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Targeting epigenetic mediators of gene expression in thoracic malignancies☆

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Abstract

Lung and esophageal cancers and malignant pleural mesotheliomas are highly lethal neoplasms that are leading causes of cancer-related deaths worldwide. Presently, limited information is available pertaining to epi-genetic mechanisms mediating initiation and progression of these neoplasms. The following presentation will focus on the potential clinical relevance of epigenomic alterations in thoracic malignancies mediated by DNA methylation, perturbations in the histone code, and polycomb group proteins, as well as ongoing translational efforts to target epigenetic regulators of gene expression for treatment of these neoplasms.

Keywords

Lung cancer; Esophageal cancer; Mesothelioma; Epigenetics; Prognosis; Clinical trials

1. Introduction

Lung and esophageal cancers and malignant pleural mesotheliomas (MPM) rank among the most lethal neoplasms worldwide [74]. In 2011, these malignancies will account for approximately 1.8 million deaths globally; in the United States, nearly 160,000 deaths will be attributed to lung cancer, 15,000 will be due to esophageal cancer, and approximately 2500 will be attributed to MPM [149,185]. A variety of genetic and environmental factors contribute to these neoplasms; of particular concern, 80% of lung cancers and 50% of esophageal carcinomas are directly attributable to tobacco smoke [1,186]. Interestingly, although not typically considered to be a tobacco-related disease, MPM occurs much more frequently in smokers [48]. These clinical observations are consistent with experimental studies demonstrating that cigarette smoke enhances asbestos-mediated development of MPM in rodents [180].

Relatively limited information is available pertaining to the frequency, mechanisms and clinical relevance of potentially reversible, epigenomic alterations that perturb gene expression in thoracic malignancies [11,14,144]. Similar to other neoplasms [9], thoracic malignancies exhibit genomic hypomethylation, primarily involving repetitive DNA

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sequences [45,155], with paradoxical site-specific DNA hypermethylation, which silences numerous homeobox and tumor suppressor genes [145]. Alterations in DNA methylation coincide with reversible modifications of core histones reflecting activated, repressed or bivalent chromatin structure within the respective genomic loci, and appear to recapitulate stem cell development paradigms [8,72,102].

Although recent studies suggest that global DNA demethylation may be linked to aberrant DNA repair [55,139], decreased DNMT1 expression [100,173], replication independent DNA demethylases [45,127], or formation of 5-hydroxymethylcytosine [37,75], the mechanisms mediating genomic demethylation in thoracic malignancies remain elusive. DNA hypomethylation appears to occur early during aerodigestive tract carcinogenesis. Liu et al. [100] exposed cdk-4/h-tert immortalized human bronchial epithelial cells to cigarette smoke condensate, and observed that hypomethylation of DNA repeats and upregulation of imprinted alleles preceded epi-genetic silencing of tumor suppressors. In an additional recent study, Alvarez et al. [3] observed that DNA hypomethylation is an early epigenetic event, which synergizes with gene amplification during esophageal carcinogenesis.

Irrespective of the mechanisms, global DNA demethylation facilitates de-repression of endogenous retroviruses [178], pseudogenes, and imprinted alleles [45,64,66,68,88–90,163,192], thereby enhancing genomic instability (Table 1). Global DNA demethylation also results in de-repression of a variety of genes that are silent in normal somatic cells, yet exhibit stage-specific expression during germ cell development in testes or ovary (Table 1). To date, more than 100 of these “cancer-testis (CT)” genes have been identified, approximately half of which map to the X chromosome [152]. Relative to autosomal CT genes, CT-X chromosome (CT-X) genes are more frequently activated in cancer cells, and particular gene families appear to be coordinately de-repressed in a tumor-specific manner.

Although several studies have suggested that de-repression of CT genes enhances the malignant phenotype of cancer cells by up-regulation of additional CT genes [67,161], activation of telomerase [130], induction of cell cycle progression and inhibition of p53 function [86,106,113,126,176], or increasing resistance to death receptor ligands, radiation or chemotherapeutic agents [4,34], limited evidence indicates that CT gene activation directly impacts prognosis of patients with thoracic malignancies. Melloni et al. [108] observed that GAGE expression correlated with diminished survival of lung cancer patients. Gure et al.[54] examined CT-X gene expression in tumors from over 500 lung cancer patients undergoing potentially curative resections. De-repression of CT-X genes coincided with tobacco use and advanced stage of disease; expression of NY-ESO-1 and/or MAGE-A3 correlated with poor survival. Consistent with these findings, Yanagawa et al. [174] observed that MAGE-A1 or A3 expression in lung cancers correlated with adverse patient outcome. In contrast, Akcakanat et al. [2] detected expression of GAGE, NY-ESO-1 and MAGE-A1 protein expression in 20%, 21% and 52% of 213 esophageal cancer specimens, respectively. De-repression of these CT genes did not correlate with tumor stage, disease progression, or patient survival. To date the clinical relevance of CT genes activation in MPM has not been established.

Unlike the effects of DNA hypomethylation, the clinical ramifications of aberrant silencing of tumor suppressor genes in thoracic malignancies are more evident (Table 2). The remaining presentation will focus on the potential clinical relevance of epigenomic alterations in thoracic malignancies mediated by DNA methylation, perturbations in the histone code, and polycomb group proteins (Table 3), as well as translational efforts to target epigenetic regulators of gene expression for treatment of these neoplasms.

2. DNA methyltransferases and methylated DNA binding proteins

DNA methylation is the major epigenetic mechanism mediating dynamic patterns of gene expression during differentiation and normal cellular homeostasis [63], as well as long-term repression of gene expression associated with imprinting and X chromosome inactivation [9,40,65]. To date, three major DNMTs (DNMT1, 3a, and 3b), with numerous isoforms have been characterized in somatic cells [29], all of which mediate transfer of a methyl group from S-adenosyl-methionine to the 5′ position of cytosine in the context of CpG. Whereas the specificities of various isoforms have not been fully elucidated, and considerable overlap exists, DNMT1 binds preferentially to hemimethylated DNA, and functions primarily as a maintenance methyltransferase; DNMT3a and 3b, interact primarily with unmethylated or hemimethylated DNA to mediate de-novo DNA methylation [168]. An additional isoform-designated DNMT3L, which is normally expressed only in germ cells[51], lacks methyltransferase activity, and functions to enhance targeting of DNMT3a and 3b to DNA [168].

Several laboratory studies indicate that aberrant expression of DNMTs contributes to the pathogenesis of thoracic malignancies [38,100]. Furthermore, increasing evidence indicates that altered expression of DNMTs may be of prognostic significance in these neoplasms. For example, Kim et al. [82] observed over-expression of DNMT1 and DNMT3b in approximately 50% of lung cancer specimens; over-expression of DNMT1 correlated with p16 promoter hyper-methylation, and diminished patient survival. Lin et al. [98] reported that coordinate expression of DNMT1, 3a, and 3b coincided with increased methylation of a variety of tumor suppressor genes including p16, FHIT and RAR-β in lung cancers, and diminished survival of patients with these neoplasms; this phenomenon was especially pronounced in smokers with squamous cell carcinomas. In a subsequent study, Lin et al. [96] examined DNMT1 expression in 124 lung cancers, and observed that high-level intranuclear DNMT1 levels correlated significantly with smoking status, and diminished patient survival. Vallbohmer et al. [159] examined DNMT1, 3a and 3b expression levels relative to methylation status of APC, DAPK, GSTP1 and MGMT in 91 lung cancer specimens and adjacent normal lung samples. Expression of the three DNMTs was higher in tumors compared to normal lung. Whereas no correlation was observed between DNMT expression and DNA methylation, hypermethylation status was an independent adverse prognostic factor. Consistent with these findings, Brock et al.[16] observed that methylation of cdk2a, p16, CDH13, RASSF1A, and APC in resected early stage (node negative) lung cancers was associated with tumor recurrence, independent of histology or stage, gender, or smoking history.

Xing et al. [170] used quantitative RT-PCR techniques to examine DMNT1 and DNMT3b, as well as methylated DNA-binding protein 2 (MBD2) expression in 148 resected NSCLCs. High-level DNMT1 expression correlated significantly with increased risk of cancer-related death in all patients, whereas increased DNMT3b expression was associated with poor outcome in patients less than 65 years of age. High-level expression of MBD2 correlated with poor survival in male patients and those with squamous cell carcinomas. Combinatorial effects of DNMT1 and 3b, DMNT1 and MBD2, and DNMT3b and MBD2 were evident in male patients, and those with squamous cell carcinomas. Collectively, aforementioned clinical data as well as laboratory experiments indicating that knock-down of DNMT1 and/or 3b induces genotoxic stress and apoptosis [79], whereas knock-down of MBD2 inhibits growth and tumorigenicity of cultured lung cancer cells [21], attest to the relevance of aberrant expression of these modulators of DNA methylation during pulmonary carcinogenesis.

Several studies have been performed to examine DNMT expression, promoter methylation, and outcome in patients with esophageal cancers. Simao et al. [150] observed over-expression of DNMT1, DNMT3a, and DNMT3b in 35%, 41%, and 65% of esophageal squamous cell carcinomas (ESCC), relative to paired normal esophageal mucosae; over-expression of DNMT3b correlated with down-regulation of p16/p14. Li et al. [95] examined promoter methylation status of 12 tumor suppressor genes (TSG) using MSP in ESCC and paired normal esophageal tissues from 47 patients. Forty-six of 47 ESCC exhibited methylation of at least one of these TSG, with methylation being most frequently observed for RAR-β, DAPK, p16 and CDH1. Over-expression of DNMT1, DMNT3a and DNMT3b was observed in 48%, 48%, and 72% of tumors, respectively. A significant increase in DNMT3b levels was observed, which correlated with hypermethylation of TSGs. No significant correlation was observed between promoter hypermethylation, nodal status, or smoking/ethanol history.

Ling et al. [99] examined promoter methylation status of 9 TSGs in 50 ESCC, 50 dysplastic lesions, and 50 normal esophageal samples. CpG island methylation phenotype (CIMP), defined as 5 or more methylated genes, was identified in 54% of ESCC compared to 8% dys-plastic tissues, and 0% of normal esophageal samples. CIMP positivity correlated with more advanced TNM stage and metastases. Furthermore, patients with CIMP-positive tumors had significantly shorter survivals relative to patients with CIMP-negative tumors following esophagectomy. These findings are consistent with data reported by Brock et al. [15] who observed that patients with esophageal adenocarcinomas exhibiting methylation of >50% of seven TSGs had significantly poorer survival and earlier tumor recurrence than patients without such methylation. Interestingly, CIMP status was a more powerful predictor of survival than tumor stage.

Limited information is available regarding DNMT expression levels and aberrant DNA methylation in MPM. Christensen et al. [31] examined methylation status of six cell cycle regulatory genes in 70 MPM specimens, and observed significant correlation between methylation status and asbestos body burden, which remained significant irrespective of gender, age or tumor histology. In a subsequent study, Christensen et al. [32] observed that extent of gene copy number alterations correlated with DNA methylation profile, an

association that appeared to be due, in part, to allelic loss of DNMT1. Fischer et al.[47] examined methylation status of nine genes in serum DNA, and observed that methylation status of any single gene had no association with survival. However, combining RAR-β with either RASSFIA or DAPK correlated significantly with decreased survival. Furthermore, patients with tumors exhibiting methylation of any two or all three of these TSGs did significantly worse than those with no methylation of these genes, suggesting that the extent of DNA methylation coincided with aggressive tumor phenotype. Destro et al. [41] evaluated methylation status of p15, p16, RASSFIA and NORE1A in 79 MPM patients. Methylation of at least one of these genes was observed in 30% of the cases (the frequency of methylation ranged from 5 to 20%). Patients undergoing extra-pleural pneumonectomy for MPM with DNA methylation had shorter median overall survivals compared to patients with tumors lacking methylation of these genes (16 vs. 35 months; p=0.06). Collectively, these data suggest that extent of DNA methylation coincides with more aggressive tumor histology and decreased survival of patients with MPM.

3. Histone acetylases/deacetylases and histone methyltransferases/ demethylases

Lysine rich tails of core histone proteins (H2A, H2B, H3, and H4), protrude from the nucleosome, providing sites for highly diverse, reversible, covalent modifications such as acetylation, methylation, phosphorylation, sumoylation, and ubiquitination that alter chromatin structure and modulate gene expression [7,94,140]. To date, alterations in histone acetylation/deacetylation and methylation/demethylation, have been the most extensively characterized histone modifications in cancer cells [10,137,141,182]. Histone acetylation/ deacetylation is mediated by a variety of histone acetyltransferases (HATs) including GNAT, MYST, and p300 families [10,42], whereas histone deacetylation is mediated by histone deacetytlases (HDACs), which are divided into four classes based on phylogenetic and functional criteria [13,141]. Histone acetylation increases net negative charge leading to repulsion of DNA, relaxation of chromatin, and activation of gene expression [5]. Many non-histone proteins including p53, Hsp90, Sp1, and HDAC1 are also targets for HATS/ HDACs [30,141,151].

Histone lysine methylation is mediated by a variety of histone methyltransferases (KMTs) including SET, MLL, SUV39H, and EZH1/2 families as well as, RIZI, G9a, ASH1, and DOT1L that mediate mono-, di- and trimethylation of specific lysine residues [50,62]; histone demethylation is mediated by histone demethyltransferases (KDMTs) comprising two groups (LSD1) and jumonji-domain containing (JHDM) proteins [35,50,119,182]. In contrast to histone acetylation, histone ly-sine methylation does not alter charge of core histones. Depending on the site, histone lysine methylation may either facilitate or inhibit gene expression. For example, methylation of histone H3K9 and H3K27 coincides with transcriptional repression; in contrast, methylation at H3K4, H3K36 or H3K79 tends to be associated with gene activation (reviewed in ref. [50,119]). A variety of non-histone proteins including p53, E2F1, and NFB are targets of HMTs and HDMTs [62,189]. Revised nomenclature and substrates for histone lysine demethylases have been reviewed recently [35].

Relatively limited information is available concerning the direct clinical implications of aberrant expression of HATs and HDACs, as well as HMTs and HDMTs in thoracic malignancies. Increased HDAC1 mRNA levels appear to coincide with advanced stage of disease in lung cancer patients [134]. Interestingly, HDAC1 deacetylates DNMT1, thereby protecting it from proteosomal degradation providing a potential mechanistic link between HDAC1 over-expression and aberrant DNA methylation during malignant transformation [43]. In a recent study, lung cancer patients with high intratumor levels of HDAC3 had significantly shorter disease-free survivals than patients whose tumors exhibited low HDAC3 expression [110]. Multivariate analysis revealed that HDAC3 over-expression was an independent prognosticator of poor survival in patients with adenocarcinomas, but not those with squamous cell carcinomas. In an additional study, Osada et al. [122] observed that reduced expression of several class II HDACs (particularly HDACs 5 and 10) correlated with adverse outcome in lung cancer patients.

Toh et al. [156] utilized immunohistochemistry techniques to examine levels of HDAC1 and histone H4 acetylation in ESCC, and observed H4 hyperacetylation in early lesions, with progressive H4 hypoacetylation in invasive lesions. HDAC1 levels also seemed to decrease with tumor invasion. In a subsequent study, Langer et al. [92] examined HDAC1 and HDAC2 levels in 180 esophageal carcinomas. Approximately 50% of tumors had no detectable or very low HDAC1 expression, whereas 30% of the tumors had no detectable HDAC2 expression. High HDAC2 expression correlated with poor differentiation and lymph node metastases. Interestingly no correlation was observed between HDAC1 or HDAC2 expression and response to therapy or survival.

Less information is available regarding roles of specific KMTs or KDMTs in the pathogenesis of thoracic malignancies. Kim et al. [85] observed up-regulation of DOT1L, which mediates methylation of H3K79 in cultured lung cancer cells and resected lung cancer specimens. Knock-down of DOT1L resulted in decreased proliferation of lung cancer cells, a phenomenon that coincided with aberrant mitotic spindles, chromosomal missegregation, and senescence; these findings suggest that increased expression/activity of this KMT enhances the malignant phenotype of lung cancer cells. Using immunohistochemistry techniques, Hudlebush et al. [69] observed over-expression of multiple myeloma SET (MMSET) histone lysine methyltransferase, which mediates di and tri methylation of H3K36 in a variety of human malignancies including lung and esophageal cancers, and pleural mesotheliomas. This study extends earlier microarray studies reported by Kassamabara et al. [78] demonstrating that numerous human malignancies including lung and esophageal cancers over-express MMSET, and that MMSET up-regulation correlates with advanced stage of disease, and in some tumor histologies, patient survival as well. Hayami et al. [57] observed over-expression of JARID1B, which demethylates H3K4Me3/Me2, in a large panel of small cell as well as non-small cell lung cancers. JARID1B over-expression correlated significantly with increased expression of E2F1 and E2F2. In subsequent studies, Hayami et al. [56] observed up-regulation of LSD1, which catalyzes demethylation of H3K4Me2/Me1 and possibly H3K9Me2/Me1, in small cell lung cancers relative to normal lung tissues; knock-down of LSD1 induced G1 arrest without apotosis in cultured lung cancer cells. Consistent with these findings, Watanabe et al. [165] observed that knock-down of EZH2, which mediates trimethylation of H3K27,or knockdown of

SETDB1, G9a, and SUV39H1, which methylate H3K9, inhibited proliferation and soft agar colony formation of immortalized or fully transformed respiratory epithelial cells, implicating these modifiers of histone methylation in the pathogenesis of lung cancer. Chen et al. [25] observed that G9a expression correlates with aggressive phenotype of lung cancer cells, and poor prognosis in lung cancer patients; over-expression of G9a coincided with decreased H3K9 methylation and repression of the cell adhesion molecule Ep-Cam.

Several recent studies suggest that the summation of perturbed acetylation and methylation of core histones observed in lung cancers may be clinically relevant. Seligson et al. [147] reported that decreased global levels of H3K4Me2 and H3K18Ac correlated significantly with reduced survival of patients with early stage lung cancers undergoing potentially curative resections. Van Den Broeck [160] observed hyperacetylation of H4K5 and H4K8, hypoacetylation of H4K12/H4K16, and decreased H4K20Me3 levels in lung cancer cells relative to adjacent normal respiratory epithelia. Whereas loss of H4K20Me3 was more common in squamous cell carcinomas, this his-tone alteration, which coincided with diminished expression of SUV4–20h2, was not associated with patient outcome. In contrast, loss of H4K20Me3 in early stage adenocarcinomas correlated significantly with decreased patient survival.

Barlési et al. [6] classified 138 lung cancer patients into seven groups based on histology, stage, and global expression levels of H3K4Me2, H2AK5Ac, and H3K9Ac; the groups exhibited significant differences in disease free, as well as overall survivals. Interestingly, the four groups comprising Stage I patients displayed dramatic differences, with a median survival of ten months for adenocarcinoma patients with high-level intratumoral H3K9Ac, compared to 147 months for non-adenocarcinoma patients with high-level intratumoral H3K4Me2 expression.

Several studies have been performed to examine histone alterations in esophageal cancers. For example, Chen et al. [24] used immunohisto-chemistry techniques to examine expression of H3/H4 acetylation and H3K4/H3K27 methylation levels in 30 ESCC patients; none had undergone chemo-or radiation therapy. In addition, qRT-PCR techniques were used to examine expression levels of the respective HATs, HDACs, KMTs, and KDMTs. H3 and H4 acetylation levels were significantly reduced, whereas H3K4 and K3K27 methylation levels were higher in tumor samples compared to adjacent normal esophageal mucosa. The extent of H3 hypoacetylation and H3K27Me3 levels correlated with advanced tumor stage. H4 hypoacetylation coincided with alcohol consumption, whereas H3 hypoacetylation as well as HDAC2 levels correlated with lymph node metastases. Levels of HDAC1, HDAC2, SIRT1 and SUV39H1 were significantly higher in more advanced tumors.

In an additional study, I et al. [71] examined the effects of histone modifications (H3K9Ac, H3K18Ac, H4K12Ac, and H4R3Me2) on recurrence free survival in 237 patients undergoing esophagectomy for esophageal squamous cell carcinomas. Patients whose tumors expressed high global levels of H3K18Ac and H4R3Me2 had significantly reduced recurrence free survivals relative to comparably staged patients with tumors exhibiting low global levels of these his-tone marks. Tzao et al. [158] examined expression status of H3K18Ac, H4K12Ac, H4R3Me2, H3K4Me2, and H3K27Me3 in 97 ESCC. A positive

correlation was observed for H3K18Ac, H4R3Me2, and K3K27Me3 and poor tumor differentiation. Low levels of H3K18Ac and H3K27Me3 were associated with improved prognosis in early stage tumors. Collectively, these studies attest to the relevance of perturbed histone marks in thoracic malignancies.

4. Polycomb group proteins

Polycomb group proteins (PcG) have emerged as critical epigenetic mediators of pluripotency and differentiation of stem cells [33,138], as well as aberrant gene repression during malignant transformation [109,136]. Two major polycomb repressor complexes, each of which contains a variety of core subunits have been identified in mammals. The initiation complex, PRC-2, containing EZH2, EED, PCL, and SUZ12 subunits, mediates trimethylation of H3K27. PRC-2 recruits the maintenance complex PRC-1, containing core subunits of PCAF, PHC, RING1, CBX, and SML, as well as SFMBT and L3MBTL proteins that mediate ubiquitination of H2AK119 [136]. These histone marks coincide with formation of heterochromatin, and repression of gene expression by mechanisms, which to date, have not been fully elucidated [46,114]. Of particular relevance regarding the role of PcG proteins in tumorigenesis are recent observations that EZH2 is increased and essential for maintenance of cancer stem cells [132,154].

Several recent studies highlight the potential relevance of aberrant Polycomb expression/activity in thoracic malignancies. For example, stem cell polycomb group targets are significantly more likely to have cancer-specific promoter hypermethylation than non-polycomb targets [76,167]. Consistent with these findings, Liu et al. [100] observed that polycomb target genes were seven-fold more likely to undergo DNA hypermethylation in cultured human bronchial epithelial cells exposed to cigarette smoke condensate.

Hussain et al. [70] observed polycomb-mediated repression of Dickkopf-1 (Dkk-1), which encodes a secreted antagonist of Wnt signaling in normal human small airway epithelial and lung cancer cells exposed to cigarette smoke. Knock-down of EZH2 abrogated CSC-mediated inhibition of Dkk-1. Furthermore, knock-down of Dkk-1 increased non-canonical Wnt signaling and increased tumorigenicity of lung cancer cells. Collectively, these data provided the first direct evidence that cigarette smoke engages polycomb machinery to silence tumor suppressor genes during pulmonary carcinogenesis.

In additional experiments, Liu et al. (unpublished) observed over-expression of EZH2 in a panel of lung cancer lines relative to cultured normal respiratory epithelial cells. Knock-down of KMT6 inhibited proliferation and tumorigenicity of lung cancer cells. Growth inhibition coincided with cell cycle arrest and senescence. Microarray analysis revealed that knock-down of EZH2 resulted in up-regulation of numerous gene regulatory networks mediating cell cycle control, differentiation and senescence; many of the genes activated by knockdown of EZH2 were stem cell polycomb targets.

Several potential mechanisms contribute to aberrant expression of PcG genes in thoracic malignancies. For example, the vast majority of lung and esophageal cancers and MPM exhibit disruption of Rb-mediated regulation of E2F; EZH2, EED, and SUZ12 are potential

E2F targets [12,36]. Inactivation of p16, which is observed in 30–40% of lung and esophageal cancers, and the majority of MPM [14,73,135], results in up-regulation of KMT6 and SUZ12 [131]. Furthermore, several micro-RNAs such as miR-101 and miR-26a, and miR98 which target the 3′ UTR of EZH2 are down-regulated in aerodigestive tract cancers [49,80,104,187].

Several recent studies have examined the prognostic significance of aberrant PcG protein expression in thoracic malignancies. Vrzalikova et al. [162] used immunohistochemistry (IHC) techniques to examine expression of BMI1 (a component of PRC-1) in 179 NSCLCs including 106 squamous cell, 58 adeno-, and 15 large cell carcinomas. Nearly 70% of samples exhibited moderate or strong nuclear immunoreactivity for BMI1. Subsequent analysis revealed that BMI1 expression correlated significantly with advanced stage of disease (stages III or IV), as well as decreased disease free survival for patients with stage I or II neoplasms. An additional study pertaining to IHC analysis of 134 resected pulmonary adenocarcinomas, demonstrated that BMI1 expression was higher in stage III tumors relative to stage I or II cancers; multivariate analysis revealed that BMI1 expression was an independent prognosticator of poor survival of adenocarcinoma patients [190].

Kikuchi et al. [81] used IHC techniques to examine BMI1 and EZH2 expression in 157 resected NSCLC specimens, including 65 squamous cell carcinomas, 82 adenocarcinomas, 7 adenosquamous carcinomas, and 2 large cell carcinomas. In contrast to aforementioned results, BMI1 expression did not appear to be associated with any clinicopatho-logic features. However, high intratumoral EZH2 expression correlated significantly with non-adenocarcinoma histology, increased proliferation index, moderate to poor differentiation, and significantly decreased patient survival in all stages; the adverse impact of EZH2 expression was particularly apparent in stage I neoplasms.

Several studies have been undertaken to examine the clinical significance of aberrant PcG protein expression in esophageal cancers. He et al. [61] observed that expression of BMI1 mRNA was significantly higher in ESCC compared to adjacent normal tissues. High level BMI1 expression correlated with lymph node metastases, poor differentiation, and advanced pathologic stage. Liu et al. [101] examined BMI1 protein expression in 171 ESCC. BMI1 was detected in 64% of these specimens. BMI1 expression was higher in more advanced tumors. Multivariate analysis suggested that BMI1 expression was an independent adverse prognostic factor. In additional studies, He et al. [59] examined EZH2 expression in approximately 100 pretreatment biopsies of patients with ESCC who received definitive chemo-radiation therapy. High level expression of EZH2 was seen in 54% of cases, and correlated with increased proliferation, high tumor grade, lymph node metastases, and lack of complete response. Multivariate analysis revealed that EZH2 expression was an independent risk factor for poor progressive free survival, and disease specific survival. Consistent with these findings He et al. [60] observed a positive correlation between EZH2 and H3K27Me3 levels, advanced pathologic stage, poor loco-regional progression free survival, and incomplete response to chemo-radiation therapy in the same patients used in the previous study. More recently, Yamada et al. [172] evaluated BMI1 and EZH2 expression in 136 ESCC specimens, and adjacent normal tissues. Approximately 15% of the specimens exhibited over-expression of BMI1 or EZH2. Aberrant expression of EZH2

correlated with larger, more invasive tumors, distant metastases and shorter disease free survival. In contrast BMI1 expression did not appear to coincide with survival.

In a recent study, Kemp et al. [80] observed over-expression of EZH2 in cultured MPM cells relative to normal mesothelia. Subsequent qRT-PCR, immunoblot and immunohistochemistry experiments demonstrated over-expression of EZH2 in 85% of MPM; in contrast, EZH2 expression was not detected in normal pleura or peritoneum. EZH2 over-expression coincided with increased global H3K27Me3 levels, and correlated significantly with diminished survival of MPM patients undergoing potentially curative resections. Knock-down of EZH2 decreased proliferation, migration, clonogenicity and tumorigenicity of mesothelioma cells. Interestingly, although EED was not over-expressed in MPM, the anti-tumor effects of knock-down of EED were more profound than EZH2 depletion, possibly due to relative knockdown efficiencies, compensation of EZH2 knock-down by EZH1, or more profound destabilization of PRC-2 by depletion of EED. The effects of PRC-2 knock-down were recapitulated by the nucleoside analog, 3-deazaneplanocin A (DZNep), which has been shown recently to deplete PRC-2 components in cancer cells [129]. Collectively, these experiments highlight the clinical relevance of EZH2 over-expression in MPM, and suggest that targeting PRC-2 expression/activity may be a novel strategy for mesothelioma therapy.

5. Translational implications

Alterations in chromatin structure secondary to aberrant expression/function of epigenetic regulators of gene expression in thoracic malignancies result in silencing of numerous tumor suppressor genes via polycomb and DNA hypermethylation mechanisms with paradoxical de-repression of CT genes in thoracic malignancies [8,145]. Because CT genes are typically expressed only in immune-privileged sites, proteins encoded by these genes induce humoral as well as cell-mediated immune responses when aberrantly expressed in somatic cells; as such, cancer-testis antigens (CTA) have emerged as highly attractive targets for cancer immunotherapy [20]. Vaccines targeting CTAs such as NY-ESO-1, MAGE-A1, and MAGE-A3 induce anti-tumor immunity, and T cells expressing native or genetically-engineered receptors recognizing these antigens mediate tumor regression in some cancer patients [22,77,133].

Whereas NY-ESO-1, MAGE-A1, and MAGE-A3 are expressed in 25–40% of lung and esophageal cancers, and MPM, [145], immune responses to these CTAs are uncommon in patients with these neoplasms [52,153], due in part to levels of antigen expression, which are below the threshold for immune recognition. Conceivably, up-regulation of CTAs expression by chromatin remodeling agents can enhance immunogenicity of lung and esophageal cancer and MPM cells, facilitating their eradication by endogenous immune mechanisms, or adoptively transferred T cells. In preclinical studies, we have demonstrated that under exposure conditions that reactivate aberrantly silenced tumor suppressor genes, DAC and the HDAC inhibitor depsipeptide (romidepsin; DP), or DAC and DZNep mediate synergistic induction of CT gene expression in cultured lung cancer cells, but not normal respiratory epithelia or lymphoid cells [67,129]. In addition, we have reported that following DAC, sequential DAC/DP, or concurrent DAC-DZNep exposure, lung and esophageal cancer and

MPM cells can be recognized by cytolytic T lymphocytes (CTL) expressing receptors specific for NY-ESO-1 or MAGE-A3 [28,129,164,166]. Furthermore, we have demonstrated that a CTA induced in tumor cells in vivo by systemic DAC administration can be effectively targeted by adoptively-transferred CTL in immunocompetent mice [53]. Collectively, these observations provide strong rationale for the use of chromatin remodeling agents as a means to simultaneously inhibit growth and enhance immunogenicity of thoracic malignancies in clinical settings.

To date, most epigenetic therapies for thoracic malignancies have focused on the evaluation of pharmacologic inhibitors of DNMT and HDAC activity [17,141]. Specifically these trials have evaluated the nucleoside analogs, 5-azacytidine (5-AC) and 5-aza-2′ deoxycytidine (DAC) as well as HDAC inhibitors (HDACi) such as depsipeptide (DP; Romidepsin), sodium butyrate, and SAHA (Vorinostat) either alone or in combination with other chromatin remodeling agents or conventional chemotherapy regimens. When evaluating results of trials utilizing chromatin remodeling agents in cancer patients, it is important to remember that the anti-tumor effects of DNA demethylating agents and HDACi are not solely attributable to re-activation of tumor suppressor genes aberrantly silenced by epigenetic mechanisms [171]. For instance, cytotoxicity mediated by DAC is attributable in part to formation of DNMT-DNA adducts (particularly those involving DNMT 3a and 3b) [120] as well as DNA damage [124]. HDACi may facilitate acetylation of a variety of non-histone proteins such as p53 and Hsp90, thereby modulating cell cycle progression and apoptosis in cancer cells [30,97]. Furthermore, DNA demethylating agents in combination with HDACi induce DNA damage [105], and inhibit removal of incorporated abases [23] in lung cancer cells. Similar issues must be kept in mind when examining mechanisms by which KMT/KDMT inhibitors mediate cytotoxicity in cancer cells.

In a phase I/II study, Momparler et al. [112] treated 15 lung cancer patients with DAC administered at various doses over 8 h, and observed prolonged survival (>5 yr) in one of nine evaluable patients. In a more recent phase I trial, Schrump et al. [143] treated 35 patients (including 22 with lung cancer, 4 with esophageal cancer and 6 with MPM) with escalating doses of DAC administered via continuous 72 h infusions. The maximum tolerated dose of DAC was 75 mg/m^2; myelosuppression was dose limiting. Steady state plasma DAC concentrations ranged from 25 to 50 ng/ml, which closely approximated threshold exposures for gene activation in cultured cancer cells. Although no objective responses were observed, two lung cancer patients exhibited prolonged stabilization of disease (>1 yr). Nearly one quarter of all patients exhibited expression of p16, MAGE-3, or NY-ESO-1 in post-treatment tumor biopsies. Serologic responses to NY-ESO-1 were observed in several patients receiving DAC infusions for >6 months.

In a phase II trial, Schrump et al. [142] treated 18 lung cancer patients with Romidepsin (~18 mg/m^2) administered as a 4 h infusion on days 1 and 7 of a 21 day cycle. Transient stabilization of disease (2–6 months) was observed in 9 patients. Steady state Romidepsin levels during infusion ranged from 384 to 1114 ng/ml (median: 667 ng/ml), decreasing to ~10 ng/ml within 4 h following the infusion. Intratumoral levels of H3Ac and p21 (a major target of HDAC inhibitors) were increased in approximately 50% of patients following

Romidepsin therapy. In addition, several patients exhibited enhanced expression of NY-ESO-1 and MAGE-A3 in tumor biopsies following Romidepsin infusions.

In a more recent trial, Otterson et al. [123] treated 16 SCLC patients with Romidepsin (13 mg/m2) administered as 4 h infusions on days 1, 8 and 16 of a 29 day cycle. No objective responses were observed; transient stabilization of disease was observed in 3 patients. Median progression free survival was less than two months, and median overall survival approximated six months. In an additional phase II trial, Traynor et al. [157] treated 16 patients with relapsed NSCLC with Vorinostat 400 mg PO daily. No objective responses were observed in 14 evaluable patients. Median time to progression was 2.3 months, which was similar to that observed for lung cancer patients receiving other targeted agents. Collectively, results of these lung cancer trials are consistent with broader experience demonstrating minimal activity of HDAC inhibitors as single agents in solid tumors [141].

Numerous pre-clinical studies have demonstrated either additive or synergistic pro-apoptotic effects of HDAC inhibitors in combination with standard cytotoxic chemotherapeutics or targeted agents in lung cancer cells [18,177,184,188]. The mechanisms underlying this phenomenon appear to be related to HDAC inhibitor-mediated induction of reactive oxygen species, depletion of oncoprotein expression, and inhibition of survival signaling [141,183,184]. Ramalingam et al. [128] randomized 94 chemo-naive patients with stage IV NSCLC to receive carboplation and paclitaxel with either Vorinostat (400 mg) on days 1–14 of a 21 day cycle, or placebo. The median number of treatment cycles was four in both arms. Median response rate was significantly improved in patients receiving Vorinostat vs placebo (34% vs12.5%). Whereas progression free and overall survivals in patients receiving Vorinostat increased 2–3 months, this survival benefit was not statistically significant.

In a recent phase I trial, Schrump et al. (manuscript in preparation) treated 31 patients with thoracic neoplasms (including 20 lung cancer, 4 esophageal cancer and 3 MPM patients) with sequential DAC/Romidepsin infusions. DAC was administered as a continuous 72 h infusion commencing on day 1 of a 35 day treatment cycle; Romidepsin was administered as a 4 h infusion immediately following DAC, and on day10. Median steady state DAC and Romidepsin levels were 13.4 ng/ml (range: 4.7–78 ng/ml), and 323 ng/ml (range: 136–923 ng/ml), respectively. Whereas no objective tumor regressions were observed, several patients exhibited stabilization of disease lasting 4–6 months. Correlative gene expression profiling experiments demonstrated that sequential DAC/Romidepsin regimens partially reversed aberrant lung cancer gene expression in vivo. The fact that these molecular responses did not translate into more prolonged disease stabilization or tumor regressions strongly suggests suboptimal drug concentrations and exposure durations.

In an ongoing phase I dose-escalation trial at the NCI, 22 patients have received sequential Romidepsin/Flavopiridol infusions, based on preclinical studies demonstrating that abrogation of p21 induction by Flavopiridol (a synthetic cdk inhibitor that also destabilizes RNA [91]) markedly enhances DP-mediated apoptosis in cultured lung and esophageal cancer and MPM cells [118]. To date, 7 patients with lung cancer, 4 with esophageal cancer, 4 with MPM, and 3 with thymoma have received 4 h DP infusions followed by 72 h Flavopiridol infusions under exposure conditions mimicking those used in preclinical

experiments. Whereas no objective responses have been observed, seven patients with refractory cancers have exhibited disease stabilization lasting 4 to greater than 12 months.

6. Conclusions

Considerable data suggest that epigenetic regulators of gene expression contribute to initiation and progression of thoracic malignancies. As such, these mediators or their co-factors are attractive targets for intervention in these malignancies. Of particular concern regarding the use of chromatin remodeling agents for these neoplasms are observations that genes aberrantly hypermethylated in cancer cells do not revert to a fully euchromatin state [107], and that genes, which have been de-repressed by DNA demethylating agents cannot be maintained in an active state by HDAC inhibitors [44]. These findings, together with results of recent clinical trials suggest that epigenetic therapies may need to be administered for prolonged periods to be ef-ficacious. Novel agents such as DZNep, which depletes PRC-2 components, as well as other histone KMT/KDMT inhibitors are currently in preclinical development. One intriguing issue regarding the development of inhibitors of KMTs and KDMTs pertains to the apparent simultaneous targeting of activation and repressive methylation marks; a critical aspect of development of these compounds will be delineation of the hierarchy of methylation marks, which coincide with anti-tumor activity, in order to improve the specificity and reduce potential toxicities of these agents.

Moving forward, it appears that epigenetic strategies will need to be combined with conventional chemotherapeutics, molecularly-targeted agents, or immunotherapy regimens to mediate regression of thoracic neoplasms in clinical settings. Further analysis of epigenetic mechanisms associated with malignant transformation, as well as the identification of novel compounds that specifically target components of the epigenetic machinery that are dysregulated in thoracic malignancies may hasten the development of more efficacious epigenetic regimens for the treatment and possible prevention of these neoplasms.

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Table 1

Genes frequently de-repressed in thoracic malignancies.

	Chromosome
Imprinted genes	
H19 [88]	11p15.5
IGF2 [90]	11p15.5
MEST [88]	7q32
Cancer testis genes	
BORIS [67]	20q13.2
MAGE A3/6 [87,146]	Xq28
NY-ESO-1 [84,87]	Xq28
LAGE-1 [87]	Xq28
GAGE [2,54,108]	Xp11.4
XAGE [115]	Xp11.22
TRAG-3 [27]	Xq28
OY-TES-1 [121]	12p13
CT-45 [26]	Xq26–3
CTSP-1 [125]	21q11.2

Table 2

Tumor suppressor genes hypermethylated with prognostic significance in thoracic malignancies.

DAPK [19,47,103]	CDH-13 [16]
FHIT [83,98,117]	TFPI-2 [169]
HIC-1 [58]	TIMP-3 [39]
APC [15,16]	$RUNX_3$ [175]
WIF-1 [181]	DLEC1 [148,191]
Cdh1 [93,117]	RAR-β [47,98]
P16/pl4ARF [16,83,98,181]	RASSF1A [16,19,47,175]

Table 3

Epigenetic regulators of gene expression implicated in thoracic malignancies.

DNMT1 [82,96,98,150]	SUV39H1 [165]
DNMT3b [82,98,150,159]	SUV39H2 [179]
MBD2 [170]	G9a [25,165]
HDAC1 [134]	KDM1 [56]
HDAC3 [110,111]	JARID1B [57]
HDAC5 [122]	BMI1 [61,101,162,190]
HDAC8 [116]	HDAC10 [122]
EZH2 [59,60,80,81,172]	