and tumor characteristics

	Total n=128 n (%)	Asbestos burden data available n (%) (n=80)
Gender		
Female	30 (23)	20 (25)
Male	98 (77)	60 (75)
Patient Age		
Range	30 – 85	30 – 80
Mean (sd)	62 (10.1)	61 (9.8)
Histology		
Epithelioid	91 (71)	60 (75)
Mixed	33 (26)	18 (22.5)
Sarcomatoid	4 (3)	2 (2.5)
Asbestos Exposure[a]		
Yes	95 (74)	59 (74)
No	33 (26)	21 (26)
Asbestos Body Count[b]		
Range (median)	NA	0 – 6211 (128)
Mean (sd)	NA	875 (1467)

[a] Self reported [b] Data for 83 cases available, three outliers removed

Survival time was defined as time from diagnosis to death or last known follow-up. Figure 2.1 shows the Kaplan-Meier survival probability plots stratified by gender, and the Log-rank test indicates a significantly reduced survival for males versus females ($P < 0.04$). Similarly, Figure 2.2 shows the Kaplan-Meier survival plots by tumor histology. These data reveal a significant survival difference between epithelioid and non-epithelioid histologies (Log-rank $P < 0.02$), as well as a significant difference among epithelioid, biphasic, and sarcomatoid histologies (Log-rank $P < 0.01$).

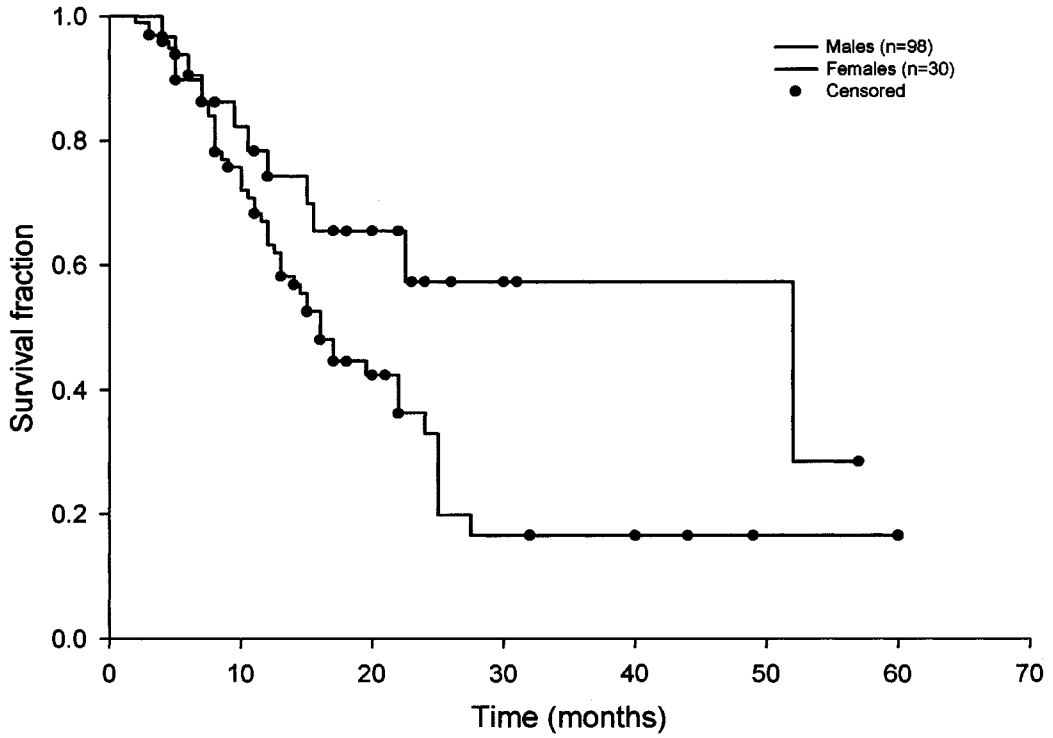
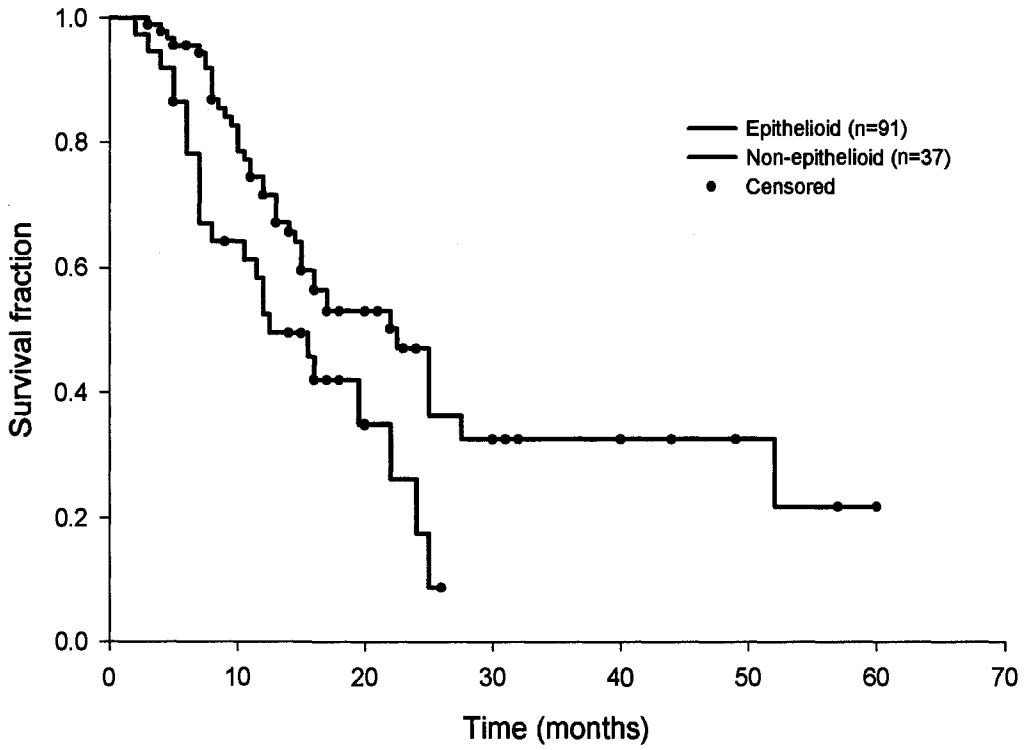


Figure 2.1. Survival by gender in MPM

Survival time is defined as time from diagnosis to death or last known follow-up and circles represent censored values. The Log-rank method was used to test for a difference between strata. Kaplan-Meier survival probability plots of males and females showing that males have significantly reduced survival versus females ($P < 0.04$, n = 128).

A)


1.0
0.8
0.6
0.4
0.2
0.0
Survival fraction
0
10
20
30
40
50
60
70
Time (months)
Epithelioid (n=91)
Non-epithelioid (n=37)
Censored

B)

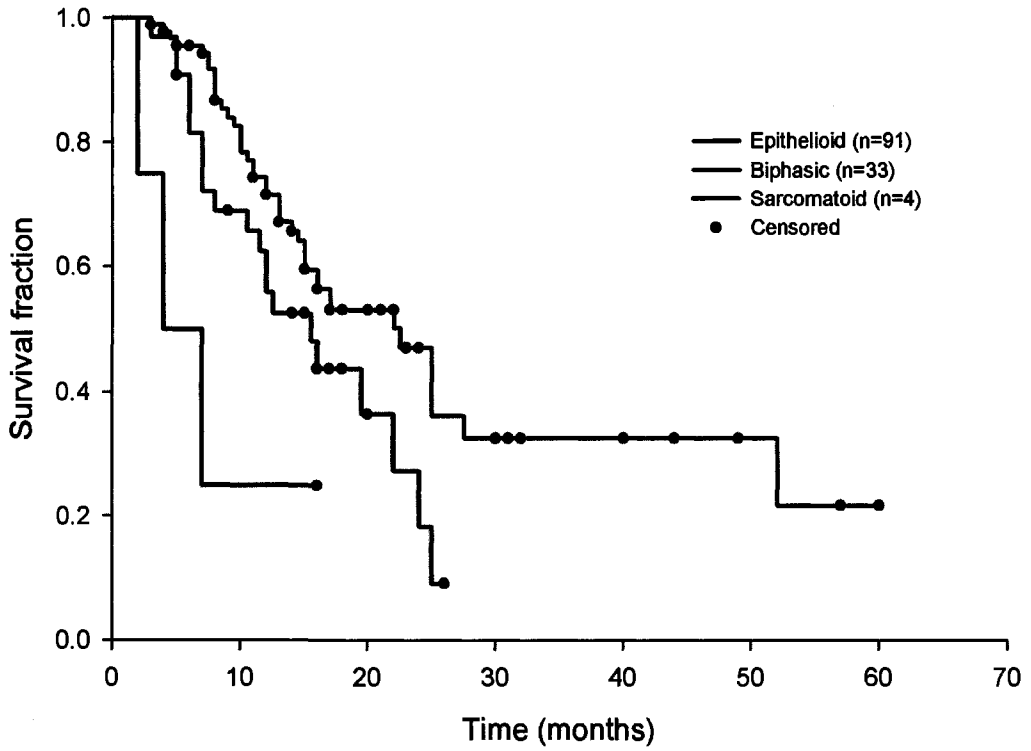

1.0
0.8
0.6
0.4
0.2
0.0
Survival fraction
0
10
20
30
40
50
60
70
Time (months)
Epithelioid (n=91)
Biphasic (n=33)
Sarcomatoid (n=4)
Censored

Figure 2.2. MPM tumor histology and survival

Survival time is defined as time from diagnosis to death or last known follow-up and circles represent censored values. The Log-rank method was used to test for a difference between strata. A) Kaplan-Meier survival probability plots of patients with an epithelioid tumor and patients with a mixed or sarcomatoid tumor. Patients with a non-epithelioid tumor have significantly reduced survival compared to those with an epithelioid tumor ($P < 0.02$, n = 128). B) Kaplan-Meier survival probability plots of patients with an epithelioid, biphasic, or sarcomatoid tumor. Survival was significantly different among patients with epithelioid, biphasic and sarcomatoid tumor types ($P < 0.01$, n = 128).

Next we examined the relationships among asbestos exposure, asbestos fiber burden, patient demographic, tumor histology, and survival data; and we found a significant difference among asbestos fiber burden levels and survival. Among all 128 cases, self-reported exposure to asbestos was not predictive of survival in MPM (Log-rank $P = 0.44$, data not shown). However, there was a significant association between self-reported asbestos exposure and older age at diagnosis (reported exposure; 62.0 ± 9.5 years, no reported exposure; 56.9 ± 9.7 years; T-test, $P < 0.05$), as well as between male gender and reported asbestos exposure (Fisher's, $P < 0.0001$; data not shown). Quantitative asbestos burden data from 80 cases showed that males (mean count = 219, range = 0 – 6211) had significantly higher asbestos burden than females (median count = 20, range = 0 – 2437, Wilcoxon Test $P < 0.0001$). Models of survival by asbestos exposure did not demonstrate a linear trend; thus, data were stratified into tertiles for subsequent analysis. Stratifying asbestos burden data into tertiles of low burden (0 – 99 asbestos bodies), moderate burden (100 – 1099 a.b.), and high

burden (>1099 a.b.), there was an association of fiber burden with survival among these groups that approached statistical significance (Figure 2.3, Log-rank $P = 0.06$).

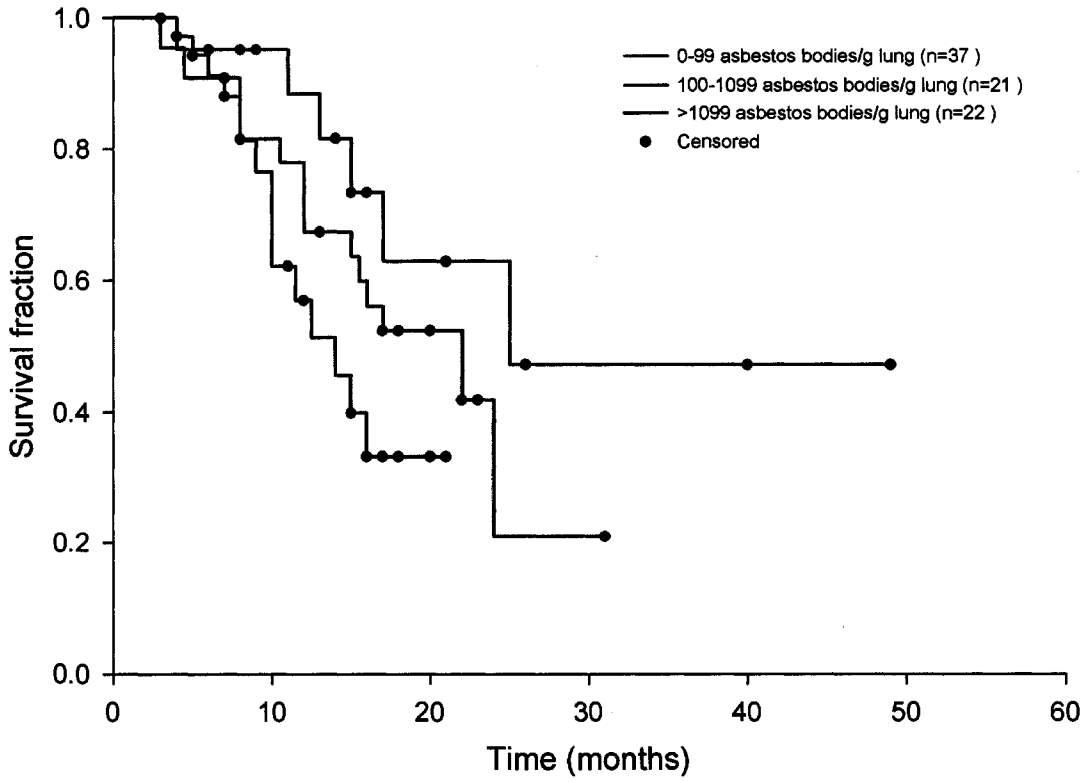


Figure 2.3. Asbestos burden and survival in MPM

Survival time is defined as time from diagnosis to death or last known follow-up and circles represent censored values. The Log-rank method was used to test for a difference among strata. Kaplan-Meier survival probability plots of patients with available asbestos body counts (n = 80). Survival differences among exposure groups approaches statistical significance ($P = 0.06$).

Using a Cox proportional hazards model to adjust for covariates, cases with low asbestos fiber burden had a 3-fold elevated risk of death (95% CI, 0.95 – 9.5, $P = 0.06$) compared to cases with moderate burden (Table 2). Patients with high asbestos fiber burden had 4.8-fold elevated risk of death (95% CI, 1.5 – 15.0, $P < 0.01$) compared to patients with moderate burden (Table 2.2). Including the three cases with extreme outlying asbestos counts in this model did not significantly alter the results (data not shown).

Table 2.2. Asbestos body burden predicts survival in MPM, Cox's proportional hazards model.

Co-variate	n (%)	Hazard Ratio (95% CI)	*P*-value
Gender			
Male	60 (75)	1.0 (reference)	
Female	20 (25)	0.72 (0.27 – 1.9)	0.94
Histology			
Epithelioid	54 (77)	1.0 (reference)	
Mixed	14 (20)	0.82 (0.38 – 1.8)	0.62
Sacromaoid	2 (3)	3.7 (0.35 – 39.1)	0.28
Asbestos body count			
0 – 99	37 (46)	3.0 (0.95 – 9.5)	0.06
100 – 1099	21 (26)	1.0 (reference)	
>1099	22 (28)	4.8 (1.5 – 15.0)	< 0.01

Model is controlled for age and all variables in the table

Discussion

In this study we evaluated the relationships among asbestos exposure, asbestos fiber burden, patient demographics, tumor histology, and survival in MPM. Similar to other groups, we found that male gender and non-epithelioid histologies predict reduced survival (Flores, Zakowski et al. 2007). Interestingly, we also demonstrated that after correcting for co-variates, low or high lung tissue asbestos burden predicted a higher risk of death compared to moderate asbestos burden.

Historically, most asbestos exposure is occupationally related and affects individuals who mined, manufactured, or applied asbestos-containing products (McDonald and McDonald 1980). Given that men are more likely employed in asbestos associated occupations, it is not surprising that they have higher levels of fiber burden, and that the ratio of men to women with MPM is between three and five to one (Zellos and Christiani 2004). Our case series follows this pattern: men have a significantly higher lung tissue asbestos burden and outnumber women more than three to one. Men are known to have both higher fiber burdens and significantly reduced survival compared to women, making it reasonable to posit that an increased asbestos fiber burden may contribute to poor survival *per se*. Consistent with this, we observed an increased risk of death among patients with high asbestos burden compared to patients with moderate asbestos burden. However, we also note an increased risk of death among patients with the lowest lung tissue asbestos burden versus those with moderate fiber burden.

The mechanism responsible for this unusual dose-response association with survival is unclear. One possibility is that cases with low asbestos burden were exposed to chrysotile asbestos or other naturally occurring mineral fibers such as erionite, that have been

associated with MPM (Carbone, Kratzke et al. 2002). Chrysotile asbestos is less biopersistent and is considered by many to be less pathogenic than amphibole asbestos. Hence, significant exposure to chrysotile could have occurred in those with lower numbers of asbestos bodies, and this might not be evident in our data. However, lung chrysotile fiber burden has been shown to correlate with asbestos body levels, arguing against significant chrysotile exposure (Butnor, Sporn et al. 2003).

Concomitantly, erionite fibers are reported to have the highest carcinogenic potential of studied fibers, and form ferruginous bodies indistinguishable from asbestos bodies (Dumortier, Coplu et al. 2001). Since erionite fibers do not form ferruginous bodies as readily as asbestos fibers the asbestos body counts in individuals with erionite exposure may underestimate their true internal dose (Dumortier, Coplu et al. 2001). Since the worldwide geographic distribution of erionite is very limited, it is unlikely that patients in this study had this exposure. However, if either of these scenarios were true (patients with low asbestos body counts having significant chrysotile or erionite exposure), it would imply that the fiber dose is directly associated with survival.

A more likely explanation of our results is related to the considerable literature that has documented both the absence of an appreciable threshold for asbestos-induced mesothelioma, and the fact that MPM can occur with very low level exposures (Hansen, de Klerk et al. 1998; Hodgson and Darnton 2000). Further, widespread exposures to asbestos, particularly environmental exposures in some parts of the globe, combined with the rare incidence of mesothelioma, suggest there may be susceptible individuals. In fact, multiple reports indicate that genetics may modify susceptibility to MPM (Li, Lokich et al. 1978; Risberg, Nickels et al. 1980; Martensson, Larsson et al. 1984; Lynch, Katz et al. 1985;

Hammar, Bockus et al. 1989; Otte, Sigsgaard et al. 1990; Precerutti, Mayorga et al. 1990; Dawson, Gibbs et al. 1992; Ascoli, Scalzo et al. 1998; Dogan, Baris et al. 2006). When closely examining our asbestos fiber burden data, most of the cases within the low asbestos burden group (0 – 99 asbestos bodies/g lung) had asbestos body counts within the general population mean of 0 – 20 bodies/g lung (Dodson and Atkinson 2006), thus these patients may have a greater inherent susceptibility. Further, our data lead to the hypothesis that patients with high susceptibility suffer from more aggressive disease. Outside of this high susceptibility group, the other two tertiles demonstrate a dose-response relationship between asbestos fiber burden and survival.

In summary, our data suggest that patient survival is associated with asbestos fiber burden in pleural mesothelioma, and this association is perhaps modified by susceptibility. Studies using larger case groups-ideally with chrysotile and erionite exposure data-are necessary to further elucidate the ability of asbestos burden to predict survival in MPM.

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Chapter 3

Asbestos exposure predicts cell cycle control gene promoter methylation in pleural mesothelioma

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Abstract

Malignant pleural mesothelioma (MPM) is a rapidly fatal tumor with increasing incidence world-wide responsible for many thousands of deaths annually. Although there is a clear link between exposure to asbestos and mesothelioma, and asbestos is known to be both clastogenic and cytotoxic to mesothelial cells, the mechanisms of causation of MPM remain largely unknown. However, there is a rapidly emerging literature that describes inactivation of a diverse array of tumor suppressor genes (TSG) via promoter DNA CpG methylation in MPM, although the etiology of these alterations remains unclear. We studied the relationships among promoter methylation silencing, asbestos exposure, patient demographics, and tumor histology using a directed approach; examining six cell cycle control pathway TSGs in an incident case series of 70 MPMs. Promoter hypermethylation of *APC, CCND2, CDKN2A, CDKN2B, HPPBP1*, and *RASSF1* were assessed. We observed significantly higher lung asbestos body burden if any of these cell cycle genes were methylated ($p<0.02$), and there was a significant trend of increasing asbestos body counts as the number of methylated cell cycle pathway genes increased from 0, to 1, to >1, ($p<0.005$). This trend of increasing asbestos body count and increasing number of methylated cell cycle pathway genes remained significant ($p<0.05$) after controlling for age, gender, and tumor histology. These data suggest a novel tumorigenic mechanism of action of asbestos, and may contribute to the understanding of precisely how asbestos exposure influences the etiology and clinical course of malignant mesothelioma.

Introduction

Malignant pleural mesothelioma (MPM) is a highly lethal neoplasm linked with asbestos exposure in approximately 70 – 80% of patients. Worldwide, the incidence of MPM is rising, with approximately 3000 cases per year reported in The United States, 2000 cases per year in Great Britain, and 500 per year in Japan (Pelin, Hirvonen et al. 1994; Price 1997; Morinaga, Kishimoto et al. 2001; Roushdy-Hammady, Siegel et al. 2001; Price and Ware 2004). The costs associated with compensation for asbestos-related disease and asbestos remediation have been estimated at $265 billion in the U.S. alone over the next forty years (Bhagavatula R 2001). In addition, serious attention has recently been given to the potential contribution of exposure to the dust from the collapse of the World Trade Center towers in New York City to an increased risk for multiple serious conditions including MPM (Landrigan, Lioy et al. 2004).

Since asbestos use has been on the decline for first world nations for 20 – 30 years, the overall incidence of MPM is expected to peak in the next few years in both the U.S. and Europe (Peto, Decarli et al. 1999; Kukreja, Jaklitsch et al. 2004). At the same time, asbestos continues to be mined, exported, and widely used in many third world countries (Robinson and Lake 2005). Many nations including China, India, and some Latin American countries are still importing vast amounts of asbestos (Joshi and Gupta 2004; Kazan-Allen 2005). This fact, combined with the long 20-50 year latency of MPM, virtually assures that the MPM epidemic will continue for decades to come. This necessitates continuing research into the molecular genetic consequences of exposure to asbestos in an effort to better understand MPM pathogenesis, hopefully translating to prevention strategies and improved patient outcomes.

The pathogenic mechanisms of asbestos contributing to the development of MPM have long been studied, though they remain incompletely characterized. Many *in vitro* studies have demonstrated both clastogenic and cytotoxic effects of asbestos fibers (Kelsey, Yano et al. 1986; Jaurand 1997). Phagocytosis of fibers by macrophages and oxido-reduction reactions on fiber surfaces are known to generate genotoxic reactive oxygen species that are capable of inducing DNA damage (Wang, Jaurand et al. 1987; Okayasu, Takahashi et al. 1999; Xu, Wu et al. 1999) and leading to genetic alterations in MPM (Xu, Huang et al. 2007). In addition to genetic alterations, the rapidly emerging literature indicates that epigenetic tumor suppressor gene (TSG) silencing via promoter methylation occurs in MPM (Ohta, Shridhar et al. 1999; Murthy, Shen et al. 2000; Toyooka, Pass et al. 2001; Hirao, Bueno et al. 2002; Toyooka, Carbone et al. 2002; Wong, Zhou et al. 2002; Lee, He et al. 2004; Shivapurkar, Toyooka et al. 2004; He, Lee et al. 2005; Shigematsu, Suzuki et al. 2005; Suzuki, Toyooka et al. 2005; Tsou, Shen et al. 2005; Tsou, Galler et al. 2007). Methylation of cytosines in the context of promoter CpG islands of TSGs is a well established mechanism of stable gene silencing in human cancers (Jones and Baylin 2002; Baylin 2006). However, the precise mechanisms underlying the induction of TSG methylation and the factors that influence tumor-specific methylation profiles are incompletely understood. Exposure to carcinogens has been associated with TSG methylation silencing, and recently, simultaneous examination of multiple TSGs involved in different cellular pathways and processes has suggested that genes are phenotypically selected for silencing. Initial studies demonstrated that there is a dose-response for methylation silencing of *CDKN2A* by tobacco smoke in lung cancer (Kim, Nelson et al. 2001; Toyooka, Suzuki et al. 2004). Indeed, in lung adenocarcinoma, methylation of TSGs *CDKN2A* and *APC* was also significantly associated

with exposure to tobacco smoke (Toyooka, Maruyama et al. 2003). Dammann *et al.* have shown that asbestos exposure is significantly associated with methylation at *CDKN2A* in non-small cell lung cancer (Dammann, Strunnikova et al. 2005). Suzuki *et al.* reported that methylation of *RRAD*, *APPBP1*, *CCND2*, *RASSF1*, and *TMS1* was significantly more prevalent in SV40 positive MPM (Suzuki, Toyooka et al. 2005). Furthermore, in a recent study of 28 TSG loci in MPM, Tsou *et al.* found a significant association between methylation of two TSGs; *MT1A* and *MT2A* with self reported asbestos exposure (Tsou, Galler et al. 2007). Taken together, these data strongly suggest that asbestos exposure may act to induce methylation silencing of TSGs. However, it remains unclear if this is a direct or indirect selection for TSG inactivation across phenotypically important pathways; if the process is stochastic and less phenotypically driven; or whether a dose-response exists between exposure and methylation extent. To examine this question we have focused our efforts upon TSGs in the cell cycle control and proliferation pathway. We studied the *APC*, *CCND2*, *CDKN2A*, *CDKN2B*, *HPPBP1*, and *RASSF1* genes for promoter hypermethylation in 70 incident cases of MPM. These genes were chosen as both a part of a larger pathway-based group of genes studied in our lab-in this and other types of human cancers-and because they are generally considered among the most important cell cycle control TSGs known to be inactivated via methylation in cancer (Kusy, Larsen et al. 2004; Agathanggelou, Cooper et al. 2005; Schulz 2006). We examined whether methylation of specific genes, methylation at any of these loci, or methylation of an increasing number of genes was associated with asbestos exposure, patient demographic variables or tumor histology. In this process we were fortunate to have quantitative asbestos burden data to explore the relationship between exposure and epigenetic gene inactivation in MPM.

Materials and Methods

Study population

Tumor material was obtained following surgical resection at Brigham and Women's Hospital through the support of the International Mesothelioma Program. All patients provided informed consent under the approval of the appropriate Institutional Review Boards. Clinical information, including pathological diagnosis was obtained from medical record review. Each patient was assessed for history of exposure to asbestos as well as additional demographic and environmental data by obtaining their medical and occupational history with an in-person questionnaire or interview. Patients were followed up for survival using the death index and last known clinic visit.

Methylation analysis

Tumor DNA was extracted from frozen tissue using the QIAamp DNA mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA). Tumor DNA was modified by sodium bisulfite to convert unmethylated cytosines to uracil using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. Methylation-specific PCR (MSP) analysis was conducted with modified template DNA as previously described (Herman, Graff et al. 1996). PCR was performed with 50ng of modified DNA in a mixture with 1 X PCR buffer (Applied Biosystems, Foster City, CA), 0.2mM dNTPs, 0.5μM primers and 1.25 units of Ampli Taq Gold (Applied Biosystems) in a total volume of 25μl. PCR products were analyzed by electrophoresis in 3% agarose gel. Sodium bisulfite modified DNA from circulating blood lymphocytes of healthy control subjects, untreated and treated with *Sss*I DNA methylase, were used as negative and positive controls respectively in each run. In addition, no template negative controls were also present in each

run. All methylation-specific PCR reactions are optimized to detect ~5% methylation in the sample, consistent with the cut-off values generally utilized in quantitative assays of methylation (Eads, Lord et al. 2000; Ogino, Kawasaki et al. 2006).

Asbestos body burden

Quantification of asbestos bodies was done using the protocol of Churg and Warnock (Churg and Warnock 1977). Portions of normal lung tissue (1-4 grams) obtained from surgery were blotted to remove excess liquid, weighed, minced and digested with sodium hypochlorite. This was mixed, vented and then sealed for 48 hours. Following digestion, samples were pelleted, resuspended in 25ml of 50% ethanol and 10ml of chloroform, vortexed, and 15ml of chloroform was added. Samples were then gently centrifuged for 10min, supernatant was aspirated, pellets resuspended in 25ml 25% ethanol, and then mixed well and filtered through a 0.45μm Milipore filter (Millipore, Billerica, MA). Sample tubes were washed twice with 25ml of 25% ethanol and filtered. Similarly, the sides of the filter funnel were washed with 25ml of 25% ethanol and filtered. Filters were dehydrated, cleared twice for one minute each in 95% ethanol, 100% ethanol and then xylene, cut in half, re-cleared in xylene for another minute, mounted on microscope slides with a counting grid using Permount™ Mounting Medium (Fisher Scientific, Hampton, NH) and dried flat. Asbestos bodies were then counted, and the asbestos bodies per (wet weight) gram of lung was calculated with the following equation: number asbestos bodies / (squares counted x 100.74 x weight in grams of the digested tissue sample).

Statistical analysis

Univariate tests for association between methylation at each of the cell cycle genes and patient demographic, tumor characteristic and exposure variables were carried out with

the appropriate statistical tests using SAS analysis software. Similarly, tests for association between methylation at zero, one or greater than one gene, and patient demographic, tumor characteristic and exposure variables were also performed. Simple linear regression was used to test for association between the number of methylated cell cycle genes and asbestos body count. Finally, an ordered logistic model (SAS PROC PROBIT), predicting the number of methylated cell cycle genes was used to control for potential confounders and evaluate the contribution of asbestos body levels to cell cycle gene methylation.

Results

A total of 83 cases had available asbestos body burden data. Among the cases with available asbestos body counts, there were 3 extreme outliers (14,870, 19,681, and 303,852 compared to the median count 158), and 10 cases with zero counts. As pleural mesothelioma arising without detectable asbestos exposure may have a distinct etiology and biology, and in an effort to avoid an analysis anchored by extreme values, we did not include tumors with zero asbestos body counts or the extreme outliers in the analysis, restricting it to the remaining 70 cases. We investigated the methylation status of six cell cycle control associated genes; *CDKN2A*, *CDKN2B*, *RASSF1*, *CCND2*, *APC*, and *APPBP1*. Exposure, demographic, and tumor characteristic data for these 70 cases are in presented in Table 3.1.

Table 3.1. Mesothelioma patient demographics and tumor characteristics

Gender, n (%)	
Female	14 (20)
Male	56 (80)
Patient Age	
Range	30 – 80
Mean (SD)	62 (9.1)
Histology, n (%)	
Epithelioid	54 (77)
Mixed	14 (20)
Sarcomatoid	2 (3)
Asbestos Exposure*, n (%)	
Yes	53 (76)
No	17 (24)
Asbestos Body Count	
Range	6 – 6211
Mean (SD)	1000 (1529)

* Self reported

The prevalence of methylation among the cell cycle control genes varied; *RASSF1*, was methylated in 33% (n = 22) of cases, *APPBP1* in 20% (n = 14), *CDKN2A* in 13% (n = 9), *APC* and *CCND2* in 9% (n = 6), and finally, *CDKN2B* was methylated in 4% (n = 3) of cases (Figure 3.1).

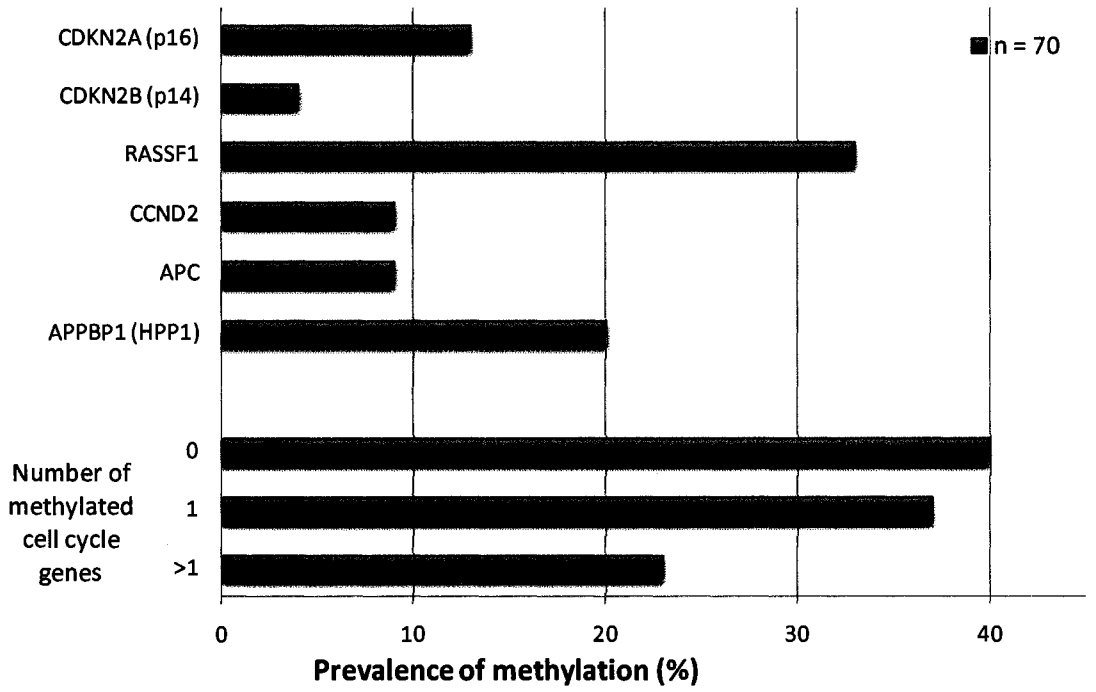

Figure 3.1. Prevalence of cell cycle control gene methylation in pleural mesothelioma Prevalence of methylation positive cell cycle control genes among pleural mesotheliomas as measured by methylation specific PCR, and prevalence of tumors with zero, one, or more than one methylation positive cell cycle control gene.

We found no significant associations between patient gender or tumor histology and methylation at any of the six individual loci examined. However, patients with *RASSF1* methylation were significantly older (65 ± 6.6 years) than patients without *RASSF1* methylation (61± 9.5 years), ($P < 0.05$). We observed a similar relationship between

methylation of *CCND2* and older age, (methylated; 69 ± 8.2 years, unmethylated; 61 ± 8.9 years), ($P < 0.05$). We then asked whether this relationship with age was a more general phenomenon, and found that methylation at any (1 or more gene) of the six TSGs was significantly associated with increased age, (methylated; 64 ± 8.1years, unmethylated; 58 ± 9.4 years), ($P < 0.01$). Since all of these genes are involved in the process of cell cycle control, we grouped cases into three categories; cases with no genes methylated, cases with one gene methylated, and cases with more than one cell cycle control gene methylated. Figure 1 displays the prevalence of methylation of zero (40%, n = 28), one (37%, n = 26), or more than one (23%, n = 16), cell cycle pathway genes.

Next, we examined the relationship between cell cycle control gene methylation and exposure to asbestos using both self-reported and quantitative asbestos body counts as exposure variables. While we found no significant associations between methylation at any one of the six genes and self-reported asbestos exposure, cases with *RASSF1* methylation did have significantly higher asbestos body counts (mean count = 698), compared to cases without *RASSF1* methylation (mean count = 409), ($P < 0.01$, Wilcoxon Test). Similarly, there was no significant relationship between methylation of any cell cycle gene (comparing samples with no genes methylated to those with any gene(s) methylated) and self-reported asbestos exposure. Notably, although we were unable to detect an association between self-reported asbestos exposure and methylation of cell cycle control related genes, we observed a significant association between reported asbestos exposure and elevated asbestos body count ($P < 0.005$). We also examined the relationships between asbestos body count and patient age, gender, and tumor histology. Although we did not find any association between asbestos body count and age or histology (data not shown), we did observe a significant difference in

asbestos body count in males (mean count = 1218) compared with females (mean count=213), ($P < 0.001$, Wilcoxon).

Figure 3.2 shows that log transformed asbestos body counts are significantly correlated with the number of cell cycle control genes methylated (linear regression F-test, $P < 0.005$).

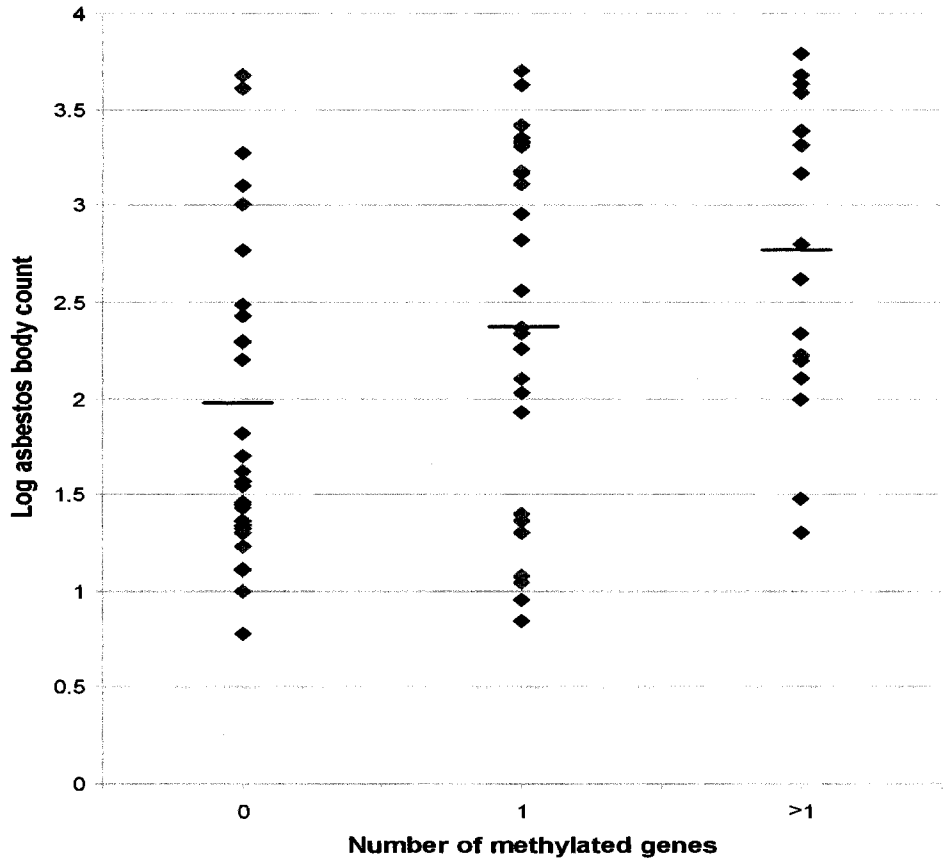

Figure 3.2. Asbestos body count versus cell cycle gene methylation

Log transformed asbestos body count (y–axis) is plotted versus the number of methylated cell cycle control genes (x–axis). Using simple linear regression there is a significant association between increasing asbestos burden and increasing number of methylated cell cycle control genes ($P < 0.005$, $R^2 = 0.12$).

In order to control for potential confounders of this relationship and to better represent the discreet ordinal nature of the methylation count, we modeled the data using an ordered logistic regression that predicts zero, one, or more than one methylated cell cycle pathway gene (Table 3.2). This model indicates that when controlling for gender and tumor histology, both age and asbestos body count are significant predictors ($P = 0.04$ and $P < 0.05$, respectively) of an increased number of methylated cell cycle control genes.

Table 3.2. Ordered logistic regression model predicting increasing number of methylated cell cycle control genes in pleural mesothelioma (n = 70).

Predictor	**Estimate**	***P*-value**
Age	0.67	0.04
Gender		
Male	1.0	referent
Female	-0.73	0.32
Histology		
Epithelioid	1.0	referent
Mixed & Sarcomatoid	0.77	0.21
Asbestos body count*	0.33	< 0.05

*Scaled to: (asbestos body count / 1000)

Discussion

We evaluated promoter hypermethylation of six cell cycle control and progression pathway genes in an incident case series of 70 MPMs examining whether methylation of specific genes, methylation of any of these loci, or methylation of an increasing number of genes was associated with patient demographic variables, tumor histology, or asbestos exposure. We chose to study these genes in part because they have been studied by our lab as a part of a pathway-based approach to investigating TSG methylation in other human cancers. Further, these genes are known to be subject to inactivation by promoter hypermethylation in cancer, and are recognized as important in cell cycle control and progression (Kusy, Larsen et al. 2004; Agathanggelou, Cooper et al. 2005; Schulz 2006). We examined these genes for methylation using methylation-specific PCR. This technique is known to be sensitive to 5% of cells with methylation, and is therefore ample in detecting aberrant methylation events of phenotypic importance (Marsit, Karagas et al. 2005).

Methylation of *RASSF1* has been observed in 32% of MPM (n = 66) and previously significantly associated with SV40 exposure (Toyooka, Pass et al. 2001). We observed essentially the same prevalence of *RASSF1* methylation among our cases (33%), and we also found that *RASSF1* methylation was significantly associated with increased asbestos body count. Furthermore, significant, independent associations between older patient age and methylation of *CCND2* and *RASSF1* were observed. The association between older age and methylation is not unexpected since it is known that CpG island hypermethylation often increases with age (Holliday 1985; Issa 2000). Also, in another report of TSG methylation in MPM, Toyooka *et al.* reported levels of methylation at the *APC*, *CDKN2A*, and *CDKN2B* genes similar to ours (Toyooka, Pass et al. 2001).

One great advantage of this study was the availability of a quantitative measure of asbestos exposure. Ferruginous asbestos bodies form as a result of the interaction of macrophages with asbestos fibers, and presence of asbestos bodies is an indicator of past exposure to asbestos. By quantifying their level, we are able to estimate the degree of asbestos fiber burden in an individual (Dodson, Atkinson et al. 2005). In our data, tumors with methylation have significantly higher asbestos exposure, using the asbestos body counts as a quantitative measure of burden. Furthermore, there was a significant trend between increasing number of methylated cell cycle TSGs (0 to 1 to >1), and increasing asbestos body count. Finally, an ordered logistic regression model controlling for gender, and tumor histology, showed that both age and an increasing asbestos body count are independent significant predictors of an increased number of methylated cell cycle pathway genes in MPM.

Hence, these data suggest that the induction of methylation in a phenotypically important pathway might occur as a result of physical interaction between asbestos fibers and the parietal pleura. However, precisely how any exposure selects TSGs for silencing has only recently begun to be explored. Maintenance of control over the cell cycle is critical to tumor suppression, but the relationship between dynamic carcinogen exposure and the targeting and induction of tumor-specific methylation profiles is likely to be highly complex. Asbestos exposure is associated with chronic inflammation (Sabo-Attwood, Ramos-Nino et al. 2005), and the physical presence of asbestos fibers at the interface of the mesothelial membrane and the lung induces a dose dependent cycle of death and re-growth of mesothelial cells in the area of fiber deposition (Adamson, Bakowska et al. 1993). Additionally, persistent mitotic stimulation of mesothelial cells after direct physical insult, and reactive oxygen species

generated by the fiber-clearance-related cellular response, may induce a reaction by mesothelial cells akin to that of cells in culture subject to repeated cycles of growth. Repeated passaging of cells in tissue culture, similar to the process of aging, is associated with the induction of TSG silencing by promoter methylation (Baylin 2002). The known decades-long latency of MPM then suggests that there is ample time for appreciable fields of clonally altered cells to accumulate, perhaps leading to malignancy through a combination of acquired genetic and epigenetic alterations enhanced by repeated mitotic selection. Additionally, asbestos fibers are known to be clastogenic and lead to genotoxic damage, and tumors with higher asbestos fiber burden may be induced to grow faster, possibly leading to the preferential selection of clones with silenced cell cycle control TSGs. While these mechanisms of clonal selection for epigenetic silencing are consistent with our data, it does not necessarily imply any direct asbestos fiber interaction with the histone / DNA methylation machinery, but instead that the chronic inflammation response and / or accelerated tumor growth related to asbestos burden may select for cells capable of continued proliferation.

In summary, using a directed pathway-based approach to methylation analysis, and a quantitative measure of asbestos exposure, we observed that methylation silencing of cell cycle TSGs is associated with both older age and asbestos exposure in MPM. Our data, using a quantitative measure of asbestos exposure, demonstrate that epigenetic gene inactivation is a crucial and novel mechanism for asbestos action in the genesis of this rapidly fatal cancer.

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Chapter 4

Epigenetic alteration profiles distinguish pleural mesothelioma from normal pleura and predict lung asbestos burden and clinical outcome

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Epigenetic alteration profiles distinguish pleural mesothelioma from normal pleura and predict lung asbestos burden and clinical outcome

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Abstract

Cancers originate through clonal alterations in both the cellular genome and epigenome. While many human carcinogens are known mutagens, the mechanisms of action of non-mutagenic carcinogens such as asbestos, remain poorly characterized. We have studied mesothelioma, a rare but extremely fatal and costly disease caused by asbestos and known to have limited numbers of genetic mutations aiming to characterize the relationships among gene-locus specific methylation silencing of tumor-associated genes, disease status, asbestos burden, and survival. 1505 CpG loci associated with 803 cancer-related genes were studied in 158 pleural mesotheliomas and 18 normal pleura. Classifying samples based upon CpG methylation profile with a mixture model approach, methylation classes discriminated tumor from non-tumor pleura (permutation test $P < 0.0001$). After false-discovery rate correction, 969 CpG loci were independently associated with disease status (Q-value < 0.05), demonstrating that utilizing profiles of methylation is a powerful method for differentiating disease from normal tissue. Among tumors (n=158), methylation class membership was significantly associated with lung tissue asbestos body burden ($P < 0.03$), and significantly predicted survival (likelihood ratio $P < 0.01$). Consistent with prior work, asbestos burden was associated with an increased risk of death (HR = 1.4, 95% CI, 1.1 – 1.8). Our results have shown that methylation profiles powerfully differentiate diseased pleura from non-tumor pleura, and that asbestos burden and methylation profiles are independent predictors of mesothelioma patient survival. We have also shown that cellular epigenetic dysregulation is a critical mode of action for asbestos in the induction of pleural mesothelioma.

Introduction

A central tenet of cancer biology states that cancer is clonal, with tumors arising as the result of expansion of increasingly dysregulated cells. This insight yielded a now well known paradigm that selective expansion of cells with a growth advantage occurs in an ordered fashion, driven primarily by genetic changes (Fearon and Vogelstein 1990). This model has expanded to now include the thesis that cancers also evolve a "mutator phenotype" and become malignant as a result of somatic genetic events (Loeb 1991). While this is almost certainly true of some cancers, particularly those induced by well characterized mutagens (e.g. tobacco smoke and ionizing radiation), other known human carcinogens are not mutagenic (or are very poor mutagens) and may be less prone to induce cancers via this mechanism. Asbestos, which is known to induce mesothelioma, is an example of a non-mutagenic carcinogen. In the case of carcinogens such as asbestos, it may be that dysregulation of the somatic epigenome is equally, if not more crucial for cancer development.

Aberrant epigenetic events, including DNA hypermethylation-induced gene silencing, are well recognized as important contributors to carcinogenesis. Methylation associated gene silencing occurs when certain cytosines in specific clustered regions primarily located in gene promoters are hypermethylated. These regulatory CpG islands often occur in tumor suppressor genes and are thought to remain largely unmethylated in noncancerous cells. Approximately half of all human genes contain CpG islands and are, therefore, potentially subject to this type of aberrant silencing (Bird 2002; Jones and Baylin 2002). Recent technologic advances allow for the simultaneous resolution of hundreds of specific,

phenotypically defined cancer-related methylation events, providing a platform for the rapid epigenetic profiling of gene silencing in human tumors (Bibikova, Lin et al. 2006).

Malignant pleural mesothelioma is a rapidly fatal malignancy associated with asbestos exposure in approximately 80% of patients (Tammilehto, Maasilta et al. 1992; Robinson and Lake 2005). In the United States, Great Britain, and Japan, over 5000 cases occur annually and median survival of patients with pleural mesothelioma is less than one year (Pelin, Hirvonen et al. 1994; Price 1997; Morinaga, Kishimoto et al. 2001; Roushdy-Hammady, Siegel et al. 2001; Price and Ware 2004). The economic burden of treating this disease and the litigation associated with asbestos exposure is estimated to exceed $265 billion over the next four decades in the United States (Bhagavatula, Moody et al. 2001). Despite the decline in asbestos use among industrialized nations, the incidence of mesothelioma continues to rise, and it is not expected to peak until 2020, as disease latency can be as long as fifty years (Peto, Decarli et al. 1999; Kukreja, Jaklitsch et al. 2004). Importantly, asbestos is currently mined and exported throughout the world, with heavy use evident in developing nations such as China, India, and Central America (Joshi and Gupta 2004; Kazan-Allen 2005). Asbestos-containing products are still imported to the U.S., and many asbestos exposure hazards remain from earlier applications; one well publicized example being dust from the World Trade Center towers collapse in New York City (Landrigan, Lioy et al. 2004). A more complete understanding the molecular-genetic consequences of asbestos exposure and the mechanism of action of these mineral fibers in inducing mesothelioma is critically needed to develop more effective approaches for identifying and treating this devastating disease.

The causal link between asbestos and pleural mesothelioma has been widely accepted since 1960 (Wagner, Sleggs et al. 1960), and the carcinogenic mechanisms of asbestos have been investigated in earnest since that time; establishing that asbestos fibers are not point mutagens, but rather both clastogenic and cytotoxic *in vitro* (Kelsey, Yano et al. 1986; Jaurand 1997). Additionally, methylation-induced tumor suppressor gene silencing has been observed in recent studies of mesothelioma (Ohta, Shridhar et al. 1999; Murthy, Shen et al. 2000; Toyooka, Pass et al. 2001; Hirao, Bueno et al. 2002; Toyooka, Carbone et al. 2002; Wong, Zhou et al. 2002; Lee, He et al. 2004; Shivapurkar, Toyooka et al. 2004; He, Lee et al. 2005; Shigematsu, Suzuki et al. 2005; Suzuki, Toyooka et al. 2005; Tsou, Shen et al. 2005; Tsou, Galler et al. 2007; Christensen, Godleski et al. 2008) leading to the hypothesis that asbestos fibers contribute to epigenetic silencing of tumor suppressor genes in this disease. Consistent with this, Tsou *et al.* observed a significant association between self-reported asbestos exposure and methylation at the *MT1A*, and *MT2A* gene loci in mesotheliomas (Tsou, Galler et al. 2007). Concomitantly, work in our laboratory, using quantitative asbestos body counts as a measure of asbestos exposure burden, revealed an association between cell cycle control tumor suppressor gene methylation and increased asbestos burden in mesothelioma (Christensen, Godleski et al. 2008).

To comprehensively investigate aberrant tumor-specific, phenotypically relevant methylation events in pleural mesothelioma, we profiled 158 tumors and 18 non-tumorigenic parietal pleura samples for methylation at 1505 CpG dinucleotides associated with 803 cancer-related genes using the Illumina GoldenGate® methylation bead array. We have definitively delineated the relationship between a comprehensive, phenotypically important

CpG methylation profile and disease status, in addition to defining the tumor methylation profiles associated with patient clinical course and asbestos exposure.

Materials and methods

Study population

Tumor material was obtained following surgical resection at Brigham and Women's Hospital through the support of the International Mesothelioma Program. Similarly, grossly non-tumorigenic parietal pleura samples were taken as residual tissue during extrapleural pneumonectomy from uninvolved anatomic sites. All patients provided informed consent under the approval of the appropriate Institutional Review Boards. Clinical information, including histologic diagnosis was obtained from pathology reports. Each patient was assessed for history of exposure to asbestos as well as additional demographic and environmental data by obtaining their medical and occupational history with an in-person questionnaire or interview. Additionally, we quantified asbestos bodies in samples of lung tissue from multiple sites in the resected lung (De Vuyst, Karjalainen et al. 1998) as previously described (Churg and Warnock 1977). Each tumor was pathologically examined and the amount of tumor in every sample estimated by direct microscopic evaluation and recorded as the percent tumor for that specimen. Patients were followed for survival using the National death index and last known clinic visit.

Methylation analysis

Tumor and non-tumor pleural DNA was extracted from frozen tissue using the QIAamp DNA mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA). DNA was modified by sodium bisulfite to convert unmethylated cytosines to uracil using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. Illumina GoldenGate® methylation bead arrays were used to simultaneously interrogate 1505 CpG loci associated with 803 cancer-related genes. Bead arrays have a

similar sensitivity as quantitative methylation-specific PCR and were run at the UCSF Institute for Human Genetics, Genomics Core Facility according to the manufacturer's protocol and as described by Bibikova *et al* (Bibikova, Lin et al. 2006).

Statistical analysis

BeadStudio Methylation software from the array manufacturer Illumina (SanDiego, CA) was used for dataset assembly. All array data points are represented by fluorescent signals from both M (methylated) and U (unmethylated) alleles, and methylation level is given by $\beta = (\max(M, 0))/(|U| + |M| + 100)$, the average methylation (β) value is derived from the ~30 replicate methylation measurements and a Cy3/Cy5 methylated/unmethylated ratio. At each locus for each sample the detection *P*-value was used to determine sample performance, three samples (2%) with >25% of loci having a detection *P*–value > 1e-5 were dropped from analysis. Similarly, CpG loci with a median detection *P*–value > 0.05 (n=8, 0.5%), were eliminated from analysis.

Subsequent analyses were carried out using the R software (R Development 2007). For exploratory and visualization purposes, hierarchical clustering was performed using R function *hclust* with Manhattan metric and average linkage. For inference, data were clustered using a mixture model (Siegmund, Laird et al. 2004) with a mixture of beta distributions (Ji, Wu et al. 2005),and the number of classes was determined by recursively splitting the data via 2-class models, with Bayesian information criterion (BIC) used at each potential split to decide whether the split was to be maintained or abandoned (Fraley and Raftery 2002; Houseman, Coull et al. 2006; Houseman, Christensen et al. 2008). Permutation tests (running 10,000 permutations) were used to test for association with methylation class by generating a distribution of the test statistic for the null distribution for comparison to the

observed distribution. For continuous variables, the permutation test was run with the Kruskal-Wallis test statistic. For categorical variables we used a Mutual information test statistic; (Yao 2003) equivalent to a likelihood ratio test comparing the saturated model for a contingency table against a model that assumes independent margins. Significant associations from permutation tests were controlled for potential confounders where appropriate using logistic regression with methylation classes and potential confounders and a likelihood ratio test of the model with and without methylation classes. For survival analyses, Cox proportional hazards models were utilized, and likelihood ratio tests were used to examine the significance of inclusion of the methylation classes in the models.

Associations between sample type, or covariates such as age or gender and methylation at individual CpG loci were tested with a generalized linear model (GLM). The beta-distribution of average beta values was accounted for with a quasi-binomial logit link with an estimated scale parameter constraining the mean between 0 and 1, in a manner similar to that described by Hsuing *et al.* (Hsiung, Marsit et al. 2007). CpG loci where an *a priori* hypothesis existed were tested independently. In contrast, array-wide scanning for CpG loci associations with sample type or covariate used false discovery rate correction and *Q*–values computed by the *qvalue* package in R.(Storey, Taylor et al. 2004)

Results

To characterize the epigenetic profile of mesothelioma and non-tumorigenic parietal pleura we used the Illumina GoldenGate® bead array that simultaneously interrogates 1505 CpG sites associated with 803 cancer-related genes to generate a methylation value based upon ~30 replicate measurements for each locus in each sample. In this study GoldenGate® arrays were used to assess methylation in 158 incident cases of mesothelioma and 18 non-tumorigenic parietal pleura specimens. Exposure, demographic and tumor characteristic data for these samples are presented in Table 4.1.

Table 4.1. Mesothelioma and non-tumor pleural sample demographic data

	Mesothelioma patients	Pleura donors
Gender, n (%)		
Female	38 (24)	4 (22)
Male	120 (76)	14 (78)
Age		
Range	30 – 80	38 – 77
Mean (sd)	62 (9.8)	58 (11.3)
Histology, n (%)		
Epithelioid	109 (69)	-
Mixed	44 (28)	-
Sarcomatoid	5 (3)	-
Asbestos exposure, n (%)		
Yes	112 (74)	13 (72)
No	39 (26)	5 (28)
Log Asbestos Body		
Available n (%)	108 (68)	-
Range	0 – 5.5	-
Mean (sd)	2.16 (1.18)	-

Array methylation data were first explored with unsupervised hierarchical clustering using Manhattan distance and average linkage for the 750 most variable autosomal CpG loci (Figure 4.1).

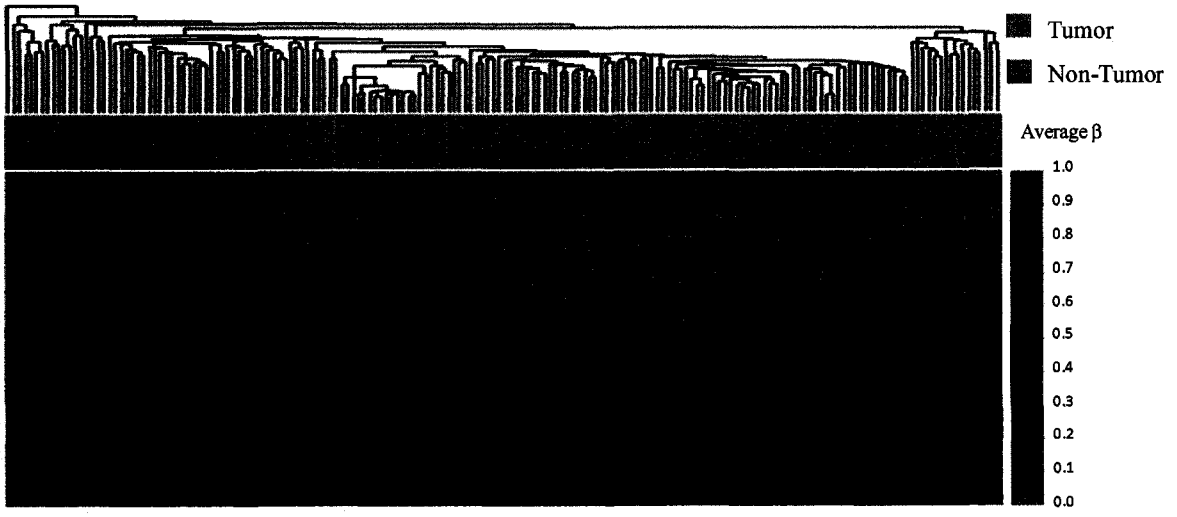

Figure 4.1. Unsupervised clustering of average beta values in tumor and non-tumor pleura. Using the R software package normal tissue sample average beta values were subjected to unsupervised hierarchical clustering based on Manhattan distance and average linkage. Each column represents a sample and each row represents a CpG locus (750 most variable autosomal loci). Above the heatmap blue indicates a tumor sample, and purple indicates a non-tumor pleural sample. In the heat map green = average beta of zero, or unmethylated, and red = average beta of one, or methylated

Striking differences between the epigenetic profiles of mesothelioma and non-tumor pleura are observed, with almost perfect clustering of epigenetic profiles based on disease status. Next, in a univariate approach, we tested all CpG loci individually for an association between methylation and disease status, and 969 CpG loci had methylation levels that differed ($Q <$

0.05) comparing tumor and non-tumor pleura following FDR correction. Of these, 727 loci associated with 493 genes had enhanced methylation in non-tumor pleura, and 242 loci associated with 153 genes had more methylation in the tumors (Supplemental Table S1). Since so many loci were differentially methylated between tumor and non-tumor pleura, we next applied a modified model-based form of unsupervised clustering known as mixture modeling. This approach built classes of samples based on profiles of methylation with data from all autosomal loci using a mixture of beta distributions to recursively split the tumors into parsimoniously differentiated classes (Siegmund, Laird et al. 2004; Shen, Toyota et al. 2007; Siegmund, Connor et al. 2007). All posterior class membership probabilities were numerically indistinct from 0 or 1. Applying a beta mixture model to methylation data from all autosomal loci in tumors and non-tumor pleura returned eleven methylation classes, their average methylation profiles, and their sample type distributions (Figure 4.2). Methylation class membership was a highly significant predictor of diseased versus non-diseased tissue (permutation $P < 0.0001$). Among the 11 classes in the model, 9 classes perfectly captured only tumor or only normal, and there were 2 methylation classes containing both tumor and normal samples.

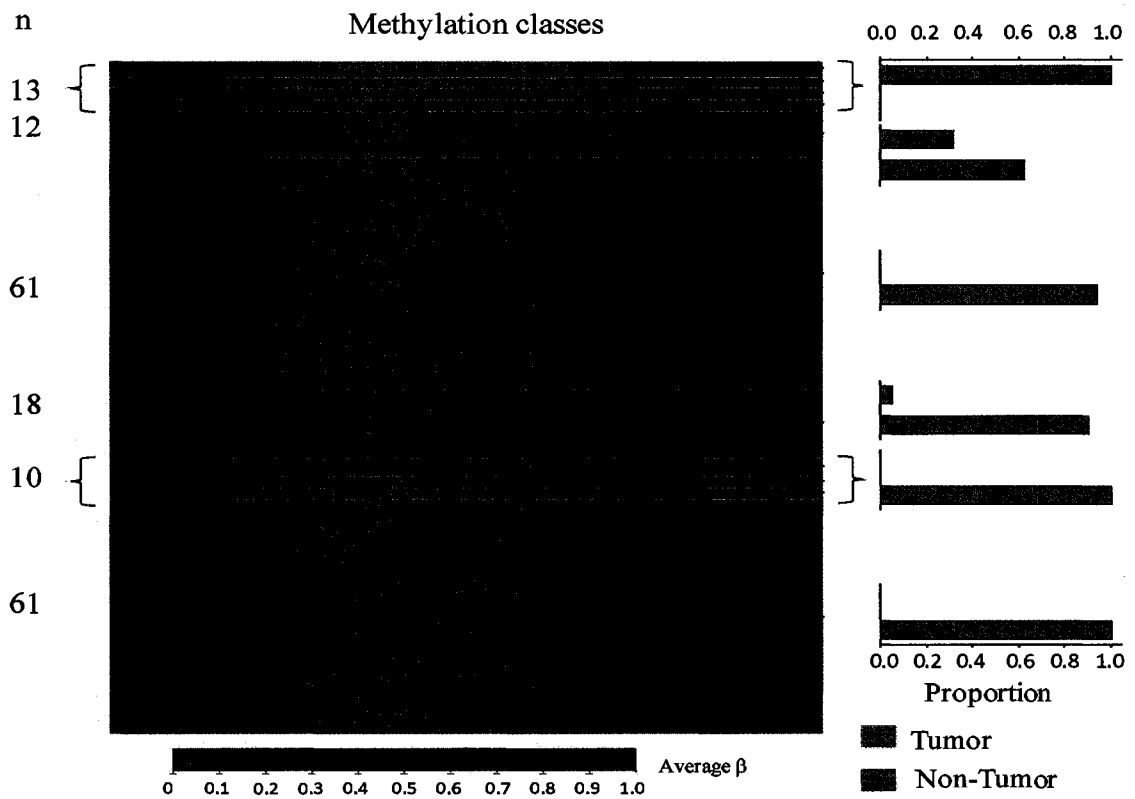

Figure 4.2. Beta mixture model of methylation profiles in mesothelioma and non-tumor pleura. Methylation average β is green for unmethylated and red for methylated. Methylation profile classes are stacked in rows separated by yellow lines, and class height corresponds to the number of samples in each class. Class methylation at each locus is a mean of methylation for all samples within a class. Bar charts display the proportion of tumors and non-tumor pleura samples in each class. Methylation profile classes differentiate tumor from non-tumor pleura ($P < 0.0001$).

We next restricted our analyses to tumors, (n = 158) first applying our beta mixture model approach, and Figure 4.3 shows the seven methylation classes that resulted. This figure also displays the distributions of gender, histology, and asbestos body counts by

methylation class. Methylation class membership was not a significant predictor of patient gender or tumor histology (data not shown).

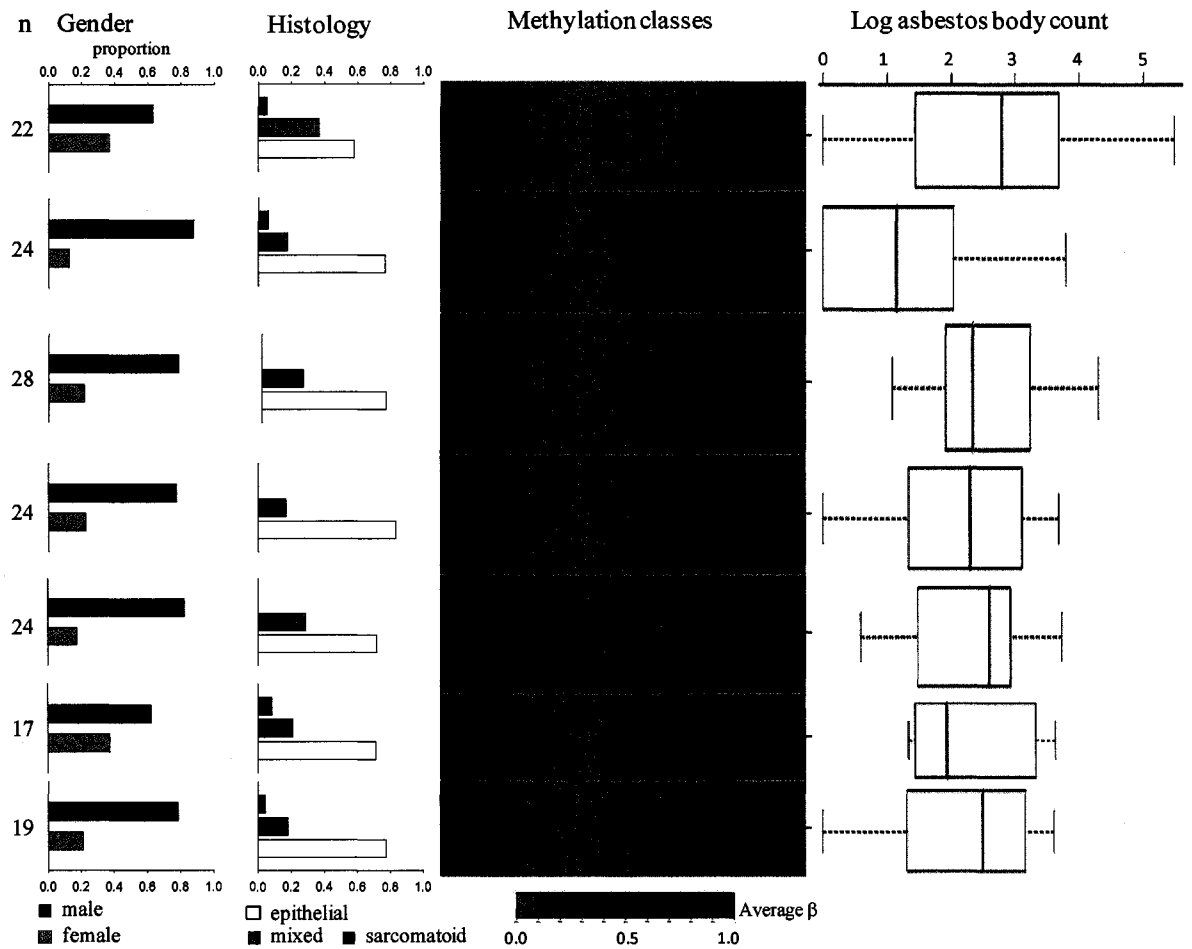

Figure 4.3. Beta mixture model of methylation profiles in pleural mesothelioma. Methylation average β is green for unmethylated and red for methylated. Methylation profile classes are stacked in rows separated by yellow lines, and class height corresponds to the number of samples in each class. Class methylation at each locus is a mean of methylation for all samples within a class. On the left, bar charts show proportions for gender and tumor histology among samples within each class. On the right, box plots of log asbestos body counts for each class. Controlling for gender, methylation class membership predicts asbestos burden ($P < 0.03$).

Again, methylation profile class membership was not associated with the amount of tumor in the sample. However, methylation class membership significantly predicted lung asbestos body count (permutation $P < 0.04$). Since men with pleural mesothelioma have higher asbestos body counts compared to women ($P < 0.0001$) (Christensen, Godleski et al. 2008) we controlled for gender, and methylation class membership remained a significant predictor of asbestos burden (likelihood ratio test $P < 0.03$). Based upon prior published work, specific CpG loci were tested for associations between methylation and asbestos body counts; consistent with our prior data, (Christensen, Godleski et al. 2008) tumor methylation average β values at *CDKN2A* ($P < 0.02$), *CDKN2B* ($P < 0.02$), and *RASSF1* ($P < 0.03$) were significantly and positively associated with asbestos body counts. In addition, methylation of *MT1A* (previously reported as asbestos exposure-associated by Tsou *et al.* (Tsou, Galler et al. 2007)) was significantly positively associated with asbestos burden; promoter associated CpG49 ($P < 0.04$), and exonic CpG13 ($P < 0.02$). When testing all autosomal loci for an association between methylation and asbestos burden using the *MTA1* promoter CpG 49 *Q*–value ($Q = 0.32$) as a cutoff, there were 110 loci with an association between methylation status and asbestos burden (Supplemental Table S2). The vast majority of these 110 loci (94%) had a positive correlation between CpG methylation and asbestos body counts, indicating gene silencing was the overwhelming dominant phenotype associated with asbestos associated epigenetic change.

Lastly, we examined the relationships between methylation profiles and patient outcome using Cox proportional hazards models of survival controlling for age, gender, and tumor histology. In a proportional hazards model including all cases (n = 158), women had half the risk of death of men (HR = 0.5, 95% CI, 0.3 – 0.96), and patients with mixed

histology tumors were at greater risk of death compared to those with epithelial tumors (HR = 2.7, 95% CI, 1.7 – 4.4). Importantly, methylation class membership was also a significant predictor of patient outcome ($P < 0.01$). In particular, membership in methylation classes four and seven were both independently associated with a significant 3-fold increased risk of death compared to the class with the lowest median asbestos count (95% CIs, class four: 1.4 – 7.0, class seven: 1.3 – 7.4) (Table 4.2).

Table 4.2. Patient gender, tumor histology and methylation profile class membership predict patient survival in pleural mesothelioma.

Co-Variate	n (%) Total n=158	HR	95% CI	p-value
Age, mean (sd)	62 (9.8)	1.02	1.0 - 1.05	0.09
Gender				
Male	120 (76)	1.0 (reference)	-	-
Female	38 (24)	0.5	0.3 - 0.96	< 0.04
Histology				
Epithelial	109 (69)	1.0 (reference)	-	-
Mixed	44 (28)	2.7	1.7 - 4.4	< 0.0001
Sarcomatoid	5 (3)	2.8	0.95 - 8.2	0.06
Methylation Class				
2	24 (15)	1.0 (reference)	-	-
1	22 (14)	1.4	0.6 - 3.4	0.47
3	28 (18)	0.9	0.4 - 2.0	0.75
4	24 (15)	3.1	1.4 - 7.0	< 0.01
5	24 (15)	1.4	0.6 - 3.5	0.44
6	17 (11)	2.0	0.8 - 5.4	0.16
7	19 (12)	3.1	1.3 - 7.4	< 0.01

Controlled for all variables in table, model log liklihood $P < 0.01$
Class numbers are 1 to 7, top to bottom from figure 4.3

Where data were available (n = 108), and after adjustment for methylation class membership, asbestos burden was associated with a significant 1.4-fold increased risk of death (95% CI, 1.1 – 1.8) (Table 4.3). In this model, membership in methylation class four remained associated with a significant, nearly 3-fold increased risk of death (HR = 2.8, 95% CI, 1.1 – 7.1). Again, in this model including asbestos exposure, likelihood ratio tests indicate that methylation classes were significant predictors of patient outcome ($P < 0.005$).

Table 4.3. Asbestos burden and methylation profile class membership predict patient survival in pleural mesothelioma.

Co-Variate	n (%) Total n=108	HR	95% CI	p-value
Age, mean (sd)	61 (9.5)	1.03	1.0 - 1.1	0.18
Gender				
Male	84 (78)	1.0 (reference)	-	-
Female	24 (22)	1.5	0.6 - 3.5	0.38
Histology				
Epithelial	74 (68)	1.0 (reference)	-	-
Mixed	31 (29)	2.1	1.2 - 3.8	< 0.02
Sarcomatoid	3 (3)	1.2	0.3 - 5.2	0.83
Asbestos burden	2.2 (1.2)	1.4	1.1 - 1.8	< 0.04
Methylation Class				
2	17 (16)	1.0 (reference)	-	-
1	10 (9)	0.5	0.1 - 2.2	0.37
3	19 (18)	0.4	0.1 - 1.2	0.11
4	24 (22)	2.8	1.1 - 7.1	< 0.03
5	17 (16)	0.9	0.3 - 2.8	0.89
6	11 (10)	1.2	0.3 - 4.8	0.79
7	10 (9)	1.7	0.6 - 5.0	0.36

Controlled for all variables in table, model log liklihood $P < 0.005$
Class numbers are 1 to 7, top to bottom from figure 4.3

Discussion

Exposure to asbestos is the single most important risk factor for pleural mesothelioma and prior research has established that somatic mutations (Sugarbaker, Richards et al. 2008) and alterations in gene expression (Gordon, Rockwell et al. 2005) are a feature of this disease. Interestingly, relatively few pathologically important mutations arise in this cancer, and there is no characteristic somatic genetic change that can be attributed to the action of asbestos (Sugarbaker, Richards et al. 2008). Further, although there is consensus that gene expression (at the mRNA level) is significantly altered in mesothelioma, there is no gene expression signature representative of the action of asbestos in this disease, and there remains debate about the clinical utility of mRNA expression profiling (Gordon, Jensen et al. 2003; Gordon, Rockwell et al. 2005; Lopez-Rios, Chuai et al. 2006). Our work sought to definitively characterize phenotypically important alterations in the epigenome of mesothelioma. We enumerated the epigenetic status of over 800 known cancer-related genes that stably control mRNA expression; comparing normal pleura with mesotheliomas. Our findings indicate that an extremely large number of loci are epigenetically altered in mesothelioma, that asbestos exposure is associated with the degree of epigenetic alteration, and finally, that profiles of gene silencing are associated with clinical outcome. This work demonstrates that the epigenome is a primary target of asbestos in the genesis of mesothelioma.

Precisely why epigenetic alterations are a prominent feature of pleural mesothelioma is not clear. However, it is well known that chronic inflammation is a primary tissue response to asbestos exposure (Moalli, MacDonald et al. 1987; Antony 2003; Sabo-Attwood, Ramos-Nino et al. 2005). Epigenetic alterations have been associated with inflammation in colon

cancer, and it has been suggested that inflammation-related epigenetic alterations are common in human cancers (Issa, Ahuja et al. 2001; Issa 2008). Infiltrating macrophages, neutrophils, and eosinophils have been observed at sites of fiber deposition; these cells are known to generate reactive oxygen, and nitrogen, and to induce the release of peroxidases (Moalli, MacDonald et al. 1987; Quinlan, Marsh et al. 1994; Macdonald and Kane 1997). Treatment of human mesothelial cells with crocidolite asbestos has been shown to increase reactive oxygen species and oxidized DNA residues such as 8-oxoguanine (Chen, Marsh et al. 1996). Further, it has been reported that 5-hydroxymethylcytosine can be generated by oxidation of 5-methylcytosine (Penn, Suwalski et al. 1972; Masuda, Shinoara et al. 1975). Both 5-methylcytosine adjacent to 8-oxoguanine, and 5-hydroxymethylcytosine have been shown to inhibit binding of methyl-CpG binding protein 2, a critical epigenetic regulator that recruits cytosine methyltransferases and histone deacetylases (Valinluck, Tsai et al. 2004). It is also known that 5-hydroxymethylcytosine is not recognized as 5-methylcytosine by the maintenance methyltransferase DNMT1, and hence, may lead to aberrant loss of methylation during cell replication (Valinluck and Sowers 2007). Additional base alterations occur via neutrophil and eosinophil peroxidase-derived HOCl and HOBr which can react with DNA to form 5-chlorocytosine and 5-bromocytosine respectively (Henderson, Byun et al. 2003). These halogenated cytosines can be mistaken by DNMT1 as 5-methylcytosine during replication, thus providing a potential mechanism for asbestos-related, inflammation-induced aberrant hypermethylation (Valinluck and Sowers 2007). This is consistent with our data suggesting that asbestos burden (and the resultant inflammatory response) is associated with epigenetic alterations. Furthermore, the decades-long latency of MPM would allow ample

time for cellular turnover and selection of cells with altered epigenetic programs that favor survival and deregulated proliferation.

We also found that epigenetic profiles differentiate tumor from non-tumor pleura ($P < 0.0001$). Similarly, a genome-wide approach profiling of gene expression at the mRNA level in mesothelioma has demonstrated significant differential expression of over 600 genes between mesothelioma and non-tumor pleura, although the precise pattern of gene expression differs in the hands of different investigators (Gordon, Rockwell et al. 2005; Lopez-Rios, Chuai et al. 2006). Epigenetic gene inactivation is inherently more stable and less prone to variability than measures of mRNA, making our approach for differentiating tumors from non-tumor pleura likely more reproducible. Although our method differentiates tumors from non-tumor pleura extremely well, there remained two methylation profile classes with a minor mixture of tumor and non-tumor samples. It is clear that "contaminating" non-tumor tissue, or samples containing a lower percent tumor cannot explain this result, as we were able to reject this based upon direct observation of the amount of tumor in each specimen. Consequently, it may be that the relative epigenetic similarity between certain tumors and non-tumor pleura is explained by a difference in the prevalence of somatic genetic aberrations (such as gene deletions) among these tumors. In other words, tumors resembling non-tumor pleura epigenetically may harbor significantly greater genetic alterations (relative to more epigenetically divergent tumors) that contribute to their malignant phenotype. An integrative genomics approach using common tumor sets for both epigenetic alteration profiling and genome-wide copy number alteration profiling will begin to address this question.

Hundreds of loci had different methylation levels between tumor and non-tumor pleura after correcting for multiple comparisons ($Q < 0.05$). Some of the pathways and processes whose genes are differentially methylated include epigenetic regulation, cell cycle control, and inflammation, among others. For instance, *HIC1* was hypermethylated in tumors, which may prevent its transcriptional repression of the histone deacetylase SIRT1, and *DNMT3B* had significantly lower methylation in tumors, suggesting epigenetic dysregulation. Cell cycle control genes *WEE1* and *RASSF1* both had significantly higher methylation in tumors, suggesting uncontrolled proliferative potential, a hallmark of cancer (Hanahan and Weinberg 2000). Another hypermethylated gene in tumors was *ASB4*; a SOCS box-containing protein that inhibits c-Jun N-terminal kinases, is thought to contribute to chronic inflammation (Johnson and Nakamura 2007; Li, Chai et al. 2007). Among tumors, *JAK2* had significantly decreased methylation, and concomitant silencing of SOCS genes may then result in increased, or constitutive STAT activation, which is a recognized inflammation-associated mechanism of tumor initiation and promotion (Yoshimura, Naka et al. 2007). In addition, *HOXA9* was hypermethylated in tumors; HOXA9 is an inhibitor of NF-κB-dependent activation of leukocyte adhesion molecules ICAM-1, VCAM-1 and E-selectin which recruit leukocytes, exacerbating the inflammatory response (Trivedi, Patel et al. 2007).

At the same time, it is tenable to posit that, although many genes harbor altered epigenetic states, only a small number of genes with epigenetic alterations are chiefly responsible for the phenotypic differences between tumor and normal tissue, and between more deadly mesothelioma compared with less deadly forms. However, if relatively few "epigenetic gatekeepers" determine phenotype, there must be a very large number of genes

that are inactivated consequent to the clonal evolution of the tumor; this may be analogous to 'hitchhiker' mutations that arise during tumor clonal expansion. Alternatively, a 'hit' at a locus essential for epigenetic regulation, the chronic inflammatory process, or altered tumor metabolism, for example, may induce a change in overall epigenetic regulation that results in the targeting and aberrant methylation of many genes in a wholesale fashion. It is not possible to directly distinguish the alternative explanations of our data.

Among the 158 mesotheliomas studied, seven distinct methylation profile classes were identified. Previous genome-wide mRNA expression analysis of mesotheliomas was able to define two distinct subclasses of tumors that loosely correlated with histology (Gordon, Rockwell et al. 2005). Although our epigenetic profiling data revealed more tumor subclasses, the genome-wide approach using mRNA expression as an outcome encompassed over ten-times as many loci, and used fewer tumors, both of which potentially decreased the power to resolve tumor subclasses compared to our larger sample set restricted to cancer-associated loci. We found that methylation profiles were associated with asbestos body burden in an analysis controlling for gender ($P < 0.03$), again suggesting asbestos-associated inflammation as a mechanism driving tumors into distinct epigenetic subclasses. There were also distinct methylation profile classes comprised of tumors with similar asbestos burden distributions, arguing that asbestos burden *per se* does not account for all of the observed epigenetic alterations in mesothelioma. However, in the context of a decades-long latency and a non-specific, asbestos-related chronic inflammation state, alterations of different epigenetic gatekeeper genes could occur at different stages in tumorigenesis and tumor progression. Furthermore, specific genes, or gene pathways, may be more likely to be selected for, or more susceptible to asbestos-related epigenetic alterations; consistent with

this, associations between CpG methylation of specific genes and asbestos previously reported by our group and others were confirmed in this study (Tsou, Galler et al. 2007; Christensen, Godleski et al. 2008). Here, we extended these findings to include several apoptosis-related genes such as *AATK*, *CASP2*, *CASP10*, and *PYCARD*.

Finally, overall methylation profile class membership was a significant, independent predictor of patient survival ($P < 0.01$), suggesting that epigenetic dysregulation is strongly associated with disease progression. Although general epigenetic deregulation may be highly correlated with disease prognosis, we cannot exclude epigenetic events at specific loci as responsible for predicting survival. Consistent with our previous finding, we confirmed that an increased asbestos burden is a significant, independent predictor of reduced survival in pleural mesothelioma (Christensen, Godleski et al. 2008).

In summary, our data show that epigenetic alterations are extraordinarily common in mesothelioma and discriminate the malignant phenotype from normal pleura. Epigenetic changes are also significantly associated with asbestos burden and significantly predict clinical outcome. Hence, our data indicate that phenotypically important somatic epigenetic modification is a major mode of action of asbestos in mesothelioma, strongly suggesting that investigation of the underlying mechanism responsible will assist in diagnosis, assessment of prognosis and design of therapies for this rare, but rapidly fatal disease.

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Table S1. Loci with signifiacntly different methylation between tumor and non-tumor pleura (Q - value ranked)

Significantly higher methylation in: Non-tumor pleura, **Tumor**

Rank[a]	*GENE*	CpG[b]	Rank[a]	*GENE*	CpG[b]
1	*ADAMTS12*	E52	34	*RARB*	P60
2	*APC*	P280	35	*RARRES1*	P57
3	*APP*	E8	36	*RIPK2*	E123
4	*BCAM*	E100	37	*SFN*	P248
5	*CARD15*	P302	38	*SHB*	P473
6	*CCND3*	P435	39	*SKI*	E465
7	*CCNE1*	P683	40	*SMARCA3*	P109
8	***CSF3***	**E242**	41	*SPARC*	E50
9	*CTNNA1*	P382	**42**	***SRC***	**E100**
10	*EPHB4*	E476	43	*TGFA*	P558
11	*EPS8*	E231	44	*TGFB2*	P632
12	*EPS8*	P437	45	*TGFBR3*	E188
13	*FANCG*	E207	46	*TJP2*	P330
14	*FER*	E119	47	*TJP2*	P518
15	*GAS1*	E22	48	*TNFRSF10B*	E198
16	*HIC1*	P565	49	*TRAF4*	P372
17	*HPSE*	P29	50	*TRIP6*	E33
18	*ID1*	P880	51	*TUBB3*	P721
19	***IL18BP***	**E285**	52	*WNT5A*	E43
20	*IL8*	P83	53	*YES1*	P600
21	*ITGA2*	P26	54	*ACVR2B*	E27
22	*JAG1*	P66	55	*BCL3*	E71
23	*LAMB1*	E144	56	*CASP8*	E474
24	*MCM2*	P241	**57**	***CPA4***	**P961**
25	*MCM2*	P260	58	*FES*	E34
26	*MLH3*	P25	59	*GATA6*	P21
27	*MUC1*	P191	60	*ISL1*	E87
28	*PCDH1*	E22	61	*ITGB4*	E144
29	*PCGF4*	P760	62	*PPARD*	P846
30	*PDE1B*	E141	63	*PTCH*	E42
31	*PDGFA*	P841	64	*TJP1*	P326
32	*PRKCDBP*	E206	65	*WNT2B*	P1185
33	*PRKCDBP*	P352	66	*APP*	P179

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Rank[a]	*GENE*	CpG[b]	Rank[a]	*GENE*	CpG[b]
67	*ITGB1*	P451	103	*FGFR2*	P460
68	*PDGFRB*	P273	104	*TSG101*	P257
69	*IGSF4C*	E65	105	*CD34*	E20
70	*TIMP2*	P267	106	*MCC*	E23
71	*CDK10*	E74	107	*PTEN*	P438
72	*INHA*	P1189	108	*NTRK3*	P636
73	*PHLDA2*	E159	109	*FHIT*	P93
74	*PLAU*	P11	110	*GAS1*	P754
75	*DHCR24*	P406	111	*EPHB1*	E202
76	*ITGA6*	P298	**112**	***IL1B***	**P829**
77	*PDGFB*	E25	113	*TIAM1*	P117
78	*ERCC1*	P354	114	*IGF1R*	P325
79	*RAD54B*	P227	115	*TMEFF1*	E180
80	*CD9*	E14	116	*DAB2IP*	E18
81	*GNMT*	E126	117	*APC*	E117
82	*HPSE*	P93	118	*EXT1*	E197
83	*CAV1*	P130	119	*CTGF*	E156
84	*INHA*	P1144	120	*RARRES1*	E235
85	*EPHB2*	P165	121	*CCND1*	E280
86	*CCR5*	P630	122	*PTPN6*	E171
87	*GSTP1*	seq 38	**123**	***MUSK***	**P308**
88	*DSP*	P36	124	*GRB10*	E85
89	*LIG4*	P194	125	*ABL2*	P459
90	*MYCN*	E77	126	*ROR2*	E112
91	*CDK6*	P291	**127**	***DDIT3***	**P1313**
92	*HOXB13*	E21	128	*IRF5*	P123
93	*IGF2R*	P396	129	*EPHB6*	E342
94	*ENC1*	P484	130	*FLT4*	E206
95	*ACVR1C*	P115	131	*DST*	P262
96	*SMO*	E57	132	*FASTK*	P257
97	*ZMYND10*	P329	133	*PSIP1*	P163
98	*EIF2AK2*	E103	134	*IGFBP5*	E144
99	*TGFBI*	P31	135	*CASP6*	P230
100	*CYP1B1*	P212	**136**	***SLC22A2***	**P109**
101	*PDGFRB*	E195	137	*SEMA3B*	P110
102	*P2RX7*	E323	138	*PKD2*	P287

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Rank[a]	*GENE*	CpG[b]	Rank[a]	*GENE*	CpG[b]
139	*TESK2*	P252	175	*PTK7*	E317
140	***ASB4***	**E89**	**176**	***MLH3***	**E72**
141	*ADAMTS12*	P250	177	*BCAM*	P205
142	*ITGB4*	P517	178	*EFNB3*	P442
143	*GADD45A*	P737	179	*BCL3*	P1038
144	*CDH3*	E100	180	*SEMA3B*	E96
145	*ROR2*	P317	181	*PDGFA*	P78
146	*CASP6*	P201	**182**	***FLJ20712***	**P984**
147	*COL6A1*	P425	**183**	***SRC***	**P164**
148	***PSCA***	**P135**	184	*HTR1B*	P107
149	***S100A4***	**P887**	185	*ABCG2*	P178
150	*THBS1*	P500	**186**	***TNFRSF10C***	**P7**
151	*FGFR1*	E317	187	*FVT1*	P225
152	*NOTCH2*	P312	188	*KLF5*	E190
153	***XRCC2***	**P1077**	189	*HBEGF*	P32
154	*BMP4*	P123	190	*TNC*	P57
155	***COPG2***	**P298**	**191**	***MT1A***	**P600**
156	*SLIT2*	E111	192	*CDH1*	P45
157	*MCM6*	E136	193	*MAF*	E77
158	*ITGA2*	E120	194	*GPX1*	E46
159	*EPHB3*	E0	**195**	***PYCARD***	**P150**
160	*VEGFB*	P658	196	*MLH1*	P381
161	*FGFR2*	P266	**197**	***CSF3***	**P309**
162	*CPA4*	E20	198	*STK11*	P295
163	*GNMT*	P197	199	*FANCF*	P13
164	*RASA1*	E107	200	*IGFBP6*	P328
165	*FYN*	P352	201	*IFNGR2*	E164
166	*PTGS1*	P2	202	*PLXDC1*	E71
167	*CDKN1A*	E101	203	*PTCH2*	P568
168	*TGFA*	P642	204	*IL16*	P93
169	***THPO***	**P585**	205	*WNT2B*	P1195
170	*PTPNS1*	E433	206	*E2F5*	P516
171	*FHIT*	E19	207	*HDAC5*	E298
172	*PLAUR*	E123	208	*YES1*	P216
173	*ST6GAL1*	P164	209	*LAMC1*	P808
174	*SPARC*	P195	**210**	***TRPM5***	**P979**

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Rank[a]	*GENE*	CpG[b]	Rank[a]	*GENE*	CpG[b]
211	*ERCC6*	P698	247	*TSG101*	P139
212	*NGFB*	E353	248	*IL8*	E118
213	*TGFBI*	P173	249	*TCF7L2*	P193
214	*DAPK1*	E46	**250**	***CTSH***	**E157**
215	*WNT5A*	P655	251	*SMARCA3*	P17
216	*TP73*	P496	252	*COL1A1*	P117
217	*DIO3*	P90	253	*BMP3*	P56
218	***CDH3***	**P87**	254	*TRIP6*	P1090
219	*WRN*	E57	255	*MGMT*	P272
220	*INSR*	E97	256	*GAS7*	E148
221	***SFTPB***	**P689**	257	*PKD2*	P336
222	*CDK2*	P330	258	*MMP14*	P208
223	*LIF*	P383	259	*ICAM1*	P119
224	***GPR116***	**P850**	260	*COL18A1*	P494
225	***CD34***	**P339**	**261**	***MCC***	**P196**
226	*CDKN1C*	P6	262	*PCGF4*	P92
227	*MLF1*	P97	263	*JAG2*	E54
228	*HLA-DOA*	P191	264	*LRP2*	E20
229	*BAX*	E281	265	*NFKB2*	P709
230	*MALT1*	P406	**266**	***IL18BP***	**P51**
231	*RUNX1T1*	P103	267	*EPHB2*	E297
232	*DST*	E31	268	*ABCA1*	P45
233	*LAMC1*	E466	269	*THY1*	P20
234	*KLF5*	P13	**270**	***HIC-1***	**seq 48**
235	*PLAT*	P80	271	*CTTN*	E29
236	*EPO*	P162	272	*FGFR1*	P204
237	*SMAD4*	P474	273	*MLF1*	E243
238	*ALK*	E183	274	*JAG2*	P264
239	*ETS1*	E253	275	*PTPN6*	P282
240	***PLAGL1***	**P334**	276	*CDC25B*	E83
241	*DDR1*	E23	**277**	***CEACAM1***	**P44**
242	***IGFBP3***	**E65**	278	*SEMA3F*	P692
243	***LMTK2***	**P1034**	279	*TIMP2*	E394
244	*OAT*	P465	280	*DUSP4*	P925
245	*CAV1*	P169	281	*NEO1*	P1067
246	*APC*	P14	282	*SMARCB1*	P220

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Rank[a]	*GENE*	CpG[b]	Rank[a]	*GENE*	CpG[b]
283	*NOTCH3*	P198	319	*BMPR2*	P1271
284	***IFNGR1***	**P307**	320	*TIMP3*	seq 7
285	*E2F3*	P840	321	*CRIP1*	P274
286	*CASP10*	P186	**322**	***GRB10***	**P496**
287	*MCAM*	P169	323	*NRAS*	P103
288	*DAB2IP*	P9	324	*IL6*	E168
289	***P2RX7***	**P597**	325	*TFPI2*	P9
290	*ABO*	E110	**326**	***SLC22A3***	**P634**
291	*NBL1*	P24	327	*SMAD2*	P708
292	*DLL1*	P386	**328**	***PTHLH***	**P757**
293	*DAPK1*	P10	329	*CAPG*	E228
294	*EPHB4*	P313	**330**	***BMPR2***	**E435**
295	***CLDN4***	**P1120**	331	*CSTB*	E410
296	*EPHA5*	E158	**332**	***ASB4***	**P52**
297	***ITGA6***	**P718**	333	*ABCA1*	E120
298	*DHCR24*	P652	334	*HHIP*	E94
299	*MTA1*	P478	335	*CSK*	P740
300	*DDB2*	P407	336	*PTK2B*	P673
301	*SMARCA4*	P362	337	*JAK2*	P772
302	*SNURF*	P78	338	*MAP3K9*	E17
303	*IRF7*	E236	339	*TNFRSF10B*	P108
304	*NKX3-1*	P146	340	*WT1*	P853
305	*ABO*	P312	341	*PTPRF*	E178
306	*p16*	seq 47	**342**	***S100A12***	**P1221**
307	***KRT13***	**P341**	343	*CHI3L2*	E10
308	*TYK2*	P494	344	*FGF12*	E61
309	*PTPRG*	P476	**345**	***DMP1***	**E194**
310	*VAMP8*	E7	**346**	***DMP1***	**P134**
311	*FOSL2*	E384	347	*TRIM29*	P261
312	*LYN*	E353	**348**	***TNFRSF10C***	**E109**
313	*VIM*	P343	349	*COMT*	E401
314	*HLA-DOB*	E432	350	*EPHA7*	P205
315	*BMP4*	P199	351	*CCND1*	P343
316	*CTSH*	P238	**352**	***PGR***	**P790**
317	*FGF9*	P862	353	*TUBB3*	P364
318	*NEU1*	P745	**354**	***TAL1***	**P594**

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Rank[a]	*GENE*	CpG[b]	Rank[a]	*GENE*	CpG[b]
355	*HDAC11*	P556	391	*BMPR1A*	P956
356	*GABRA5*	P1016	392	*PTPNS1*	P301
357	*DAPK1*	P345	393	*EGFR*	P260
358	*PPP2R1B*	P268	394	*CDK6*	E256
359	*FAT*	P279	395	*PLXDC2*	P914
360	*PCDH1*	P264	396	*ERG*	E28
361	*CHFR*	P501	397	*EPHA7*	E6
362	*CSPG2*	E38	398	*DDR2*	E331
363	*AHR*	P166	399	*LOX*	P313
364	***SFTPC***	**E13**	400	*CASP3*	P420
365	*IL1RN*	E42	401	*EPM2A*	P113
366	*FGF9*	P1404	402	*BMP2*	E48
367	***JAK3***	**P156**	**403**	***EYA4***	**P794**
368	*NGFB*	P13	404	*TNK1*	P221
369	*BMP3*	E147	**405**	***HOXA9***	**E252**
370	*ITK*	P114	**406**	***SERPINA5***	**P156**
371	***MAS1***	**P469**	407	*GLI3*	P453
372	*ACVR2B*	P676	408	*TRIM29*	P135
373	*SEMA3F*	E333	409	*AREG*	E25
374	*RARB*	E114	410	*GPX3*	E178
375	*SNRPN*	seq 18	**411**	***LEFTY2***	**P719**
376	*EFNA1*	P7	**412**	***NTRK1***	**E74**
377	***PTHLH***	**E251**	**413**	***BCR***	**P422**
378	*CRIP1*	P874	414	*MUC1*	E18
379	*C4B*	P191	415	*F2R*	P88
380	*SHH*	P104	416	*CTNNA1*	P185
381	***EDN1***	**E50**	417	*GALR1*	P80
382	***SFTPD***	**E169**	418	*RAD50*	P191
383	*ZMYND10*	E77	419	*ESR2*	P162
384	*DDR2*	P743	420	*MMP9*	P237
385	***SEPT9***	**P374**	421	*MLLT3*	E93
386	*EGR4*	E70	422	*ETS1*	P559
387	*ABL1*	P53	423	*LOX*	P71
388	***GFAP***	**P1214**	424	*ODC1*	P424
389	***WEE1***	**P924**	425	*PTCH2*	E173
390	*BIRC5*	E89	426	*FLI1*	E29

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Rank[a]	*GENE*	CpG[b]	Rank[a]	*GENE*	CpG[b]
427	*AHR*	E103	463	*ALK*	P28
428	***CSF1***	**P217**	464	*ACVR1B*	P572
429	*FAS*	P322	465	*COL4A3*	P545
430	*C4B*	E171	466	*SH3BP2*	E18
431	*DAB2*	P35	467	*TRIM29*	E189
432	*PALM2-AKAP2*	P183	468	*IL12B*	P1453
433	*S100A2*	P1186	469	*MXI1*	P75
434	*EFNB3*	E17	**470**	***CHD2***	**P667**
435	*RARRES1*	P426	471	*IL1B*	P582
436	*JUNB*	P1149	472	*HBII-13*	E48
437	***FOLR1***	**E368**	473	*RRAS*	P100
438	***GLI2***	**P295**	474	*CHFR*	P635
439	*MYCN*	P464	475	*EGFR*	E295
440	*FGF5*	E16	476	*IL12B*	E25
441	***NAT2***	**P11**	**477**	***GLI3***	**E148**
442	***NDN***	**E131**	**478**	***RAB32***	**P493**
443	***TNFSF10***	**E53**	479	*TYRO3*	P366
444	*HIC1*	E151	480	*TMEFF1*	P626
445	*APOC1*	P406	**481**	***SLC22A3***	**P528**
446	***PLAGL1***	**P236**	482	*DNAJC15*	P65
447	***APBA1***	**E99**	483	*ABCC5*	P444
448	***HPN***	**P374**	484	*EMR3*	P1297
449	***HS3ST2***	**E145**	485	*TNFRSF10C*	P612
450	***KRT13***	**P676**	**486**	***HOXA5***	**P1324**
451	*IGF2AS*	E4	487	*MGMT*	P281
452	*GABRG3*	P75	488	*NRG1*	E74
453	***PEG3***	**E496**	**489**	***SLC22A18***	**P472**
454	*TBX1*	P520	490	*EFNA1*	P591
455	*RIPK3*	P124	491	*SMO*	P455
456	*NQO1*	E74	492	*HRASLS*	E72
457	*SNCG*	P98	**493**	***TAL1***	**E122**
458	*PROM1*	P44	494	*PTHR1*	P258
459	*ICAM1*	E242	495	*MMP7*	E59
460	*PTGS1*	E80	**496**	***THBS2***	**P605**
461	*MYLK*	E132	497	*COL1A1*	P5
462	*DLC1*	P695	498	*DSG1*	E292

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Rank[a]	*GENE*	CpG[b]	Rank[a]	*GENE*	CpG[b]
499	***TMPRSS4***	**E83**	535	*EMR3*	E61
500	*GSTP1*	P74	536	*IAPP*	E280
501	***TSP50***	**E21**	537	*MAPK4*	E273
502	***ASB4***	**P391**	538	*IHH*	P246
503	*TP73*	E155	539	*RIPK4*	P172
504	*LMO2*	P794	**540**	***ERBB2***	**P59**
505	*PLA2G2A*	E268	541	*EMR3*	P39
506	*FAS*	P65	542	*EPM2A*	P64
507	***CD81***	**P211**	543	*TUSC3*	P85
508	***DES***	**E228**	544	*CDKN2B*	seq 50
509	*GABRA5*	P862	545	*PPARG*	E178
510	*CPA4*	P1265	**546**	***CD34***	**P780**
511	*NTSR1*	E109	547	*TES*	E172
512	*DSC2*	E90	**548**	***CDKN1B***	**P1161**
513	*PTGS2*	P308	549	*KDR*	E79
514	*PHLDA2*	P622	550	*SH3BP2*	P771
515	*CTLA4*	P1128	551	*MATK*	P190
516	*CPNE1*	P138	**552**	***SEPT9***	**P58**
517	***NCL***	**P1102**	**553**	***HOXA9***	**P1141**
518	***HOXA11***	**P698**	554	*MPO*	P883
519	*CDH11*	P354	555	*VAV2*	E58
520	*MBD2*	P233	556	*MAGEL2*	E166
521	*IGFBP7*	P297	557	*COL18A1*	P365
522	*TCF4*	P175	558	*IL1RN*	P93
523	*PADI4*	P1011	559	*RAN*	P581
524	*S100A2*	E36	560	*TGFB2*	E226
525	*RIPK3*	P24	561	*CDH11*	E102
526	*CHI3L2*	P226	562	*FRK*	P258
527	*GRB10*	P260	563	*DDB2*	P613
528	*HIF1A*	P488	564	*PLAGL1*	E68
529	*BLK*	P668	565	*HLA-F*	E402
530	***AXL***	**P223**	**566**	***TNF***	**P1084**
531	*SMAD2*	P848	**567**	***POMC***	**P400**
532	*FLI1*	P620	568	*LRRK1*	P39
533	***MAS1***	**P657**	**569**	***WNT10B***	**P993**
534	*RAF1*	P330	570	*CCKBR*	P361

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Rank[a]	*GENE*	CpG[b]	Rank[a]	*GENE*	CpG[b]
571	***NNAT***	**P544**	607	*NOTCH4*	P938
572	***HSPA2***	**P162**	608	*ICAM1*	P386
573	***SLC22A18***	**P216**	609	*HGF*	E102
574	*COL6A1*	P283	**610**	***DNAJC15***	**E26**
575	***HBII-13***	**P991**	**611**	***HDAC9***	**P137**
576	*HOXB13*	P17	612	*TEK*	P479
577	*CYP1B1*	E83	613	*SPDEF*	P6
578	*EGF*	E339	614	*IGF1*	E394
579	*FGF7*	P44	615	*MMP3*	P16
580	*LCN2*	P141	**616**	***HOXB2***	**P99**
581	*B3GALT5*	E246	**617**	***HPN***	**P823**
582	*ABCB4*	E429	618	*OPCML*	P71
583	***ARHGAP9***	**P260**	619	*KRT5*	E196
584	***ACTG2***	**P346**	**620**	***CALCA***	**P171**
585	*VAMP8*	P114	621	*TYRO3*	P501
586	***HLA-DQA2***	**P282**	**622**	***ALPL***	**P433**
587	*LMO1*	P169	623	*ITPR2*	P804
588	*GDF10*	E39	624	*KIT*	P405
589	*CCL3*	P543	625	*MAP2K6*	E297
590	***SPI1***	**P929**	626	*MEG3*	P235
591	*MYB*	P673	**627**	***SERPINA5***	**E69**
592	*ZIM3*	P718	628	*NRG1*	P558
593	***TNFRSF10D***	**P70**	629	*RET*	seq 54
594	***SNRPN***	**seq 12**	630	*ETV1*	P515
595	***HLA-DPA1***	**P28**	**631**	***TNFRSF10D***	**E27**
596	***ASCL2***	**E76**	632	*ZNF215*	P71
597	***PDGFRA***	**E125**	633	*ERBB4*	P255
598	***FN1***	**P229**	634	*NOTCH1*	P1198
599	*MMP9*	P189	635	*DNMT3B*	P352
600	*PI3*	P274	636	*ER*	seq a1
601	***HOXC6***	**P585**	**637**	***GFAP***	**P56**
602	*EGF*	P413	638	*LCN2*	P86
603	*PYCARD*	P393	639	*IL12A*	E287
604	*NQO1*	P345	640	*APBA1*	P644
605	*CTSD*	P726	641	*AGXT*	P180
606	***KCNQ1***	**P546**	**642**	***PPAT***	**E170**

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Rank[a]	*GENE*	CpG[b]	Rank[a]	*GENE*	CpG[b]
643	*CCL3*	E53	679	*IGF2*	P36
644	*DUSP4*	E61	680	*LRRC32*	E157
645	***ABCG2***	**P310**	681	*OSM*	P188
646	***JAK3***	**P1075**	682	*ITK*	E166
647	*BCL6*	P248	683	*MAPK12*	E165
648	***DLC1***	**P88**	**684**	***ABCC2***	**E16**
649	*EPHA8*	P256	**685**	***KRT1***	**P798**
650	*WNT2*	P217	686	*FGF5*	P238
651	*SOD3*	P460	687	*TMEFF1*	P234
652	*PROK2*	E0	688	*UNG*	P170
653	***BSG***	**P211**	689	*PWCR1*	P357
654	*AKT1*	P310	690	*MMP8*	E89
655	*MFAP4*	P10	**691**	***PECAM1***	**P135**
656	***HOXA9***	**P303**	692	*MAF*	P826
657	*NFKB1*	P336	**693**	***SEMA3C***	**P642**
658	***EPHX1***	**P1358**	694	*KLK10*	P268
659	*ACVR1B*	E497	695	*TIAM1*	P188
660	*ATP10A*	P524	696	*FES*	P223
661	*RUNX1T1*	E145	697	*TUBB3*	E91
662	*TNFRSF1B*	P167	698	*RAB32*	E314
663	*GABRG3*	E123	699	*CASP10*	E139
664	*NBL1*	E205	700	*PTPRH*	E173
665	***SLC5A8***	**P38**	701	*ERBB4*	P541
666	*CTSL*	P264	**702**	***FZD9***	**E458**
667	***UGT1A1***	**P564**	703	*EPHA5*	P66
668	***TGFB1***	**P833**	704	*PLAUR*	P82
669	*IGF2*	P1036	705	*TDGF1*	P428
670	***HDAC9***	**E38**	706	*PEG10*	P978
671	***PENK***	**P447**	707	*HLA-DRA*	P77
672	*GALR1*	E52	708	*HSD17B12*	E145
673	*INS*	P804	**709**	***IGF1***	**P933**
674	*DDR1*	P332	710	*CSPG2*	P82
675	***IGF2AS***	**P203**	711	*PTHR1*	E36
676	*OGG1*	E400	712	*PRSS1*	P1249
677	***CSF2***	**P605**	713	*BCL2L2*	E172
678	*IL6*	P611	714	*SIN3B*	P607

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Rank[a]	*GENE*	CpG[b]	Rank[a]	*GENE*	CpG[b]
715	*IGSF4*	P454	751	*NR2F6*	E375
716	*MMP2*	E21	752	*IL4*	P262
717	***RIPK1***	**P744**	753	*TFPI2*	P152
718	***FGF1***	**E5**	754	*FABP3*	P598
719	***ETV6***	**E430**	**755**	***SPI1***	**E205**
720	***FANCE***	**P356**	**756**	***FGFR4***	**P610**
721	*MMP1*	P460	**757**	***HIC2***	**P498**
722	***PECAM1***	**E32**	758	*MMP19*	P306
723	***MYOD1***	**E156**	759	*IFNG*	P188
724	***CD9***	**P504**	760	*TNFRSF10A*	P91
725	*PALM2-AKAP2*	P420	761	*HHIP*	P307
726	*IGF2*	E134	762	*KRAS*	E82
727	*ZIM2*	P22	**763**	***GSTM2***	**E153**
728	*WNT1*	E157	764	*ERCC3*	P1210
729	*NKX3-1*	P871	765	*CDKN2A*	E121
730	*MSH3*	E3	766	*SFN*	E118
731	***MSH2***	**P1008**	**767**	***SOX17***	**P303**
732	***MT1A***	**E13**	768	*CD86*	P3
733	***PRSS8***	**E134**	769	*AREG*	P217
734	***TFDP1***	**P543**	770	*AATK*	E63
735	*CSF1R*	E26	771	*SIN3B*	P514
736	*EDN1*	P39	772	*TMEFF2*	P210
737	*PWCR1*	E81	773	*SHB*	P691
738	*LTA*	E28	**774**	***WNT10B***	**P823**
739	***SRC***	**P297**	775	*RBL2*	P250
740	*IHH*	P529	776	*MYCL1*	P502
741	***CDH17***	**E31**	777	*TNFRSF1B*	E5
742	*GJB2*	P791	**778**	***CD81***	**P272**
743	*ABCB4*	P51	779	*PLXDC1*	P236
744	***THBS1***	**E207**	**780**	***HCK***	**P858**
745	*PDE1B*	P263	**781**	***SOX1***	**P294**
746	*GPX1*	P194	**782**	***MMP7***	**P613**
747	*CSF1*	P339	**783**	***LEFTY2***	**P561**
748	***DNMT2***	**P199**	**784**	***FGF6***	**P139**
749	*TGFB3*	E58	785	*MAP2K6*	P297
750	***MT1A***	**P49**	786	*APBA2*	P305

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Rank[a]	*GENE*	CpG[b]	Rank[a]	*GENE*	CpG[b]
787	*RUNX3*	E27	**823**	***CEACAM1***	**E57**
788	*SLC14A1*	E295	824	*MMP9*	E88
789	*SEMA3A*	P343	825	*IL10*	P85
790	***HOXC6***	**P456**	826	*POMC*	E254
791	***MOS***	**E60**	827	*RARA*	E128
792	*LMO1*	E265	828	*CXCL9*	E268
793	***HOXA11***	**E35**	829	*ZIM3*	E203
794	*DES*	P1006	830	*EIF2AK2*	P313
795	***SLC5A5***	**E60**	831	*CDH11*	P203
796	***ASCL2***	**P360**	832	*TFAP2C*	P765
797	***USP29***	**P205**	**833**	***MDS1***	**E45**
798	*CCNA1*	P216	834	*GJB2*	E43
799	*LRRK1*	P834	**835**	***PAX6***	**P1121**
800	*TJP1*	P390	836	*EDNRB*	P709
801	***TIMP3***	**P690**	837	*FZD9*	P15
802	***ERN1***	**P809**	**838**	***TAL1***	**P817**
803	***MMP2***	**P303**	**839**	***CD40***	**P372**
804	*TPEF*	seq 44	**840**	***SPDEF***	**E116**
805	*CDH1*	P52	841	*NOS3*	P38
806	***SLC22A2***	**E271**	842	*FZD9*	P175
807	*OSM*	P34	843	*ESR2*	E66
808	***WNT8B***	**E487**	844	*HTR2A*	P853
809	*MST1R*	E42	845	*MCAM*	P265
810	*PTPRG*	E40	**846**	***IPF1***	**P750**
811	***FN1***	**E469**	847	*MC2R*	E455
812	***COL1A2***	**P48**	**848**	***DCC***	**E53**
813	*SEMA3C*	E49	**849**	***NEFL***	**P209**
814	*UGT1A7*	P751	850	*NTRK2*	P395
815	*SERPINE1*	E189	**851**	***DSP***	**P440**
816	*IL17RB*	P788	852	*SERPINB5*	P19
817	*MAPK14*	P327	**853**	***MAD2L1***	**E93**
818	***GJB2***	**P931**	**854**	***CD40***	**E58**
819	*NFKB1*	P496	855	*TFF2*	P178
820	*CDKN2B*	E220	856	*EPHA3*	E156
821	***GNG7***	**P903**	857	*FRK*	P36
822	*DIRAS3*	E55	**858**	***LTB4R***	**P163**

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Rank[a]	*GENE*	CpG[b]	Rank[a]	*GENE*	CpG[b]
859	*EPHA2*	P340	**895**	***H19***	**P1411**
860	*FGFR3*	P1152	**896**	***EVI1***	**E47**
861	*GP1BB*	E23	897	*PTCH2*	P37
862	***HRASLS***	**P353**	**898**	***SYK***	**E372**
863	***LTB4R***	**E64**	899	*ZP3*	P220
864	*MET*	E333	900	*EGF*	P242
865	*RUNX3*	P247	901	*LTA*	P214
866	*TFF2*	P557	**902**	***TCF4***	**P317**
867	*TNC*	P198	903	*CSF1R*	P73
868	*GUCY2D*	P48	**904**	***COL1A2***	**P407**
869	*ALOX12*	P223	**905**	***JAK3***	**E64**
870	*UGT1A1*	E11	**906**	***GAS7***	**P622**
871	*TFF1*	P180	907	*EDNRB*	P148
872	*DSC2*	P407	908	*SNCG*	P53
873	*CFTR*	P372	**909**	***FLT1***	**P615**
874	*NTRK3*	P752	910	*INS*	P248
875	***TFAP2C***	**E260**	**911**	***GML***	**E144**
876	*NPY*	E31	**912**	***SCGB3A1***	**E55**
877	*APOA1*	P75	913	*APOC2*	P377
878	*CYP2E1*	P416	**914**	***HTR1B***	**E232**
879	*SNURF*	E256	915	*MYBL2*	P354
880	*NGFR*	E328	916	*EPHA3*	P106
881	*GPATC3*	P410	917	*FGF8*	E183
882	***NOTCH1***	**E452**	918	*SFRP1*	P157
883	*ERBB3*	E331	919	*GNAS*	E58
884	***NOTCH4***	**E4**	**920**	***DBC1***	**P351**
885	*MLLT6*	P957	921	*NPR2*	P1093
886	***NGFR***	**P355**	922	*MKRN3*	E144
887	***ZP3***	**E90**	923	*WT1*	E32
888	*APBA2*	P227	**924**	***TMPRSS4***	**P552**
889	*IFNGR2*	P377	925	*CHGA*	P243
890	*ISL1*	P379	926	*HS3ST2*	P546
891	*SGCE*	E149	927	*RIPK4*	E166
892	*ISL1*	P554	928	*EPHA1*	E46
893	*GABRB3*	E42	929	*NTRK2*	P10
894	*FLT1*	E444	**930**	***SNURF***	**P2**

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Rank[a]	*GENE*	CpG[b]
931	*DSG1*	P159
932	***CHGA***	**E52**
933	*FGF1*	P357
934	***HLA-DRA***	**P132**
935	***PROK2***	**P390**
936	*CCNC*	P132
937	***PARP1***	**P610**
938	*HOXA5*	E187
939	*DBC1*	E204
940	***EPO***	**E244**
941	***GSTM1***	**P266**
942	*SLIT2*	P208
943	***RASSF1***	**P244**
944	*ZNFN1A1*	P179
945	*TK1*	P62
946	*IL6*	P213
947	***ESR1***	**P151**
948	***MOS***	**P27**
949	***GNG7***	**E310**
950	***PPARG***	**P693**
951	*NID1*	P677
952	*ROR1*	P6
953	***GSTM2***	**P109**
954	*BMPR1A*	E88
955	***BCR***	**P346**
956	*NEFL*	E23
957	*LRRC32*	P865
958	***FANCA***	**P1006**
959	*TRIP6*	P1274
960	*PLXDC2*	E337
961	*PRDM2*	P1340
962	*IL13*	E75
963	***USP29***	**E274**
964	*TDG*	E129
965	***IGFBP2***	**P306**
966	*TMEFF2*	E94
967	*SPP1*	P647
968	*PSCA*	E359
969	***COL1A2***	**E299**

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Table S2. Loci with significantly different methylation by asbestos burden in MPM

Increased methylation is associated with:
Increased asbestos burden, **Decreased asbestos burden**

Rank[a]	*GENE*	CpG[b]	Rank[a]	*GENE*	CpG[b]
1	*CASP10*	E139	31	*CDKN1C*	P626
2	*TDGF1*	P428	32	*EPM2A*	P64
3	*DES*	E228	33	*VAMP8*	P114
4	*BSG*	P211	34	*HOXB2*	P99
5	*GP1BB*	P278	35	*PAX6*	P1121
6	*FZD9*	E458	36	*PYCARD*	P150
7	*SHB*	P691	37	*SLC5A8*	P38
8	*ASCL2*	P609	38	*ASCL2*	E76
9	*PAX6*	P50	39	*ABL1*	P53
10	*GP1BB*	E23	40	*CD81*	P272
11	*IGF2AS*	E4	41	*PAX6*	E129
12	*CD40*	P372	42	*TAL1*	P594
13	*ALOX12*	E85	43	*NOS3*	P38
14	*ASCL2*	P360	44	*SLC5A8*	E60
15	*ALOX12*	P223	45	*SOX17*	P303
16	*ID1*	P659	46	*DIO3*	E230
17	*HOXA9*	E252	47	*TNFSF10*	P2
18	*DLC1*	E276	48	*HOXA11*	E35
19	*PTPN6*	P282	49	*ZIM2*	P22
20	*IRF5*	E101	50	*AATK*	P709
21	*AATK*	P519	51	*SEMA3F*	P692
22	*VAMP8*	E7	52	*HS3ST2*	E145
23	*EPO*	E244	53	*MYBL2*	P354
24	*CHGA*	E52	54	*CYP1B1*	E83
25	*PENK*	E26	55	*LMO2*	E148
26	*TAL1*	E122	56	*E2F3*	P840
27	*JAK3*	E64	57	*CDKN2A*	E121
28	*IGF2AS*	P203	58	*SOX17*	P287
29	*TAL1*	P817	59	*SOX1*	P1018
30	*HOXA11*	P698	60	*SCGB3A1*	E55

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Rank[a]	*GENE*	CpG[b]
61	*MYLK*	E132
62	***SFTPB***	**P689**
63	*ASCL1*	P747
64	*MEST*	P4
65	*IRAK3*	P185
66	*MT1A*	E13
67	*CCL3*	E53
68	*ZNFN1A1*	E102
69	*CD81*	P211
70	*CSK*	P740
71	*MAPK12*	E165
72	*LIMK1*	P709
73	*CDKN2B*	E220
74	***TIMP3***	**P690**
75	*MMP9*	P189
76	*TSP50*	P137
77	*CHI3L2*	P226
78	*MBD2*	P233
79	*THY1*	P20
80	***ACVR1***	**E328**
81	*EPO*	P162
82	*APOC1*	P406
83	*MYBL2*	P211
84	*MSH3*	E3
85	*CD40*	E58
86	*JAK3*	P156
87	***EPHB6***	**P827**
88	*CASP10*	P186
89	*TYRO3*	P366
90	*ZIM2*	E110
91	*RASSF1*	E116
92	*EFNB3*	E17
93	***MYH11***	**P236**
94	*ZNF215*	P129
95	*PARP1*	P610
96	*IGF2*	P1036
97	*CRIP1*	P874
98	*ETS2*	P684
99	*NPY*	P295
100	***HLA-DPB1***	**E2**
101	*TNFSF10*	E53
102	*IRF5*	P123
103	*IGFBP7*	P297
104	*TFF1*	P180
105	*GUCY2D*	E419
106	*TNFRSF10C*	E109
107	*FZD9*	P175
108	*CASP2*	P192
109	***ASB4***	**P52**
110	*MT1A*	P49

[a] Based on Q- value [b] Relative to transcription start site, E - exon, P - promoter

Chapter 5

Discussion and directions

For over one hundred years, exposure to asbestos has been a known health hazard, and in this time significant progress has been made toward understanding the relationship between asbestos exposure and pleural mesothelioma. Nonetheless, further clarification of the associations among asbestos burden, somatic alterations, and patient survival in the context of this rapidly fatal malignancy remain necessary and have the potential to improve prevention, treatment and patient outcomes. In 1900, Dr. H. Montague Murray of Charing Cross Hospital in London performed an autopsy on the last living member of a group of workers in an asbestos textile factory and concluded the 33-year-old, 14 year-long employee of the factory died due to his occupational exposure to asbestos (Tweedale and Hansen 1998). The first case of malignant pleural mesothelioma (MPM) documented in the modern medical literature was published in 1931; and in 1960 the link between asbestos exposure and MPM was confirmed (Klemperer P 1931; Wagner, Sleggs et al. 1960). In the time since, there has been an ongoing effort to understand the molecular mechanisms by which this fibrous mineral carcinogen contributes to the development of this otherwise extremely rare malignancy. Although significant advances have been made in establishing asbestos as a carcinogen and characterizing many of its pathogenic mechanisms, the devastating outcome for patients with the disease is little changed, and most patients do not survive more than a year following diagnosis.

Asbestos is a group of crystalline-hydrated silicate minerals that occur in a naturally fibrous form, and is the single most important risk factor for MPM, with 70-80% of MPM patients reporting a known exposure to asbestos (Tammilehto, Maasilta et al. 1992). Derived from the Greek word for inextinguishable, asbestos was used centuries ago as a textile for clothing and in oil lamp wicks (Cugell and Kamp 2004). Inhalation of asbestos fibers leads to deposition at alveolar duct bifurcations and eventual migration to the pleural membranes (Brody,

Hill et al. 1981; Viallat, Raybuad et al. 1986). There are two main groups of asbestos fibers; serpentine asbestos, also known as chrysotile, is comprised of shorter, curved fibers; and amphiboles, which are long and straight and have several forms such as crocidolite, amosite, anthophylite, tremolite, and actinolite. Each type of asbestos has a specific mineralogy, and these fibers often undergo treatment in mining and processing that can further alter their physical chemistry (Mossman, Kamp et al. 1996). In 1981, Stanton et al. presented compelling evidence from extensive experiments in rats that argued a more important feature of asbestos fiber carcinogencity is size, rather than chemisty (Stanton, Layard et al. 1981). More specifically, the inability of fibers with large length:width ratios (amphiboles) to be cleared by phagocytic immune cells was associated with increased pathogenicity compared to fibers with small aspect ratios (chrysotile) (Stanton, Layard et al. 1981). Other labs have presented additional evidence that amphibole asbestos fibers have increased pathogenic potential for pleural mesothelioma, and the biopersistence of these fibers are generally considered an important aspect of their pathogenic potential relative to shorter fiber types (Moalli, MacDonald et al. 1987; Goodglick and Kane 1990). However, it is critical to note that no form of asbestos is free from disease risk (Dodson, Atkinson et al. 2003; Suzuki, Yuen et al. 2005), and concomitant exposure to multiple fiber types is common (Butnor, Sporn et al. 2003).

An unusual and important feature of this research, compared with essentially all prior work of this type, is the use of a quantitative asbestos burden assessment for most patients. Asbestos burden was quantified by digesting lung tissue from multiple sites per patient and counting asbestos bodies with light microscopy in the laboratory of participating pathologist Dr. John Godleski, according to the procedures described in (Churg and Warnock 1977; De Vuyst, Karjalainen et al. 1998). Ferruginous asbestos bodies-asbestos fibers with iron

mucopolysaccharide deposits-are formed through the long-term interaction of asbestos fibers with iron:protein complexes in macrophages that attempt to engulf and clear these fibers from the lung and airways. Quantifying asbestos bodies is a powerful method to estimate past exposure to asbestos and their presence in the lung is considered a hallmark of asbestos exposure (Craighead, Abraham et al. 1982). At the same time, there are important considerations when interpreting asbestos body count data in any given asbestos exposed group. Amphibole asbestos fibers with larger length:width ratios are known to form asbestos bodies more readily than shorter fibers such as chrysotile (Churg and Warnock 1980), and there is believed to be inter-individual variation in asbestos body formation (Dodson, Williams et al. 1985). Nonetheless, unlike chrysotile asbestos fibers which are easier for the body to clear (for example, they can undergo transverse breakage, becoming more amenable to macrophage mediated removal from the parenchyma and airways), amphibole fibers are biopersistent. Therefore, asbestos body counts may bias exposure estimates toward amphiboles, but this may also represent a more biologically relevant dose when one is considering the action of asbestos fibers in inducing MPM.

For this thesis, using a quantitative assessment of asbestos burden, a more precise understanding the relationship between asbestos exposure and patient survival in MPM was sought. Further characterizing the epigenetic aberrations in these tumors and their relationships with asbestos exposure is critical to understanding disease pathogenesis; this project was designed specifically to determine whether asbestos exposure contributes to aberrant epigenetic events in MPM. The ability of epigenetic alterations to differentiate diseased and non-diseased pleura was assessed utilizing non-tumor pleural tissues, and the investigation of epigenetic alterations included over one thousand loci. In addition, the relationships among asbestos

exposure, epigenetic alterations, and patient survival were also examined. Integrating these analyses will hopefully result in clinically applicable tools for improving disease diagnosis and prognosis that benefit patient outcomes.

In Chapter 2, an incident case series of MPM patients enrolled through the international mesothelioma program (IMP) at Brigham and Women's Hospital in Boston, Massachusetts forms the base population for the research. Over 125 consecutive patients who underwent surgical resection for the disease were enrolled and most had detailed demographic and exposure histories, making this a relatively large and well-annotated group of MPM patients. As this study group was selected based upon patients most likely to benefit from surgery, it has an inherent bias toward younger patients with non-sarcomatoid tumors; however, it closely resembles other MPM surgical cohorts (Pass, Wali et al. 2008). Among all patients, those with non-epithelioid histologies had significantly poorer survival than those with epithelioid tumors, consistent with the findings of others (Flores, Zakowski et al. 2007; Flores, Pass et al. 2008). In addition, and again consistent with the findings of Flores *et al.*, males in this study did not survive as long as females. Exposure to asbestos generally occurs in an occupational setting, placing a higher asbestos burden on men, resulting in 3 – 5 times as many men with MPM (Robinson and Lake 2005). Our study group had a similar ratio of males to females, and in addition, men were found to have significantly higher asbestos burden than women. This may, in addition, suggest that asbestos burden independently contributes to poor survival. When this potential association was examined, individuals in the lowest and highest asbestos burden tertiles were found to have significantly increased risk of death compared to patients with moderate asbestos burden. While this pattern of dose-response was not expected, it suggests a potential susceptibility to the carcinogenic effects of asbestos may exist among these patients.

Pleural mesothelioma can occur in the context of very low-level asbestos exposure, and there is evidence from several studies that genetics may modify susceptibility to MPM (Hansen, de Klerk et al. 1998; Hodgson and Darnton 2000). Substitution of a single base in the genome with a minor allele frequency of 1% or greater (polymorphisms) are the most common type of genetic variation, and known to number over 10 million in humans, with perhaps 5 million loci occurring in over 10% of the population (Risch 2000; Lander, Linton et al. 2001; Venter, Adams et al. 2001; Botstein and Risch 2003; Erichsen and Chanock 2004). Specific variations have the potential to modify disease risk, including risk of MPM. For instance an increased risk among asbestos workers with *GSTM1* homozygous deletion or with the slow acetylator phenotype in *NAT2* have been reported (Hirvonen, Pelin et al. 1995). In a more recent study, individuals with *GSTM1* homozygous deletion were found to have a significant, 1.7 fold increased risk of MPM (95% CI, 1.04 – 2.74) (Landi, Gemignani et al. 2007). Further, DNA repair gene sequence variations may predispose individuals to an increased risk for MPM; *XRCC1-399Q* genotype (OR = 2.1, 95% CI, 1.1 – 4.3), *XRCC3-241T* genotype (OR = 4.1, 95% CI, 1.3 – 13.2) (Dianzani, Gibello et al. 2006). Additionally, a study of over 500 individuals in two Turkish villages that spanned six generations suggested an autosomal dominant transmission of MPM risk (Roushdy-Hammady, Siegel et al. 2001). Based on blood-line segregation of MPM cases in erionite-exposed populations in Turkey, and numerous reports of familial MPM in the U.S. and Europe the potential for genetics to modify the susceptibility to MPM is high, further supporting the hypothesis that there may be individuals with a high susceptibility to asbestos (Risberg, Nickels et al. 1980; Martensson, Larsson et al. 1984; Lynch, Katz et al. 1985; Hammar, Bockus et al. 1989; Otte, Sigsgaard et al. 1990; Precerutti, Mayorga et al. 1990; Dawson, Gibbs et al. 1992; Ascoli, Scalzo et al. 1998; Musti, Cavone et al. 2002; Bianchi, Brollo et al. 2004; Picklesimer,

Zanagnolo et al. 2005; Dogan, Baris et al. 2006; Ohar, Ampleford et al. 2007; Ugolini, Neri et al. 2007). In fact, most of the patients in our study who were in the low asbestos burden tertile had asbestos body counts similar to those seen in the general population (Churg and Warnock 1977).

An additional explanation for the unusual dose-response that we observed is that there are individuals in the low asbestos burden tertile that do not form ferruginous deposits after exposure to asbestos fibers that persist in the lung. Evidence for this possibility has been described in an analysis of mesotheliomas associated with low asbestos burdens. Among 18 cases with asbestos body counts in the range of those from the general population, approximately one-third of these patients had fiber burdens (as measured by electron microscopy) that exceeded levels seen in the general population (Srebro, Roggli et al. 1995). If a similar portion of our cases with low asbestos body counts follow this pattern it suggests that their asbestos burden may be higher than estimated by counting asbestos bodies. Nonetheless, while these cases imply that there is an even stronger link between increased asbestos burden and poor survival in MPM, it is likely that most patients in the low asbestos burden tertile would form asbestos bodies in a manner similar to the general population. As such, most patients in the low asbestos burden tertile have disease that truly occurred in the context of low asbestos exposure, suggesting that they harbor a susceptibility to the carcinogenic effects of asbestos.

Examining epigenetic inactivation of tumor suppressor genes in pleural mesothelioma and the potential contribution of asbestos burden to theses alterations was the focus of Chapter 3. We found that having an increased number of methylated cell cycle control related genes was associated with increasing asbestos body counts among pleural mesothelioma patients after controlling for potential confounders. Part of a larger, pathway-based approach to tumor suppressor gene (TSG) methylation, this chapter presents evidence that induction of methylation

is associated with asbestos exposure, a crucial, novel mechanism of asbestos carcinogenicity. However, it is not clear that the asbestos fibers themselves are a direct cause of these alterations; interaction between asbestos fibers and the epigenetic regulatory machinery is not implied, and it is therefore important to more carefully consider the basis for the observed association. Asbestos exposure is associated with chronic inflammation, as well as a dose-dependent death and re-growth of mesothelial cells in areas of fiber deposition (Adamson, Bakowska et al. 1993; Sabo-Attwood, Ramos-Nino et al. 2005). Chronic inflammation has been associated with an increased prevalence of epigenetic TSG silencing in colon cancer, and is thought to be a generalizable phenomenon (Issa, Ahuja et al. 2001; Issa 2008). More specifically, the combination of direct asbestos-induced cell injury or death, and inflammation-associated reactive oxygen species over the decades-long latency of MPM would allow ample time for the selection of clones capable of continued proliferation. It is likely that dynamic carcinogen exposure and its relationship to the targeting and induction of specific epigenetic alterations in highly complex, and future work is necessary to determine the relationship between asbestos burden and phenotypically selected epigenetic pathway inactivation events.

The final chapter of research comprising this thesis introduced additional samples to the surgical cohort and included several non-tumorigenic pleural tissue samples. To combine and expand the exploration of the relationships between asbestos burden and survival, as well as that between asbestos burden and epigenetic alterations, an array-based technique to simultaneously interrogate hundreds of cancer-related genes for methylation alterations was used. In this manner, epigenetic profiles were found to be highly significant predictors of disease status. Further, epigenetic profiles were also associated with asbestos burden, and finally, both asbestos burden and epigenetic profile class membership were significant, independent predictors of

survival in pleural mesothelioma. These findings both confirmed and extended the results from previous chapters, and reveal the potential clinical utility of epigenetic biomarkers of disease to assist in diagnosis and prognosis of pleural mesothelioma.

Using an unsupervised clustering technique and locus-by-locus analysis, overall methylation profiles were highly predictive of disease status. However, hundreds of CpG loci had significantly altered methylation in tumor versus non-tumor pleura, making it difficult to determine which, and how many loci are necessary to differentiate tumors from non-tumor pleura with optimal sensitivity and specificity. Evidence for the necessity of using a large number of loci comes from our model, where it is clear that the number and identity of loci with alterations versus normal pleura will vary from tumor to tumor. To better estimate the optimal number of loci for correctly differentiating tumor from non-diseased tissue, a plot of the error in differentiating sample types versus the number of loci used to estimate the error (ranked by highest variance between sample types) was generated. To this end, the error in ability to differentiate MPM from normal pleura was as low as 2% when using between 400 and 550 loci. While these results need to be verified in an additional set of samples, there is promise for use of this method in a clinical setting to assist in the differential diagnosis of pleural mesothelioma.

In an analysis of tumor samples, asbestos burden was significantly predictive of methylation class membership, previously reported loci with differential methylation based on asbestos burden were confirmed, and these findings were extended to include several additional loci. As previously discussed, it is a distinct possibility that asbestos-induced inflammation may contribute to the selection of clones with epigenetic alterations that favor their ability to persist. Chronic inflammation is one of the most well documented tissue responses to asbestos exposure (Sabo-Attwood, Ramos-Nino et al. 2005), and again, Issa et al. have presented evidence of

chronic inflammation induced hypermethylation (Issa, Ahuja et al. 2001; Issa 2008). As inflammation is known to be associated with the production of reactive oxygen species, there is additional evidence for the relationship between inflammation and epigenetic regulation. Normal cellular histone 3 lysine demethylation by lysine demethylase (LSD1) results in reactive-oxygen-species that generate 8-oxo-guanine lesions leading to the recruitment of 8-oxoguanine-DNA glycosylase and topoisomerase IIβ. Recruitment of these DNA repair enzymes was shown to be necessary for chromatin-conformation changes that allowed estrogen-induced gene transcription to occur (Perillo, Ombra et al. 2008). In this manner then, inflammation-induced aberrant reactive oxygen DNA damage may result in improper activation of gene transcription related to DNA repair activity, one possible mechanism of inflammation-related epigenetic deregulation. However, epigenetic alterations are not the only somatic changes in these tumors. Asbestos is also known to induce chromosome damage, and numerous gene deletion events have been described in MPMs (Bjorkqvist, Tammilehto et al. 1997; Murthy and Testa 1999).

Characterizing the dose-response between asbestos burden and gene copy number alterations would allow investigation of the potential association between asbestos burden and copy number alterations. Further, combining copy number alteration data with the data presented here would allow investigation into whether the extent of copy number alterations is related to the extent of epigenetic alterations, or modifies the association between asbestos burden and methylation profile class membership. Since some tumors appear more epigenetically similar to non-tumor pleura, it is reasonable to suggest that these tumors may harbor a significant number of other somatic alterations such as gene deletions. In an effort to begin understanding these potential relationships, copy number alteration data for a subset of the incident cases studied here are now available, and preliminary research into this question has commenced. Following an

analysis of potential associations between copy number alteration and demographic, exposure and tumor characteristics; using an index of copy number alteration together with an index of epigenetic alteration, relationships among these indices and asbestos burden can begin to be investigated.

In addition to the TSG methylation events researched here, the original link between aberrant methylation events in cancer was made in a description of global hypomethylation (Feinberg and Vogelstein 1983). The result of loss of methylation in repeat elements of the genome, global methylation contributes to increased genomic instability (Feinberg and Vogelstein 1983; Yoder, Walsh et al. 1997). The extent to which hypermethylation profile class membership may be associated with global hypomethylation is not known. Nonetheless, it is likely that the same epigenetic deregulation that contributes to aberrant promoter CpG hypermethylation also contributes to global hypomethylation, and that hypomethylation may also be related to carcinogen exposures. In fact, evidence for an association between smoking and hypomethylation at the repeat element LRE1 has been described in squamous cell carcinoma of the head and neck (Hsiung, Marsit et al. 2007). A significant relationship between low dietary intake of the methyl donor folate and a variant allele of the methyl-folate reductase gene *MTHFR* with decreased global methylation was also described (Hsiung, Marsit et al. 2007). These findings suggest diet and genetic variation contribute to aberrant epigenetic events and may be modified by carcinogen exposure.

Constitutional genetic variation at certain loci may be associated with methylation profile class membership, and may modify the association between asbestos burden and methylation class membership. For instance, variant alleles in epigenetic regulatory genes may enhance the likelihood of selection for particular aberrant epigenetic profiles . Using available genotyping

data from epigenetic regulatory genes from approximately 40% of cases, work aimed at addressing this question has begun. Initial tests for association between genotypes and methylation profile class membership are promising, and the distribution of cases with variant alleles in certain genes may be predictive of class membership. *SIRT2* is a histone H4 deacetylase that has been reported to be down-regulated in gliomas; among MPM cases with available genotyping data, the distribution of *SIRT2* alleles approaches statistical significance as a predictor of methylation profile class membership (permutation $P = 0.09$), (Inoue, Hiratsuka et al. 2007). Similarly, distribution of *SIRT3* alleles across methylation profile classes also approaches statistical significance ($P = 0.09$). In contrast, other genes such as the DNA methyltransferase enzymes do not exhibit similar putative associations, though these data are only from a subset of cases, and genotyping needs to be completed for the entire cohort. Furthermore, additional candidate genes such as those involved in the inflammatory response, cell cycle control, apoptosis, and methyl donor group metabolism, among others, should be considered for this type of analysis.

Another somatic alteration that may be associated with epigenetic profiles or modify the association between asbestos burden and methylation profile class membership is aberrant MicroRNA (miRNA) expression. MiRNAs are single-stranded, non-coding short oligonucleotide sequences of about 22 bases that inhibit mRNA translation in plants and animals (Bartel 2004). Alterations in the expression of many miRNAs have been linked to multiple types of human cancer, and it is believed that miRNAs can function both as oncogenes and tumor suppressors. Over 50% of known miRNAs are located in chromosomal regions that are frequently altered in human cancer such as fragile sites, frequent breakpoints, and areas of amplification or deletion (Calin, Ferracin et al. 2005). Importantly, recent work has shown that certain miRNAs can be

aberrantly silenced by hypermethylation, as exemplified in cancers of the oral cavity, breast, and colon (Grady, Parkin et al. 2008; Kozaki, Imoto et al. 2008; Lehmann, Hasemeier et al. 2008). In addition, there is also evidence for hypomethylation resulting in the aberrant over-expression of miRNAs (Brueckner, Stresemann et al. 2007; Iorio, Visone et al. 2007; Lujambio, Ropero et al. 2007). An initial study of miRNA expression profiles in this case series has been completed for a subset of cases, and will soon be extended to include additional cases at specific target miRNAs. Although statistical power to detect associations is low due to small sample size, preliminary results indicate that expression of several miRNAs may be independent predictors of tumor histology. Another important experiment is to profile the miRNA expression in non-tumor pleural samples, and this approach may reveal biomarkers for differential diagnosis of pleural mesothelioma in addition to those presented in this thesis. Finally, once these initial investigations are complete, integration of the miRNA,epigenetic, and genetic copy number profiling datasets will hopefully provide a more complete picture of the true genomic context of human MPM tumors.

Although it is a source of considerable debate, there is evidence that infection with simian virus 40 (SV40) may contribute to mesothelioma risk (Barbanti-Brodano, Sabbioni et al. 2004). Between 1955 and 1963 hundreds of millions of humans in North America, Europe, Asia and Africa were exposed to SV40 virus in contaminated polio vaccines (Carbone, Rizzo et al. 1997). Reviews from (Butel and Lednicky 1999; Minor, Pipkin et al. 2003) note a seroprevalence of SV40 in the general population that ranges from 2-20%. Importantly, shedding of infectious SV40 virus is detectable in stool for at least five weeks post polio vaccination, indicating the potential for horizontal viral transmission (Melnick and Stinebaugh 1962). Whereas SV40 grows poorly in human fibroblasts, it is capable of productively infecting human

fetal tissues, newborn kidney cells and mesothelial cells without cell killing (Shein and Enders 1962; O'Neill and Carroll 1981; O'Neill, Xu et al. 1990; Vilchez and Butel 2004). SV40 encodes large T-antigen, a protein that facilitates viral transformation by binding and inactivating the tumor suppressor p53 and the Rb family of TSGs (Bargonetti, Reynisdottir et al. 1992; Ahuja, Saenz-Robles et al. 2005). A review that conducted meta-analysis of SV40 infection in human cancer reported that 262/528 (50%) cases of MPM had SV40 infection, compared to 26/468 (6%) controls, a significant OR of 16.8 (Vilchez and Butel 2004). Strengthening this evidence of a role for SV40 in MPM, a more recent report found the same prevalence of SV40 positive tumors (49%) in their case series of 63 tumors (Suzuki, Toyooka et al. 2005). These authors also examined methylation at 12 TSGs in these tumors and found a higher prevalence of methylation among SV40 positive tumors at all 12 TSGs, suggesting a potential association between SV40 infection and TSG hypermethylation in MPM. However, many contend that the widespread use of plasmids containing portions of the virus has led to the contamination of PCR reactions designed to detect viral sequences in cancers. In an effort to determine whether SV40 infection is more prevalent among MPM cases; investigate its potential association with asbestos burden; and determine if viral infection is associated with methylation class profiles; we have developed a novel method for SV40 detection. Putative viral miRNAs have been described in the SV40 genome and are thought to assist the virus in immune evasion by down-regulating expression of large T-antigen (Sullivan, Grundhoff et al. 2005). Stem-loop RT-PCR assays aimed at detecting mature SV40 miRNAs have been designed, and are a promising approach for determination of SV40 infection in pleural mesothelioma.

The research and analytic methods presented here, and the discussed directions have the potential to be applied in studies of other exposure-related human cancers. Certainly, the

relationships among constitutional genetics, somatic alterations, carcinogen exposure, and patient outcome in human cancer are incredibly complex. As such, it is not surprising that there are currently several challenges to the integrative genomics approaches alluded to above; software and analysis tools to integrate and analyze data from multiple array-based platforms are lacking. In addition, one cannot ignore the substantial expertise and funds necessary for experiments of this scale and complexity. It is of paramount importance that well designed epidemiologic studies are the basis of these investigations such that there is ample genetic material and annotation of demographic, exposure and tumor characteristics for such comprehensive study. Despite these challenges, disentangling these dynamic and complex interactions holds incredible potential for appraising the pathogenic mechanisms of this and other cancers, as well as for translating into effective prevention, screening, diagnostic, and treatment strategies that may dramatically improve patient quality of life and survival.

Surprisingly, asbestos has not been completely banned in the United States, and serious, high-level exposures persist in many nations that continue to import and utilize asbestos (Joshi and Gupta 2004; Kazan-Allen 2005). Despite a partial ban on asbestos in the U.S. there are still approximately one thousand asbestos citations issued by the occupational health and safety administration annually (Castleman 2001). Further, some provisions of the original measures to reduce asbestos use had loopholes that allowed products such as spray-on fireproofing to be sold and marketed as "asbestos free" as long as the asbestos content was less than 1% for years after the EPA ban on these products (Castleman 2001). Surprisingly, in 2000, over two hundred metric tons of asbestos-laden clothing, yarn and other textile products were imported to the U.S., along with over 50,000 metric tons of asbestos-cement products (Castleman 2001). Leading nations of occupational health and safety regulations in Europe banned asbestos before the turn

of the century, and the European Union had a deadline for all its aspiring member nations to ban asbestos by 2005 (Castleman 2001). However, the asbestos industry has effectively managed to work around these bans, simply exporting asbestos to poorer nations. Import and use of asbestos remains high in developing countries like India, China, and Central American nations. Combined with the fact that asbestos-containing products are still imported, and other exposure sources still exist in the U.S., pleural mesothelioma and other asbestos-related diseases are sure to continue presenting in patients for the foreseeable future; arguing for advancing the understanding of how exposure contributes to disease. As of spring 2008, there is a renewed push to ban asbestos in the United States in the form of the "Ban Asbestos in America Act" which has passed the U.S. Senate and awaits consideration by the House of Representatives.

In summary, these results presented herein have contributed to the understanding of the relationships among asbestos burden, epigenetic alterations, and patient survival in pleural mesothelioma. It is important to reiterate the clinical implications of these findings as they may truly serve to improve diagnostic procedures and translate to increased quality of life and survival for patients with this devastating disease.

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