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Transl Res. Author manuscript; available in PMC 2016 January 01.

Published in final edited form as:

Transl Res. 2015 January ; 165(1): 74–90. doi:10.1016/j.trsl.2014.03.001.

Epigenetics of Lung Cancer

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Abstract

Lung cancer is the leading cause of cancer-related mortality in the United States. Epigenetic alterations, including DNA methylation, histone modifications, and non-coding RNA expression, have widely been reported in the literature to play a major role in the genesis of lung cancer. The goal of this review is to summarize the common epigenetic changes associated with lung cancer to give some clarity to its etiology, and provide an overview of the potential translational applications of these changes, including applications for early detection, diagnosis, prognostication, and therapeutics.

Keywords

DNA methylation; histone modifications; microRNA; lnc-RNA; non-coding RNA; biomarkers; NSCLC; small cell carcinoma; pulmonary carcinoma

INTRODUCTION

Despite large-scale reductions in cigarette consumption over the past several decades, lung cancer remains the leading cause of cancer-related mortality in the United States and is the second leading cause of death overall, after heart disease [1]. While lung cancer rates have steadily declined among men since the 1980s and seem to have plateaued among women, there still remain an estimated 228,190 new cases and 159,480 deaths each year [1]. This high mortality rate is driven by the high incidence of this disease coupled with its dismal 5-year survival rate of only 17% [1].

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All authors have all read the journal's authorship agreement and policy on conflicts of interest and have no conflicts to declare.

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The vast majority of lung cancer is can be characterized as small cell (neuroendocrine) carcinoma (SCLC) or non-small cell carcinoma (NSCLC), which broadly includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma subtypes [2]. NSCLC is by far the more common of the types, accounting for approximately 85% of all lung cancer cases [3]. While these histologies share a common organ of origin and some molecular attributes, they also exhibit unique molecular traits and represent distinct diseases, and it has become clear that it is important to make the distinction between the histologies for the purpose of treating the disease.

While smoking remains the major risk factor for all histologies (especially small cell and squamous cell carcinoma), it is important to note that only some 10% of smokers will ultimately develop lung cancer [4]. Moreover, not all lung cancer patients have a smoking history, as there are other risk factors for the disease. Adenocarcinoma, which accounts for approximately 37% of all lung cancers in the United States, is the most common form among non-smokers [5]. Globally, an estimated 15% of men and 53% of women with lung cancer are never-smokers [6]. Other risk factors for the disease include radon, asbestos, and environmental/occupational exposure to polycyclic aromatic hydrocarbons and other pollutants [2]. However, as with smoking, not everyone who is exposed to these environmental factors will go on to develop lung cancer.

The carcinogenic process is driven by the accumulation of genetic and epigenetic alterations that result in dysregulation of key oncogenes, tumor suppressor genes, and DNA repair/housekeeping genes. The probability of incurring these pathologically important events is largely dependent upon the individual exposome in conjunction with interpersonal phenotypic variability. While genetic heterogeneity accounts for some of the variable risk, it does not explain this phenomenon *in toto* [7]. *Epi*genetic variability, including DNA methylation, histone modifications, and non-coding RNA expression, also contribute the phenotypic makeup of an individual (e.g. xenobiotic metabolism, DNA repair capacity, immunity, etc.) and, accordingly, risk of malignancy.

This underscores the importance of enhancing our knowledge of lung cancer epigenetics (in addition to genetics) to fully comprehend the pathogenesis of this disease. Moreover, continued expansion our understanding of various epigenetic events involved with different types of lung cancer expands the potential battery of diagnostic and prognostic biomarkers available to clinicians, as well as introducing new avenues for the discovery of novel therapeutic targets. The primary objective of this review is to summarize the common epigenetic events in lung cancer and provide an overview of the potential translational applications of these events for the management of this disease.

EPIGENETICS & ETIOLOGY

Tumorigenesis

Lung cancer involves an accumulation of genetic and epigenetic events in the respiratory epithelium [8]. While somatic genetic aberrations, such as mutations and copy number alterations, play a well-known role in oncogenesis, epigenetic alterations are in fact more frequent than somatic mutations in lung cancer [9].

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Tumor suppressor gene inactivation through promoter methylation, often referred to as hypermethylation, is a hallmark of lung cancer and is an early event in the carcinogenic process [10, 11]. Promoter methylation of specific tumor suppressor genes, along with the overall number of hypermethylated genes, increases with neoplastic progression from hyperplasia to adenocarcinoma [12, 13]. Promoter methylation can couple with mutation or deletion events to inactivate a tumor suppressor gene (i.e. a different inactivation event for each allele). This is because inactivation of one allele for dominantly acting suppressor gene loci is generally insufficient to lead to clonal selection, since the protein can still be produced from one normal allele. However, there is also evidence that, at some gene loci, both copies do not necessarily need to be inactivated to adversely impact the cell, but rather that partial inactivation of one allele, giving rise to haploinsufficiency, (i.e. one wild-type allele is insufficient to provide full functionality) can contribute to carcinogenesis [14]. In these cases, inactivation of one allele by promoter methylation would be sufficient for clonal selection.

Many of the tumor suppressor genes that are hypermethylated in lung cancer are also frequently hypermethylated in other types of solid tumors [6]. Some are specific, although many are not, similar to what is observed for somatic mutations. In premalignant and malignant states, promoter methylation is frequently observed in genes involved with crucial functions, including cell cycle control, proliferation, apoptosis, cellular adhesion, motility, and DNA repair.

Some of the most oft-studied genes in the context of promoter methylation in lung cancer include *p16INK4a*, *RASSF1A*, *APC*, *RARβ*, *CDH1*, *CDH13*, *DAPK*, *FHIT*, and *MGMT*. While *p16INK4a* is frequently methylated, mutated, or deleted in NSCLC, with estimates for the prevalence of alteration of this gene are around 60%, *p14arf*, which is also encoded on the *CDKN2A* gene, is much less commonly inactivated (~8–30% of NSCLC) [15, 16]. Moreover, although a common event in NSCLC, *p16INK4a* is disrupted in less than 10% of SCLC [15]. Additionally, *RASSF1A* is deleted or methylated in 30–40% of NSCLC and 70–100% of SCLC [15], *FHIT* is deleted or methylated in 40–70% of NSCLC and 50–80% of SCLC [15], and *TSLC1* is methylated in an estimated 85% of NSCLC [15]. A more extensive list of known, commonly hypermethylated genes in lung cancer is provided in Table 1.

Hypermethylation of *CDKN2A* may occur early in the genesis of some lung cancers, having been identified in premalignant lesions [69]. Promoter methylation of *RASSF1A*, *APC*, *ESR1*, *ABCB1*, *MT1G*, and *HOXC9* have been associated with Stage I NSCLC [70], suggesting that they too may occur relatively early on in the development of the cancer. CpG island methylation of homeobox-associated genes is also common Stage I lung cancer, appearing in nearly all early-stage tumors [71]. Conversely, other commonly hypermethylated genes, such as *hDAB2IP*, *H-Cadherin*, *DAL-1*, and *FBN2*, have been associated with advanced stage NSCLC [64, 72, 73], suggesting that these changes may occur at a later point in the carcinogenic process. It is important to note, however, that later involvement does not preclude the importance of the modification in the development of the disease, as they may play key roles in the ability of the cancer to continue to flourish in its advanced state, evade host immunity or exogenous cancer treatments, or to metastasize

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locally and/or systemically. Further, it is critical that these generalized "temporal" observations are kept in perspective, as lung cancer is a very heterogeneous disease and each tumor is unique; an early event in one tumor may not occur until later on in another. Moreover, it must be considered that most of these observations are based on cross-sectional measurement of malignant or premalignant tissue, and that linear consideration of the sequential timing of molecular aberrations likely represents a gross oversimplification of cancer development and progression.

There is some evidence for CpG island methylator phenotype (CIMP), a tumor phenotype characterized by widespread hypermethylation, in lung cancer [74–77]. This is not wholly surprising, given that DNA methyltransferases (DNMT), the group of enzymes that catalyze the covalent attachment of the methyl group to the cytosine base, are upregulated in NSCLC [68, 78, 79].

In contrast to gene-specific hypermethylation, which can occur early on in cancer development, genomic hypomethylation, a genome-wide loss of methylation, may be a late event in the genesis of lung cancer, at least for adenocarcinoma [5]. However, there is not presently a clear consensus on the timing, as Anisowicz found that hypomethylation was associated with NSCLC progression from normal to lung cancer [80]. Regardless, widespread hypomethylation has been associated with genomic instability in NSCLC [81] and can result in oncogene activation [82, 83] and loss of imprinting [84]. In lung cancer, hypomethylation tends to occur at nuclear elements, LTR elements, segmental duplicates, and subtelomeric regions (loss of methylation is much less common at non-repetitive sequences) [85]. In addition to the genomic loss of methyl content, gene-specific hypomethylation has been reported for several loci, including *MAGEA* [86, 87], *TKTL1* [86, 88], *BORIS* [89, 90], *DDR1* [31] *14-3-3σ* [91, 92], and *TMSB10* [83, 93]. *MAGE* overexpression with an associated loss of methylation has been observed in 75–80% of NSCLC [94].

CpG methylation can also induce point mutations through deamination of 5-methylcytosine (5-meC) or enhancement of exongenous carcinogens. Methylated cytosine can undergo hydrolytic deamination causing a C to T transition [95]. More than 30% of disease-related germline point mutations occur at CpG dinucleotides [95]. Furthermore, nearly half of all somatic and one-third of all germline p53 mutations take place at methylated CpGs, and many common p53 mutations that manifest in somatic cells are caused by C to T transitions, including "hot spot" mutations at codons 248, 273, and 282 [96]. The risk of p53 mutation at 5-meC is 10-fold that of unmethylated cytosine, and CpG dinucleotides in these regions have been observed to be methylated in normal tissue [96]. Secondly, DNA methylation can enhance the mutagenic effect of exogenous carcinogens [95]. An example of this is the affinity of benzo(α)pyrene diol epoxide (BPDE) for adduct formation on guanine bases adjacent to 5-meC, resulting in G to T transversions in aerodigestive tract cancers in smokers [97–99]. Similarly, acrolein has an affinity for binding 5-meC, which can induce C to T transitions [100].

Histone deacetylases (HDAC) are overexpressed in lung cancer [101–103]. HDACs catalyze the removal of acetyl groups on the histone tail resulting in a transcriptionally inactive

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heterochromatic state [104]. Likewise, SIN3A (part of an HDAC repressor complex) is downregulated in NSCLC [105]. Cross talk occurs, mediated by proteins with methyl-binding domains that can recruit histone modifying enzymes (MBDs, KAISO, MeCP2), such as HDAC, that closely couples DNA methylation and histone modifications [9]. Relative to normal lung, lung cancer undergoes H4K5/H4K8 hyperacetylation, H4K12/H4K16 hypoacetylation, and H4K20me3 [106]. Lower global levels of H4K20me3 can be detected in precursor lesions and is particularly common in squamous cancers [106].

Other post-translational modifiers that have been reported as overexpressed in lung cancer relative to normal lung tissue include polycomb group genes (PcG), which are crucial epigenetic regulators of stem cell (and cancer stem cell) survival and pluripotency. Specifically, upregulation has been observed for PcGs that form the Polycomb repressive complexes (PRC1 and PRC2) [107–118], particularly for the respective catalytic subunits, B-cell-specific Moloney Murine Leukemia Virus Integration Site 1 (BMI-1), which is involved in gene silencing through ubiquitylation of histone 2A (H2A) [119], and Enhancer of Zeste Homologue 2 (EZH2) [111–114, 116–118], which controls gene expression through histone H3 Lysine 27 trimethylation or by interacting with DNMTs to signal transcriptional repression [119].[109] BMI-1 is overexpressed at higher levels in SCLC relative to NSCLC [109], although, unlike EZH2, it is constitutively expressed in normal lung tissue [120, 121]. Upregulation of Polycomb repressive complex genes have been associated with lung cancer proliferation, survival, and epithelial-mesenchymal transition (EMT) [108, 109, 112, 122, 123].

Non-coding RNA are a class of RNA sequences that are transcribed but do not encode proteins. MicroRNA are small non-coding RNA molecules (~18–22 nt) that can negatively regulate the expression of hundreds of mRNA targets and are frequently dysregulated in lung cancer. Two recent meta-analyses have reported on microRNAs for which expression is commonly reported to be dysregulated in lung cancer [124, 125], which are in summarized in Table 2. In particular, miR-196a and miR-200b are highly overexpressed in lung cancer, with estimated fold-changes exceeding 23- and 37-times, respectively [124]. Much less is presently known about the precise functions of long non-coding RNA (lncRNA), although there is evidence that they function as protein regulators and structural organizers, in addition to regulation of gene expression [126]. Two lncRNA in particular that have been implicated in lung cancer are *metastasis-associated lung adenocarcinoma transcript 1* (*MALAT1*) and *Hox transcript antisense intergenic RNA* (*HOTAIR*). *MALAT1* is a highly conserved lncRNA sequence of ~8000 nt in length [127] that is overexpressed in NSCLC (including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma) [128, 129] and is thought to play a role in cell motility, invasion, and metastasis, at least in part through posttranslational inhibition of expression of genes regulating these functions [130–132]. *HOTAIR* is also a highly conserved lncRNA (at least in mammals) that is also reported to be overexpressed in lung cancer [133–135] and interacts with PRC2 and histone regulatory complexes, and regulates *HOXD* expression [126].

There have also been several well-documented examples of interaction between DNA methylation and microRNAs in lung cancer. For example, miR-124a [136], miR-34b/c [137, 138], miR-886-3p (in SCLC) [139], miR-9-3 [140] and miR-193a [140] have all been

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reported as frequently hypermethylated in lung tumors, while let-7a-3 [141] is often hypomethylated. Moreover, miR-29 targets the transcripts of *de novo* DNMT (DNMT3a/b). Additionally, expression of miR-29 is inversely associated with DNMT3a/b expression in lung tumors, and has been shown to restore normal DNA methylation patterns in experimental models (*in vitro* and *in vivo*) [142].

Some epigenetic alterations reported for lung cancer may be smoking-specific (i.e. only found in lung tumors of smokers). Genes reported to undergo smoking-specific promoter methylation include *APC*, *FHIT*, *RASSF1A*, and *CCND2* [21, 35, 40, 143, 144]. Also, the frequency of promoter methylation of *p16INK4a*, *MGMT*, *RASSF1A*, *MTHFR*, and *FHIT* is higher in the NSCLC tumors of smokers relative to non-smokers [21, 22, 145–147]. Moreover, *RARβ*, *p16INK4a*, *FHIT*, and *RASSF1A* promoter methylation increases with increasing smoking intensity [21, 148–150]. Interestingly, genes whose silencing is associated with duration or amount of tobacco smoking are likely later stage contributors to this disease, as long-term exposure to carcinogenic stimuli would imply a later selection of existing clones. Experimentally, genomic hypomethylation and promoter methylation of *RASSF1A* and *RARβ* were observed when normal small airway epithelial cells and immortalized bronchial epithelial were exposed to cigarette smoke condensate [151]. There is also experimental evidence indicating that cigarette condensate decreases nuclear levels of H4K16ac and H4K2me3 in respiratory epithelial cells [152]. Conversely, *RASSF2*, *TNFRSF10*, *BHLHB5* and *BOLL* have been reported to be more frequently hypermethylated in NSCLC from never-smokers [153, 154].

DNMT1 expression is particularly high in lung cancer tumors of smokers [155]. The tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), has been associated with increased levels of DNMT1. Experimental evidence indicates that NNK does not affect the actual DNMT1 mRNA level but rather inhibits DNMT1 degradation via Akt signaling, which stabilizes the DNMT1 protein by decreasing ubiquitylation, resulting in elevated accumulation and, accordingly, aberrant tumor suppressor gene methylation [151, 155, 156]. More specifically, NNK stimulates Akt, which inactivates GSK3β ser/thr kinase, which would otherwise phosphorylate DNMT1 and recruit ubiquitylases. This is similarly observed for laryngeal [157] and esophageal squamous cancers [158]. Corroborating this point, NNK has been shown to induce promoter hypermethylation of tumor suppressor genes in lung tumors in mice and rats [159–161].

Moreover, chronic inflammation, which occurs in response to cigarette smoking, also plays an important role in lung cancer development, stimulating cellular turnover and proliferation. Inflammation has long been associated with DNA methylation in lung cancer [162–164]. There is evidence that reactive oxygen species (ROS), such as are generated during chronic inflammation, target transcriptional repressors, giving rise to increased levels of DNA methylation [165]. Cigarette smoke also inhibits the metabolism and storage of folate [166]. Nitrates, nitrous oxide, cyanates and isocyanates found in tobacco smoke have been shown to transform folate, a major source of methyl groups for one-carbon metabolism, into a biologically inactive compound in experimental models [167, 168]. In further support of this, reduced serum folate levels have been observed in smokers relative to non-smokers [169, 170]. One-carbon metabolism is a critical pathway in the DNA

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methylation process, and depletion of folate can negatively impact the availability of s-adenosylmethionine (SAM), the primary methyl donor in the cytosine methylation reaction. Consequently, folate deficiency can result in chromosomal damage through impaired nucleotide synthesis and aberrant DNA methylation [171, 172].

To this point, any discussion in this review relating epigenetics to cancer risk factors has primarily focused on tobacco-smoke associated events. However, as discussed earlier, not all lung cancer patients, particularly adenocarcinoma patients, have a history of smoking. Exposure to radon gas is another major risk factor for lung cancer. Experimentally, radon-exposed transformed bronchial epithelial cells exhibit altered microRNA expression profiles [173]. Further, a study of Chinese uranium miners found a dose-response between radon exposure and promoter methylation of *p16INK4a* and *MGMT* in sputum samples [174], although this was not observed in a similar study of uranium miners by Pulling et al [175].

Tissue specificity

Historically, adenocarcinoma and squamous cell carcinoma have been considered together, along with large cell carcinoma, in a heterogeneous class of lung cancers referred to as “non-small cell carcinoma’. However, this broad categorization may no longer be appropriate given our understanding of the biological and clinical differences between the histologic subtypes. Making the distinction between squamous cell carcinoma and adenocarcinoma based on morphology alone can be a challenge, necessitating additional markers for accurate discrimination.

The importance of discriminating NSCLC by histology was highlighted by data from a clinical trial of the VEGF antibody bevacizumab, in which elevated toxicity (severe pulmonary hemorrhage) was observed among patients with pulmonary squamous cell carcinoma but not adenocarcinoma [176]. Bevacizumab, in combination with platinum-based chemotherapy, has shown to be efficacious in non-squamous NSCLC in two Phase III trials (E4599 and AVAiL) [177, 178]. Further, Pemetrexed, an antimetabolite, is more efficacious in terms of overall and progression-free survival among patients with non-squamous NSCLC relative to other histologies [179].

Distinct DNA methylation patterns have been identified for adenocarcinoma and squamous cell carcinoma, respectively [180–183]. Kwon and colleagues identified a methylation panel that could distinguish histologic class of NSCLC [180]. In another regard, Christensen and colleagues were able to accurately discriminate lung adenocarcinoma from mesothelioma, which can also be challenging but is very important to differentiate diagnostically, based on the DNA methylation profile [184]. There are no significant large-scale differences in overall DNA methylation levels by lung cancer histology [32], although there are locus-specific differences. Additionally, there are also reports of histology-specific microRNA expression patterns. For example, miR-205 is reported to be SCC-specific [185, 186], while miR-124a has been reported as specific for adenocarcinoma [136].

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Early detection and diagnosis

Lung cancer mortality could be significantly reduced with earlier detection of the disease. However, only about 15% of lung tumors are localized at diagnosis, with the majority presenting at an advanced stage [2]. Five-year survival for lung cancer is markedly better for early-stage patients, with a less than 10% 5-year survival for advanced stage patients versus better than 70% for early-patients [187]. Spiral computed tomography (CT) has shown promise for the early detection of lung cancer but has a high false-positive rate [188], with up to up to 30% of indeterminate nodules identified by CT ultimately found to be benign [189], indicating a need for development of additional markers to enhance the specificity.

As previously discussed, promoter hypermethylation can be an early event in lung carcinogenesis, and as such, may have utility in early detection of the disease. For instance, promoter hypermethylation of *p16INK4a* has been observed in NSCLC precursor lesions [13, 18], and *PTPRN2* promoter methylation is reported to be an early event in pulmonary adenocarcinoma, with detectable changes in the premalignant atypical adenomatous hyperplasia [5].

Importantly, some of these early epigenetic events can be detected by non- or minimally-invasive sample collection techniques, an important characteristic for cancer screening applications. For example, aberrant DNA methylation can be detected in sputum [190, 191], bronchoalveolar aspirate/lavage [39, 145, 192–194], and saliva [195, 196] in lung cancer patients. Furthermore, *CDKN2A* and *MGMT* promoter methylation was detected in sputum up to 3-years *prior* to lung cancer diagnosis [20], and promoter methylation of *p16INK4a*, *MGMT*, *PAX5b*, *DAPK*, *GATA5*, and *RASSF1A* was detected in sputum 18 months prior to lung cancer diagnosis in another study [197]. However, specificity can be an issue for some of these early markers, as they can also be detected in individuals who will not ever go on to develop disease. To this point, promoter methylation of *p16INK4a* has been detected in sputum from former and current smokers [10], which underscores the importance of multimarker panels. However, not all single markers are non-specific, as exemplified by *SHOX2* promoter methylation, which has demonstrated good sensitivity (68–78%) and specificity (95–96%) for NSCLC in bronchial aspirates (AUC = 86–94%) [51, 193].

Additionally, cancer-specific DNA methylation [38, 198], microRNA [199, 200], and lncRNA [201] profiles have been identified in the blood of lung cancer patients. Meta-analysis on the diagnostic value of circulating microRNAs for lung cancer reports a meta-ROC of 0.92 [202], highlighting the potential diagnostic capacity.

PROGNOSTIC BIOMARKERS

Identification of novel biomarkers to aid in prediction of outcome and tumor response is of paramount importance to optimize therapeutic efficacy and prevent over- or under-treatment of lung cancer patients. Epigenetic biomarkers, in particular DNA methylation and microRNA expression, have unique properties that make them well-suited as potential prognostic markers, and accordingly have been widely studied and reported in the literature.

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Not surprisingly, many of the hypermethylated tumor suppressor genes that are implicated in lung cancer oncogenesis have also been reported to be associated with prognosis. Promoter methylation of *RASSF1A* [16, 36, 203], *PTEN*, *DAPK* [29], *p16INK4a* [21, 24, 203–206], *Wif-1*, *CXCL12* [207], *DLEC1* [208], *MLH1* [208], *CDH1* [58, 204], *CDH13* [58], *APC* [41, 209], *RUNX3* [36], *SPARC*, and *DAL1* have all been associated with NSCLC outcome [22, 36, 64, 207, 208, 210–212]. Additionally, *DNMT1* overexpression in NSCLC is associated with decreased survival [78, 79, 213]; and *DNMT3b* only in patients <65 years old [213]. Along similar lines, CIMP has also been correlated with prognosis in NSCLC [74].

Relative to advanced stage lung cancers, chemotherapeutic recommendations are not as clear for early stage disease, with no true consensus as to the optimal approach [214]. Early stage lung cancer can be locally controlled but exhibits a high recurrence rate. Completely resected Stage IB and II tumors have a nearly 50% recurrence rate with a median time to recurrence of 1-year [215]. Fewer recur in Stage IA tumors, although certain IA subsets have high recurrence rates [216]. Methylation of *p16INK4a*, *RASSF1A*, *CDH13*, and *APC* has associated with early recurrence in surgically treated Stage I NSCLCs [210]. The combination of *FHIT* and *p16INK4a* promoter methylation has also been associated with recurrence in Stage I NSCLC [205].

Several global histone modifications have been associated with lung cancer survival; in particular decreased levels of H3K4diMe have been associated with poorer outcome [217, 218]. Additionally, the combination of several histone modifications have been reported to predict survival (H3K4me2, H3K9ac, H2AK5ac) [217], and H4K20me3 down-regulation has been associated with poorer survival in Stage I lung adenocarcinoma patients [106].

Higher expression of the PRC2 protein, EZH2, in NSCLC tumor tissue has been associated with worse overall [112, 118, 219] and disease-free survival [112] in several studies[117]. Likewise, BMI-1 expression has also been correlated with poorer patient outcomes [107, 121, 220], and amplification and concordant overexpression of the PcG, *PHC3*, has recently been associated with poorer outcomes in NSCLC [221].

Noncoding RNA have also been associated with lung cancer outcomes. A number of miRNA profiles of varying size and combination have been reported in tumor tissue [222]. In particular, mir-21and mir-155 have been the most oft studied as prognostic markers. In a recent meta-analysis, high miR-21 expression was associated with poorer overall survival in NSCLC (metaHR = 2.32, 95% CI: 1.17–4.62; 6 studies) and recurrence-free survival in adenocarcinoma (metaHR = 2.43, 95% CI: 1.67–3.54; 3 studies) [222]. Likewise, in the same meta-analysis, miR-155 was reported to be associated with poorer recurrence-free survival in NSCLC (metaHR = 1.42, 95% CI: 1.10–1.83; 5 studies). Additionally, expression of the lncRNA *MALAT1* has been associated with metastasis and poorer survival in NSCLC patients [128, 129], and *HOTAIR* expression has been associated with aggressive behavior, poorer outcomes in NSCLC [133–135, 223].

MicroRNA are often released from tissue during apoptosis or in exosomes or microvesicles and can be detected in the blood stream [224]. Circulating microRNA have been associated with prognosis. Several studies have reported an association between microRNA expression

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in blood or plasma and lung cancer outcome [225–232]. In particular, high-levels of circulating miR-21 have been consistently reported to be associated with outcome [225, 229, 231, 232].

Methylation of circulating DNA may also be of use in outcome prediction, although it is important to distinguish free-circulating DNA from leukocytic DNA, since DNA methylation marks are tightly coupled to cellular differentiation and vary by cell type [233]. Thus, without careful study design, blood-based methylation profiles can be confounded by variation in relative circulating proportions of leukocyte types associated with outcome, such as immune response [7]. Ramirez and colleagues found that *14-3-3 sigma* methylation in pretreatment serum may be an important predictor of NSCLC outcome in patients treated with platinum-based chemotherapy [234]. Similarly, Ponomaryova and colleagues found that increased methylation of *RARβ2* in circulating DNA was associated with lung cancer progression [235].

Recent evidence suggests that epigenetic mechanisms may be, at least in some instances, major players in treatment resistance. A number of studies have reported an association between altered expression of a variety of miRNA and sensitivity to gefitinib [236–241], erlotinib [242, 243], cisplatin [244–259], and radiotherapy [253, 260–265]. Elevated expression of *HOTAIR* [223] and *EZH2* [117] have also each been associated with cisplatin-resistance.

In a very elegant study, Sharma et al. demonstrated the capacity of a small subset of stem-like cells in two NSCLC cell-lines to undergo chromatin remodeling after treatment with erlotinib and cisplatin treatment to acquire treatment-resistance [266]. The authors found that the treatment-resistant tumor cells overexpressed the histone demethylase, KDM5A, and exhibited reduced H3K4 di- and trimethylation, and that these resistant cells were sensitive to HDAC inhibitors. These findings are extremely important, as they (1) demonstrate a major role for epigenetic plasticity in acquired drug-resistance, and (2) suggest the potential for histone post-translational modifications as therapeutic targets in drug-resistant NSCLC cells.

Intratumoral heterogeneity and clonal selection of treatment-resistant subpopulations of tumor cells has long been considered as another possible mechanism that can drive acquired treatment resistance in tumors. This notion of intratumoral genetic heterogeneity has recently been confirmed in renal cell carcinoma [267], and very likely is the case in other solid tumor types as well. Moreover, although not assessed in the study, cancer cells are presumably also epigenetically heterogenous within the tumor, as epigenetic alterations are common activating/inactivating events in carcinogenesis, and thus would be susceptible to stochastic selection as well. However, at this point, more research on this topic is needed to determine the extent and generalizability of these findings.

PHARMACOLOGIC TARGETS

Since DNA methylation and transcriptionally inactive heterochromatin conformation are frequent causes of tumor suppressor gene inactivation, therapeutic strategies that globally target epigenetic inactivation of genes have been pioneered for cancer treatment.

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Experimentally, HDAC [268–273] and DNMT [273, 274] inhibitors can restore tumor suppressor gene expression and, consequently, critical pathway function for small-cell and NSCLC patients. Other inhibitors of post-translational modifying proteins have also demonstrated promising antitumor properties in studies of lung cancer *in vitro*, including the Bromodomain and extra terminal domain (BET) protein inhibitor, JQ1 [275], and EZH2 inhibitors, 3-Deazaneplanocin A (DZNeP) [116, 276] and GSK126 [116].

Clinically, two classes of epigenetic regimens have been clinically tested for NSCLC [277]: (1) DNMT inhibitors and (2) HDAC inhibitors. The HDAC inhibitors vorinostat and romidepsin are each currently approved by the FDA for treatment of cutaneous T-cell lymphoma [278]. However, none are currently approved for treatment of lung cancer, although a recently completed clinical trial demonstrated promising results from combining 5-azacytidine, a DNA demethylating agent, with entinostat, an HDAC inhibitor, for the treatment of advanced chemorefractory NSCLC [279]. Additionally, other epigenetic treatment strategies are currently in clinical trials. The majority of these strategies combine HDAC inhibitors, with or without the DNMT inhibitor 5-azacytidine, with cytotoxic drugs, although one phase II trial is presently underway to test the efficacy of fluoro-2-deoxycytidine (FdCyd), a DNMT inhibitor, in conjunction with tetrahydrouridine, a cytidine deaminase inhibitor that prevents FdCyd from being metabolized, in the treatment of NSCLC. A complete list of ongoing trials (as of December 31, 2013) is presented in Table 3 [140].

CONCLUSIONS

Epigenetics play a powerful role in the etiology of lung cancer and have potential utility as diagnostic and prognostic biomarkers. This may be particularly true for DNA methylation marks, due to their relative stability and amenability to PCR-based measurement, and microRNAs, which tend to be much more stable than mRNA and can each regulate up to hundreds of different gene transcripts. Expanding our understanding of how epigenetic events contribute to the genesis of lung cancer, and how these can be translated into clinically-relevant biomarkers and therapeutic targets, will enhance our ability to properly manage lung cancer and ultimately reduce the heavy global burden of this devastating disease.

Acknowledgments

This work was supported by the National Institutes of Health (R01CA100679 and R01CA126939 to KTK; and K22CA172358 to SML).

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Table 1

Select genes frequently reported to undergo promoter methylation in lung cancer.

Gene	Locus (Ensembl)	Frequency in NSCLC	Gene function(s)	References
CDKN2A/p16INK4a	9.21.3	22–47% [a]	Cyclin Dependent Kinase Inhibitor 2A/p16: key TSG involved in cell cycle arrest at the G1/S-phase checkpoint through inhibition of CDK4/6	[5, 16–31]
FHIT	3p14.2	34–47% [a]	Fragile Histidine Triad: member of the histidine triad family involved in purine metabolism; TSG that regulates genes essential for cell proliferation and induction of apoptosis	[16, 23, 27, 34–37]
APC	5q22.2	30–96% [a]	Adenomatous Polyposis Coli: TSG that acts as a negative regulator of Wnt and also is involved in cell migration and adhesion, transcriptional activation, and apoptosis	[16, 17, 27, 28, 38–41]
RASSF1A	3p21.31	25–45% [a]	Ras Association (RalGDS/AF-6) Domain Family Member 1: putative TSG involved in apoptosis and cell cycle control	[5, 16, 17, 27, 28, 31, 36–38, 42–45]
DAPK	9q21.33	16–45% [a]	Death-associated protein kinase: putative TSG involved in apoptotic signaling	[16, 28, 29, 44, 46]
RAR β	9p24.2	26–45% [b]	Retinoic acid receptor beta: involved in cell growth and differentiation	[16, 17, 37, 40, 47]
MGMT	10q26.3	11–38% [a]	O-6-methylguanine DNA methyltransferase: DNA repair enzyme that removes alkyl lesions from the O6 position of guanine	[17, 22, 48–50]
SHOX2	3q25.32	91–95% [c]	Homeobox family gene involved in gene transcription with putative involvement in cell growth and differentiation	[51, 52]
RUNX3	1p36.11	25% [d]	Runt-domain containing transcription factor that functions as a TSG	[36, 53–57]
CDH13	16q23.3	28–30% [b]	H-cadherin: TSG involved in regulation of cell growth, proliferation, and apoptosis	[5, 28, 31, 40, 58]
CDH1	16q22.1	12–58% [a]	E-cadherin; TSG that negatively regulates cell-cell adhesion, growth, motility, and proliferation; loss of function in cancer is thought to increase proliferation, invasion, and metastasis	[17, 28, 38, 42, 58]
TSLC1	11q23.3	37–44% [d]	Cell adhesion molecule 1: TSG involved in cell-cell adhesion	[59–61]
ASC/TMS1	16p11.2	35–47% [a]	PYD And CARD Domain-Containing Protein: crucial mediator of apoptosis and inflammation	[62, 63]
DAL1	18p11.31	55–57% [d]	Erythrocyte Membrane Protein Bank 4.1-Like 3; TSG involved in cell cycle arrest and apoptosis	[59, 64]
PTEN	10q23.31	26% [b]	Phosphatase And Tensin Homolog: TSG involved in cell cycle control and survival by acting as a negative regulator of the AKT/PKB pathway	[22, 65, 66]
GSTP1	11q13.2	15% [b]	Glutathione S-Transferase P1: functions as a phase II xenobiotic detoxification enzyme	[17, 27, 67, 68]

Abbreviations: NSCLC = non-small cell lung cancer; SCLC = small cell lung cancer; TSG = tumor suppressor gene

[a] Estimate based on [32, 33]

[b] Estimate based on [32]

[c] Estimate includes NSCLC and SCLC [51, 52]

[d] Estimate based on [33]

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Table 2

MicroRNAs commonly dysregulated in lung cancer.

microRNA[a]	Locus (Ensembl)	Top 10 putative targets (TargetScanHuman v.6.2)
Upregulated		
miR-21	17q23.1	ZNF367; GPR64; YOD1; PHF14; PLEKHA1; PIKFYVE; PBRM1; GATAD2B; SCML2; VCL
miR-31	9p21.3	RSBN1; ARHGEF2; IDE; NR5A2; SH2D1A; ZNF512; PRKCE; PIK3C2A; PEX; SATB2
miR-92b	1q22	CD69; FNIP1; SLC12A5; MAN2A1; ACTC1; BFXW7; ASPH; DCAF6; SYN2; EFR3A
miR-182	7q32.2	RGS17; MITF; MFAP3; CTTN; TMEM20; EDNRB; ACTR2; CAMSAP1L1; EPAS1; NPTX1
miR-183	7q32.2	ABAT; AKAP12; PIGX; PFN2; PTPN4; REV1; ITGB1; KCNK10; C20orf177; FRMD6
miR-193b	16p13.12	ABI2; IL17RD; SLC10A6; DCAF7; FLI1; JUB; SON; ERBB4; FHDC1; IDE
miR-196a	17q21.32	HOXC8; HOXA7; SLC9A6; HOXA9; KLHL23; PHOSPHO2-KLHL23; ZMYND11; PACRGL; HOXB8; MAP3K1
miR-200b	1p36.33	ZEB1; FAM122C; ZEB2; LRP1B; WIPF1; ELL2; MCFD2; RECK; SEC23A; C7orf58
miR-203	14q32.33	ZNF281; CAMTA1; B3GNT5; LIFR; ABCE1; NUDT21; AFF4; PRPS2; SEMA5A; SMAD9
miR-205	1q32.2	ZNF606; CMTM4; DMXL2; BTBD3; LPCAT1; SECISBP2L; YES1; C16orf52; CHN1; DLG2
miR-210	11p15.5	THSD7A; ISCU; ZNF462; NR1D2; DIMT1L; FAM116A; ARMC1; NEUROD2; ELFN2; SYNGAP1
miR-708	11q14.1	GON4L; SEMA4C; KIAA0355; HOXB3; ALG9; FOXJ3; EFR3B; DCUN1D5; MPL; RNF150
Downregulated		
miR-30a	6q13	PPARGC1B; ANKRA2; MKRN3; LMBR1; EED; LHX8; KLHL20; SNX16; SCN2A; CELSR3
miR-30b	8q24.22	PPARGC1B; ANKRA2; MKRN3; LMBR1; EED; LHX8; KLHL20; SNX16; SCN2A; CELSR3
miR-30d	8q24.22	PPARGC1B; ANKRA2; MKRN3; LMBR1; EED; LHX8; KLHL20; SNX16; SCN2A; CELSR3
miR-101	1p31.3	FAM108C1; GLTSCR1; ZNF654; EYA1; FLRT3; CYDL; TNPO1; LCOR; MYCN; TET2
miR-126-3p	9q34.3	PTPN9; PLXNB2; RGS3; KANK2; EFHD2; CAMSAP1; ZNF219; SPRED1; LRP6; RNF165
miR-126-5p	9q34.3	[not available in the TargetScanHuman database]
miR-138	3p21.32	GPR124; CREB3L2; RMND5A; NFIX; WWC1; RARA; PPIP5K1; SLC35F1; SYT13; CLEC1A
miR-139-5p	11q13.4	TMF1; USP6NL; TBX1; SCAPER; NDRG2; DCBLD2; SLC9A2; PRDM16; EBF1; MORN4
miR-140-3p	16q22.1	GOCP; LOC221710; STAG2; ACVR2B; VGLL2; CBL; UBAP2L; FAIM; PITPNM3; MARCKS
miR-143	5q32	DENND1B; VASH1; ASAP3; SLC30A8; ABL2; TTPA; SLC25A15; MSI2; EPM2AIP1; AKAP6
miR-145	5q32	TPM3; FSCN1; SRGAP2; FAM108C1; NAV3; ABCE1; KCNA4; PLDN; FLI1; SEMA3A
miR-451	17q11.2	OSR1; PSMB8; TTN; TSC1; C11orf30; AEBP2; S1PR2; MEX3C; CAB39; SAMD4B
miR-486-3p	8p11.21	NAT15; RGAG4; SF3A1; TMEM132E; GDI1; FLOT2; LPPR2; DMBX1; FYCO1; CNP
miR-486-5p	8p11.21	RAB8B; EFHC1; ST3GAL1; SSH3; RBM4; TTBK1; UNC45A; NUCB1; TAOK1; SDC3

microRNA[a]	Locus (Ensembl)	Top 10 putative targets (TargetScanHuman v.6.2)
let-7a[b]	22q13.31	C14orf28; FIGNL2; MHGA2; LIN28B; TRIM71; IGDCC3; ARID3B; STARD9; PTAFR; THRSP

[a]Based on two recent meta-analyses of microRNA expression in human lung cancer [124, 125]

[b]Although not considered in either meta-analysis, this is an important and historic tumor suppressor microRNA that has been oft-studied in the context of lung cancer and thus the authors have decided to include it in this table

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Table 3

Ongoing clinical trials involving epigenetic therapeutic agents for treatment of lung cancer patients, as of December 31, 2013.

Clinical Trial	Phase	Protocol IDs	Intervention(s)
HDAC Inhibitors			
Chidamide in Combination With Carboplatin and Paclitaxel in Advanced Non-small Cell Lung Cancer	II	CDM204, NCT01836679	Evaluate the efficacy and safety of **chidamide** (CS055/HBI8000; benzamide class HDI) combined with paclitaxel and carboplatin in patients with advanced NSCLC
Chemotherapy With or Without Epigenetic Priming in NSCLC Patients	II	J1309, NCT01846897	To evaluate the efficacy of epigenetic priming by **5-azacytidine** (U-18496; DNMT inhibitor) and **entinostat** (MS 275/SNDX-275; benzamide class HDI) prior to treatment with standard chemotherapy for NSCLC patients
Phase II Anti-PD1 Epigenetic Priming Study in NSCLC	II	J1353, NA_00084192, NCT01928576	Determine the response rate to Nivolumab following epigenetic priming with **5-azacitidine** (U-18496; DNMT inhibitor) with or without **entinostat** (MS 275/SNDX-275; benzamide class HDI) for NSCLC patients
Treatment of Locally Advanced Non-Small Cell Lung Cancer (NSCLC)	I	FER-TH-031, NCI-2010-01913, NCT01059552	Determine the maximum tolerated dose of the combination of **vorinostat** (L-001079038; suberoylanilide hydroxamic acid HDI), cisplatin, pemetrexed, and radiation therapy in patients with unresectable stage IIIA/IIIB NSCLC
Study of Cisplatin and Pemetrexed in Combination With Panobinostat in Solid Tumors	I	UCDCC#220, NCT01336842	Determine if **panobinostat** (LBH589; cinnamic hydroxamic acid analogue HDI) can be safely combined with cisplatin and pemetrexed without increasing side effects and that the combination will be more efficacious than platinum-based doublet chemotherapy alone in solid tumor patients (including NSCLC)
A Trial of Oral 5-azacitidine in Combination With Romidepsin in Advanced Solid Tumors, With an Expansion Cohort in Non-small Cell Lung Cancer	I	J11102, NA_00052054, NCT01537744	Determine if **5-azacitidine** (U-18496; DNMT inhibitor) combined with intravenous **romidepsin** (FK228/FR901228/NSC 630176; depsipeptide HDI) is effective in the treatment of advanced solid tumors (including NSCLC)
DNMT Inhibitors			
A Multi-Histology Phase II Study of 5-Fluoro-2-Deoxycytidine With Tetrahydrouridine (FdCyd + THU)	II	090214, 09-C-0214, NCT00978250	Evaluate the efficacy of **fluoro-2-deoxycytidine** (FdCyd; DNMT inhibitor) combined with tetrahydrouridine for treatment of solid tumor patients (including NSCLC)

Abbreviations: HDAC = histone deacetylase; DNMT = DNA methyltransferase; NSCLC = non-small cell lung cancer; HDI = histone deacetylase inhibitor