

# Epigenetics in non-small cell lung cancer: from basics to therapeutics

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**Abstract:** Lung cancer remains the number one cause of cancer-related deaths worldwide with 221,200 estimated new cases and 158,040 estimated deaths in 2015. Approximately 80% of cases are non-small cell lung cancer (NSCLC). The diagnosis is usually made at an advanced stage where the prognosis is poor and therapeutic options are limited. The evolution of lung cancer is a multistep process involving genetic, epigenetic, and environmental factor interactions that result in the dysregulation of key oncogenes and tumor suppressor genes, culminating in activation of cancer-related signaling pathways. The past decade has witnessed the discovery of multiple molecular aberrations that drive lung cancer growth, among which are epidermal growth factor receptor (EGFR) mutations and translocations involving the anaplastic lymphoma kinase (ALK) gene. This has translated into therapeutic agent developments that target these molecular alterations. The absence of targetable mutations in 50% of NSCLC cases and targeted therapy resistance development underscores the importance for developing alternative therapeutic strategies for treating lung cancer. Among these strategies, pharmacologic modulation of the epigenome has been used to treat lung cancer. Epigenetics approaches may circumvent the problem of tumor heterogeneity by affecting the expression of multiple tumor suppression genes (TSGs), halting tumor growth and survival. Moreover, it may be effective for tumors that are not driven by currently recognized druggable mutations. This review summarizes the molecular pathology of lung cancer epigenetic aberrations and discusses current efforts to target the epigenome with different pharmacological approaches. Our main focus will be on hypomethylating agents, histone deacetylase (HDAC) inhibitors, microRNA modulations, and the role of novel epigenetic biomarkers. Last, we will address the challenges that face this old-new strategy in treating lung cancer.

**Keywords:** Lung cancer; biomarkers; microRNA; DNA methylation; histone deacetylases (HDACs); epigenetic therapy

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## Introduction

Lung cancer is a cancer of the modern man and only few cases date before the 20<sup>th</sup> century. By the mid-twentieth century it had swept the world, due to increased worldwide tobacco consumption (1). Even after widespread awareness about smoking in the last 4–5 decades, the lung cancer incidence has only plateaued and in the developed world, it still accounts for approximately 25% of cancer-related

deaths. Detection of lung cancer at an early stage leads to a better prognosis; the 5-year survival for localized lung and bronchus cancer is 54.8%, compared to 27.4% for regional, and 4.2% for widely disseminated disease (1). Lung cancers are classified into small cell and non-small cell lung cancers (NSCLC) and approximately 80% are comprised of NSCLCs, which are classified into adenocarcinoma (AdC), adenosquamous carcinoma, squamous cell carcinoma

**Table 1** FDA-approved epigenetic therapy

Agent	Class	Approval date	Indication
Azacitidine	DNMT inhibitor	2004	Myelodysplastic syndrome
Decitabine	DNMT inhibitor	2006	Myelodysplastic syndrome
Vorinostat	Pan-HDAC inhibitor	2006	Cutaneous T-cell lymphoma
Romidepsin	Class I HDAC inhibitor	2009	Cutaneous T-cell lymphoma
Panobinostat	Pan-HDAC inhibitor	2015	Multiple myeloma
Belinostat	Pan-HDAC inhibitor	2014	Peripheral T-cell lymphoma

HDAC, histone deacetylase; DNMT, DNA methyltransferases.

(SqCC), and large cell carcinoma based on tumor histology, with 80% of NSCLC being either AdC or SqCC (2,3). Based on the molecular pathogenesis patterns and histologic classification, AdCs are the most common type to show common recurrent genomic gains and losses, and somatic mutations. These driver mutations have been extensively studied in AdCs and the most common ones mutated are the epidermal growth factor receptor (EGFR), KRAS, and anaplastic lymphoma kinase (ALK) oncogenes (2,3).

Over the past decade, epigenetics changes have been increasingly studied and used as markers for early cancer detection. Jean-Baptiste Lamarck first introduced the concept of epigenetics in his book *Philosophie Zoologique* more than 200 years ago, in which he called it “soft inheritance”. In 1939, Waddington defined epigenetics as “the causal interactions between genes and their products, which bring the phenotype into being” (4). Later Holiday defined epigenetics as heritable changes in gene expression that are not due to any DNA sequence alterations (5). Epigenetics consists of heritable modifications to the chromatin that influence gene expression and other DNA-dependent processes without directly altering the DNA coding sequence (5). These complex processes involve DNA methylation, microRNA regulation, and histone/nucleosome modifications. Mutations in epigenetic regulatory mechanisms and epigenetic pattern perturbations are implicated in many tumor types, including lung cancer, occurring through tumor suppressor gene silencing and oncogene activation. Epigenetic alterations are further linked to chemotherapy resistance (6,7). Unlike genetic mutations, epigenetic dysregulation is reversible and can be reversed by different pharmacologic approaches, the most common being hypomethylating agents and histone deacetylase inhibitors (HDACIs). Interestingly, while tumor heterogeneity represents a major challenge with targeted molecular therapies, broad re-modulation of the epigenome

may address this problem through affecting multiple signaling pathways. The six FDA approved drugs that target the epigenome, their approval dates, and indications are summarized in *Table 1*.

### Epigenetic dysregulation in NSCLC

The initiation and progression of lung cancer is the result of permanent genetic alterations that include point mutations, deletions, translocations, amplifications, and epigenetic modifications that affect different aspects of chromatin-dependent processes, such as histone modifications, DNA methylation patterns, and microRNA regulation (8,9). DNA methylation plays a critical role in repressing gene expression and maintaining genomic stability by preventing recombination events between repetitive sequences (10). In eukaryotes, DNA methylation occurs in CpG dinucleotide islands which comprise roughly 1% of the human genome, but are present in over half of all human gene promoter sequences (11). In cancer cells, there is a dramatic reduction in cytosine methylation at these repetitive sequences, causing increased mitotic recombination and subsequent chromosomal instability (12-14). In addition, CpG islands of tumor suppression genes (TSGs) promoters are highly methylated leading to transcriptional repression, while other genes involved in processes such as DNA repair, apoptosis, the epithelial-mesenchymal transition (EMT), cellular movement and invasion, and metastasis are dysregulated by aberrant cytosine methylation (10,15). Further, hypomethylation of transposable element DNA can cause increased transposition within the genome, activating oncogenes and increasing chromosomal anomalies through insertional mutagenesis (16). Using the Illumina Infinium HumanMethylation27K array platform, Selamat *et al.* (17) interrogated the DNA methylation status of 27,578 CpG dinucleotides spanning 14,475 genes and identified

more than 700 common differentially methylated genes. Additionally, accumulation of repressive histone markers is another hallmark of carcinogenesis, leading to chromatin compaction and gene expression repression. Histone deacetylases (HDACs) are often overexpressed in cancers and have become a major therapeutic target in recent years (18,19). HDAC overexpression can lead to TSG silencing and aberrant transcription due to altered expression/mutation of the genes encoding histone acetyltransferase (HAT) or HDAC enzymes or their binding partners, are clearly linked to carcinogenesis (20). This occurs in many human cancers, indicating that aberrant epigenetic acetylation activity is associated with cancer development (21-24).

### DNA methylation

DNA methylation is the most studied epigenetic regulatory mechanism. CpG island methylation is completed by different DNA methyltransferases (DNMTs) that can lead to gene silencing. Three active DNMTs (DNMT1, DNMT3a, and DNMT3b) mediate the transfer of a methyl group from S-adenosyl-L-methionine to the CpG islands 5'-cytosine carbon (25-27). DNMT1 binds essentially to hemimethylated DNA and is primarily involved in the maintenance methylation after DNA replication. DNMT3a and b binds preferentially to unmethylated or hemimethylated DNA, and are responsible of de novo DNA methylation (28-30).

### DNA methylation in lung cancer

DNMT overexpression is implicated in the pathogenesis of lung cancer. Elevated DNMT levels in lung cancer can result from transcriptional activator overexpression, loss of microRNAs that down-regulate the DNMTs, and/or impaired proteasomal DNMT degradation by hsp90 (21-23). Clinically, there is evidence that DNMT1 overexpression is associated with diminished survival in surgically resected lung cancer (31,32). Consistent with these findings, different TSGs are silenced by promoter hypermethylation in lung cancer (21). Many of these TSGs are involved in normal cellular function, such as cell cycle regulation (p16), DNA repair (MGMT), apoptosis (DAPK, caspase-8), regulation of Wnt signaling (APC), cell adhesion and invasion (E-cadherin, H-cadherin and tissue inhibitor of metalloproteinase-3), and suppression of invasion (CDH13, TIMP-3). For example, Brock *et al.* (22) observed that methylation of cdk2A, p16, CDH13, RASSF1A and

APC correlated with recurrence following surgical resection of stage I NSCLC regardless of histology, stage, gender, or smoking history. Similarly, another study found that methylation of p16 and concomitant p16 expression loss coincides with reduced survival after early stage NSCLC resection (23). In parallel, IGFBP-3 methylation is linked to NSCLC cisplatin resistance (24).

Besides its role as a prognostic and predictive biomarker, DNA methylation has become a therapeutic target through DNMT enzyme inhibition. The two main DNMTs inhibitors that have been largely tested in the clinic are 5-azacitidine and decitabine (25). Following phosphorylation, 5-azacitidine is incorporated into DNA and RNA, followed by the covalent trapping of DNMTs to the DNA, leading to proteasomal degradation, and subsequent global DNA methylation reduction. The DNA damage and impaired DNA synthesis resulting from DNA-DNMT adducts is responsible for the direct cytotoxicity of these agents when used at higher doses. Unlike 5'-azacitidine, decitabine is not incorporated into the RNA and is specific only for DNA (26,27,33). The hypomethylating effects of these agents are best achieved at lower doses with a more prolonged administration (34). Pre-clinical models have shown antitumor activity for both agents through de-methylation and removal of repression on numerous TSGs, including p16 (35). Unfortunately, their use as single agent in clinical trials has showed limited success in lung cancer (36).

In a phase I/II trial, 15 patients with untreated advanced NSCLC were treated with high dose decitabine (200 to 660 mg/m<sup>2</sup>) administered as a continuous infusion over 8 hours. Although no objective response was seen, four patients experiences stable disease for more than 6 months, and three patients had a survival of at least 15 months, with one patient surviving 81 months. Due to hematopoietic toxicities, only one patient completed more than one cycle, which may have impacted the treatment efficacy (37,38). Another dose-escalation phase I trial conducted on 35 patients with solid tumor including 22 with lung cancer, investigated decitabine given at lower dose, administered over 72 hours continuous infusion. No objective response was seen, although three patients with squamous cell lung cancer had stable disease. Interestingly, pharmacodynamics studies revealed increased expression of p16, MAGE-3, and NY-ESO-1 in one-third of the patients (39). Further research will analyze the best sequence, dosage, treatment duration, and combination with other antineoplastic agents, as well as look for clinically relevant pharmacodynamic and

predictive response biomarkers.

### **Smoking and DNA methylation**

Some epigenetic alterations in lung cancer occur at greater frequency in smokers (i.e., p16, FHIT, RASSF1A mutations) and increase with increasing smoking duration/intensity (40-42). DNMT1 expression is elevated in smokers with lung cancer, likely due to tobacco-specific nitrosamines that reduce DNMT1 ubiquitination and degradation (21,43). Additionally smoking-induced chronic inflammation and increased reactive oxygen species generation, leading to increased DNA methylation (44). Damiani *et al.* (21) developed an *in vitro* model that mimics the field cancerization observed in chronic smokers and identified different epigenetic changes and their kinetics. Immortalized normal human bronchial epithelial cells (HBECs) were exposed for 12 weeks to two cigarette carcinogens; methylnitrosurea (MNU) and benzo(a)pyrene-diolepoxide 1 (BPDE). Stable knockdown of DNMT1, but not DNMT3 prevented cell transformation after exposure to these carcinogens. HBECs take a fibroblast-like mesenchymal appearance after 4 weeks of carcinogen exposure. Significant reductions in miR-200b and miR-200c, were observed at 4 weeks exposure and was sustained upon cell transformation at 12 weeks. Interestingly, these microRNAs are involved in regulating and inhibiting the EMT (35). Further studies revealed that expression of these EMT-regulating microRNAs are initially reduced by transcriptionally inactive chromatin at 4 weeks, followed later by cytosine methylation-mediated repression at their promoters (21,45).

### **On-going clinical trials**

CC-486 is a novel oral azacitidine. An ongoing trial is examining the safety and efficacy of CC-486 in combination with pembrolizumab compared to pembrolizumab alone in previously treated advanced NSCLC (46). Another clinical trial is currently testing the combination of nab-paclitaxel with CC-486 when used in second line in advanced nonsquamous NSCLC (47). RRx-001 is a novel, broad-spectrum epigenetic anticancer agent that inhibits HDACs, DNMT1, and DNMT3a expression. Currently, it is being tested in lung cancer for its ability to sensitize the tumor to re-administration of a platinum doublet chemotherapy regimen (48). Another phase I trial is examining the tolerability and minimum effective dose of inhaled

azacytidine (AZA) in NSCLC (49).

### **Histone modifications**

Nucleosomes are chromosomal building blocks containing two molecules each of the core histones H2A, H2B, H3, and H4. DNA wraps around the nucleosomes octameric core approximately 1.8 times (50). The histone amino termini extend from the core, where they can be modified post-translationally by acetylation, phosphorylation, or methylation (51). Acetylation is regulated by opposing actions between HATs and HDACs (51). In humans there are 18 HDACs classified into four classes (I, II, III, IV) based on their homology to yeast HDACs, their subcellular localization, tissue specificity, and enzymatic activities (51).

HDAC inhibitors are emerging as novel anti-cancer agents due to their ability to kill cancer cells by inducing apoptosis, autophagy, cellular necrosis, ROS, cell cycle arrest, suppressing tumor angiogenesis, and exerting immunomodulatory effects. They activate both death-receptor and intrinsic mitochondrial pathways, lowering the overall tumor cell apoptotic threshold. They up-regulate pro-apoptotic genes involved in the death receptor pathway (i.e., TRAIL and DR5) and/or the intrinsic apoptotic pathway (Bax, Bak, and APAF1) and downregulate pro-survival genes (BCL-2 and XIAP). They also cause selective activation or induction of BH3-only proteins and hence initiate the intrinsic apoptotic pathway (52-54).

In addition to their direct anti-cancer effects, HDACIs strengthen the immune system by up-regulating the expression of MHC class I and II proteins, and co-stimulatory/adhesion molecules such as CD80, CD86, human leukocyte antigen (HLA)-DR, HLA-ABC, and intracellular adhesion molecule-1 (ICAM-1,28). HDACIs can also inhibit angiogenesis, a critical factor in tumor invasion and metastases (52,53). HDAC inhibitors may also enhance immune responses by altering the activities of immune cells, either directly or indirectly through cytokine secretion modulation (54).

The idea of HDAC inhibitors came through empirical screens, when molecular targets of agents that induce tumor differentiation were discovered, such as like butyrate, trichostatin A (TSA), and suberoylanilide hydroxamic acid (vorinostat) (52-54). By this theory, one can also deduce that HDAC in itself may be oncogenic, but there is no data demonstrating this. In contrast, knocking down of HDACs produces a range of antitumor effects. HDACIs inhibit the growth of a wide variety of malignant cells *in vitro*, including

lymphoma, myeloma, leukemia, and NSCLC, and inhibit the growth of a variety of solid tumors and hematological malignancies by both parenteral and oral administration, including prostate and breast cancers, leukemia, glioma, and lung cancer (52-54).

### *Histone modifications in lung cancer*

Over the last decade, many studies have revealed epigenetic aberrations involving histone lung cancer modifications. Miyanaga *et al.* (54) tested 16 NSCLC cell lines with HDAC inhibitors including TSA and vorinostat, and both displayed antitumor activities in 50% of the NSCLC cell lines. They also conducted gene expression profiling and created a nine-gene classifier which predicts HDAC inhibitor drug sensitivities (54). Van Den Broeck *et al.* (46) showed that histone epigenetic modifications play a crucial role in lung carcinogenesis. Compared to normal lung cells, lung cancer cells displayed aberrant histone H4 modification patterns with hyperacetylation of H4K5/H4K8, hypoacetylation of H4K12/H4K16, and loss of H4K20 trimethylation. Their findings indicate an important role for histone H4 modifications and highlight H4K20me<sub>3</sub> as a potential biomarker for the early detection of and therapeutic approaches to lung cancer (46). Seligson *et al.* (55) demonstrated that lower global levels of histone modifications are predictive of a more aggressive cancer phenotype in lung AdC. Additionally, the differential expression pattern of HATs and HDACs in the tumor samples, compared to the normal counterparts, can have potential therapeutic implications, such as eventual early tumor detection, prognostication, and the guiding of epigenetic-targeted therapies (56). HDAC1 gene expression appears to correlate with lung cancer progression, with strong HDAC1 and HDAC3 gene expression correlating with a poor prognosis in pulmonary AdC patients (57-59). HDAC3 was also seen elevated in 92% of tumor with SqCC histology using antibody microarrays for detection of target proteins (60). HDI-treated NSCLC cells down-regulated TNF-receptor-1 mRNA, protein levels, and surface protein expression, and consequently responded to TNF-treatment with attenuated NF- $\kappa$ B nuclear translocation and DNA binding. HDIs, therefore, might beneficially contribute to tumor treatment, by reducing the responsiveness of tumor cells to the TNF-mediated activation of the NF- $\kappa$ B pathway (61).

Treatment with TSA resulted in a dose-dependent reduction of H157 lung cancer cells by apoptosis with nuclear fragmentation and an increase in the sub-G<sub>0</sub>/G<sub>1</sub> fraction. TSA initiated apoptosis by activation of the

intrinsic mitochondrial and extrinsic/Fas/FasL system death pathways (62). TSA is also a powerful NSCLC cell radiosensitizer, enhancing G<sub>2</sub>/M cell cycle arrest, promoting apoptosis, and interfering with DNA damage repair, and synergistically triggering cell death when combined with other HDAC inhibitors, such as vorinostat (63,64). Vorinostat inhibits telomerase activity by reducing H-tert expression in A549 lung cancer cells (65). To explore the mechanisms by which vorinostat slows growth of lung cancer cells, changes of several key cell cycle and apoptosis proteins were examined. The cyclin-dependent kinase inhibitor p21 was upregulated in NCI-H520 and NCI-H460 cells treated with vorinostat. Prominent p21 induction was associated with G<sub>0</sub>-G<sub>1</sub> cell-cycle arrest in vorinostat treated human lung cancer cells. p53 levels increased in NCI-H520 cells, partly explaining the increased p21, since p21 is a p53 transcriptional. C-myc levels decreased in both cell lines, indicating vorinostat exerts antiproliferative activity. Vorinostat also decreased bcl-2 expression in NCI-H460 cells (66).

### **Smoking and histone modifications**

Cigarette smoke exposure to respiratory epithelia may also influence the histone modifications. Nickel cations present in tobacco smoke induce histone deacetylation and increase histone H3K9 dimethylation. Zhou *et al.* and others (67-69) found that smoke carcinogenic elements including nickel, chromate, and arsenite also induce H3K4 methylation.

### *Novel HDAC inhibitors*

The novel cyclic amide-bearing hydroxamic acid-based HDAC inhibitors, SL142 and SL325, have shown greater HDAC inhibitory activity and lung cancer cell line viability-inhibition than vorinostat. These small molecules induce significant caspase-3 activity, indicating that they could induce lung cancer apoptosis (70). N-Hydroxy-4-(4-phenylbutyryl-amino) benzamide (HTPB), another novel HDAC inhibitor, caused significant lung cancer cell growth suppression by inducing cell cycle arrest, mitochondrial mediated apoptosis, disruption of F-actin dynamics, and inhibition of mitochondrial membrane potential (MMP)2 and MMP9. The effect was seen *in vitro* and *in vivo* (71). CG0006 a newly synthesized HDAC inhibitor, was assessed in a NCI-60 cancer cell panel and induced cell death by increased p21 and p27 expression (72). Other novel agents such as MGCD0103, OSU-HDAC 44, CI-944, MS-275,

and LAQ824 were tested and show significant cytotoxic effects on lung cancer cells (73-80).

### MicroRNA and microRNA silencing

MicroRNAs (miRs) are small, endogenous, single-stranded, noncoding RNAs of 20–22 nucleotides that regulate gene expression. More than 1,000 miRs have been identified and regulate more than one-third of coding mRNAs and each one can regulate hundreds of target mRNAs (81). Thus silencing of miRs by methylation can profoundly modulate tumor development and progression (82). Heller *et al.* (83) identified 33 miRs whose expression was increased in A549 cells (lung AdC) following demethylation treatment (83). miR-9-3, miR-34b and miR-126 are methylated in NSCLC and this is associated with an altered prognosis (83,84). miR-487b is commonly silenced by methylation in primary lung tumors and is reduced in respiratory epithelial cells and lung tumor-derived cell lines following tobacco smoke exposure (85). This finding reinforces past data which showing that smoking influences methylation and promotes lung cancer.

### Combinatory epigenetic therapy

In normal cells, chromatin in the region of an actively transcribed tumor suppressor gene is typically in an open configuration, allowing transcription factor access. Carcinogenesis is associated with the epigenetic silencing of tumor suppressor genes, which may be secondary to DNA CpG island methylation and/or a closed chromatin configuration (6,86). Combined epigenetic therapy aims to reverse these alterations. Hypomethylating agents cause CpG island demethylation, allowing enhanced TSG transcription. On the other hand, HDAC inhibitors shift the chromatin to a more open configuration, favoring TSG transcription. While targeting each process alone has had disappointing effects on lung cancer, targeting both processes simultaneously may result in therapeutic synergism and enhanced TSG expression (87).

### Combinatory hypomethylation and HDAC inhibition

Cameron *et al.* (88) demonstrated that while treatment with either TSA (an HDAC inhibitor) or decitabine alone has no effect on the TSG transcription, the drugs combined lead to synergistic reactivation of the TSG expression in colorectal carcinoma cells. Similarly, Boivin

*et al.* (89) demonstrated that in lung cancer cell lines, AZA combined with the HDAC inhibitor phenylbutyrate exerted a greater DNA synthesis inhibition than either agent alone. Zhu *et al.* (90) 2001 found that lung cancer cell lines pre-treated with decitabine, show enhanced HDAC inhibitor-induced apoptosis and further enhanced histone acetylation. Similarly, AZA combined with the HDAC inhibitor entinostat inhibited lung cancer growth in an orthotopic mouse model, and caused the re-expression of p16, p21, and the pro-apoptotic gene PRC2. These results provide a sound scientific rationale for exploring combining hypomethylating agents with HDAC inhibitors in patients with advanced lung cancer (78,90).

Initial clinical trials using a combined approach failed to show significant response in patients with lung cancer:

- Decitabine and valproic acid, Chu *et al.* (91);
- 5-AZA and sodium phenylbutyrate, Lin *et al.* (92);
- Hydralazine and magnesium valproate, Candelaria *et al.* (93);
- Decitabine and vorinostat, Stathis *et al.* (94).

However, a more recent clinical trial combining AZA and entinostat in heavily pre-treated advanced NSCLC has revived interest in the combinatory approach. Strikingly, the achieved median overall survival was 6.4 months. Among the 34 evaluable patients, ten had stable disease for at least 12 weeks, one patient had complete response lasting 14 months and another patient had partial response lasting 8 months (87).

### Role of epigenetic priming followed by conventional therapy

Interestingly, demethylation of four epigenetically silenced genes associated with lung cancer (APC, RASSF1a, CDH13, and CDKN2A) was detectable in serial plasma circulating DNA samples in these patients and was associated with improved progression free and overall survival (87). Another intriguing observation from this trial was a persistent clinical response after epigenetic therapy cessation and a notable clinical response to subsequent next anti-cancer treatments (cytotoxic chemotherapy and anti-PD1 monoclonal antibody) in many of these patients. Four out of 19 patients who received subsequent salvage therapy exhibited a major objective response; two patients survived 44 and 52 months respectively, after failing epigenetic treatment (95). This observation has raised an important hypothesis; epigenetic therapy may reprogram cancer cells and render them more susceptible to subsequent treatments. The above findings illustrate a new paradigm in cancer treatment—

epigenetic priming. It consists of epigenetic modulator pretreatment prior to antineoplastic agent treatment. Currently many clinical trials are addressing this promising concept. An ongoing trial is currently randomizing patients with pretreated NSCLC to second line chemo alone *vs.* priming with 5-AZA and entinostat followed by second line chemotherapy. Another phase II trial is investigating the efficacy of nivolumab, a monoclonal antibody inhibitor of PD-1, after pre-treatment with 5-AZA and entinostat in patients with advanced NSCLC. This ongoing trial is based on recent findings that PD-L1 expression may be upregulated following treatment with AZA, increasing potentially the efficacy of anti-PD1 therapy (95). The combination of 5-AZA and entinostat is also being explored in the adjuvant setting with patients with resected stage I NSCLC, and comparing it to observation, the current standard of care after surgical resection in stage IA NSCLC (96).

#### ***Combinatory HDAC inhibition with other agents***

Similarly, HDAC inhibitors show a more prominent effect when used in combination with other agents. Vorinostat has shown significant benefit when combined with carboplatin and paclitaxel in advanced lung cancer (96). Combinatorial TSA and etoposide treatment induced caspase-mediated AIF-dependent apoptotic cell death in non-small cell lung carcinoma cells. Genistein and carotene as nutritional factors in combination with TSA enhanced the cell growth arrest effect on A549 NSCLC cells (97). Combined treatment with low-dose vorinostat enhanced 5-FU drug-mediated cytotoxicity and resulted in synergistic effects, especially in 5-FU-resistant NSCLC cells. Vorinostat may overcome 5-FU resistance by down-regulating thymidylate synthase expression and up-regulating p21waf1/cip1 expression via histone acetylation at its promoter. This is the first report that vorinostat enhanced 5-FU sensitivity via the modulation of 5-FU metabolism in lung cancer cells and will facilitate future clinical investigations of combined chemotherapy and vorinostat in patients with NSCLC (66).

Millward *et al.* (98) combined vorinostat with a novel bicyclic proteasome inhibitor marizomib and tested advanced solid tumors, including NSCLC cells, and found highly synergistic antitumor activity. Although no responses were demonstrated using RECIST criteria, 61% of evaluable patients demonstrated stable disease with 39% having decreases in tumor measurements (98). Chien *et al.* (99) showed that vorinostat when combined with arsenic trioxide (ATO) acts synergistically to enhance *in vitro* and *in vivo*

death of H1299 NSCLC cell. Seo *et al.* (100) combined suboptimal doses of Sulindac (NSAID) with vorinostat which resulted in growth suppression of A549 human NSCLC cells primarily via enhanced MMP collapse, release of cytochrome C, and caspase activation (100). Several on-going clinical trial using hypomethylating agents and HDAC inhibitors for the treatment of lung cancer are summarized *Table 2*.

#### ***Novel therapeutic strategies: aerosol vidaza***

After subcutaneous or intravenous administration, 5-AZA and decitabine are catabolized by cytidine deaminase in the liver, reducing the bioavailability of the drug to the lung. Aerosol delivery of these drugs may achieve higher concentrations in the pulmonary tissue by bypassing the hepatic first pass. Pharmacokinetic mice model had shown that aerosolized 5-AZA administration resulted in significant reduction of lung tumor burden and induction of global DNA demethylation at one-third of the comparable effective systemic dose (101).

#### **Epigenetic biomarkers in lung cancer**

Epigenetic changes as possible biomarkers for early lung cancer detection, diagnosis, prognostication, and the guiding of therapeutic options has recently been intensely studied, with heavy focus on DNA cytosine methylation, miR alterations, and histone modifications (6,8,9,46,50-52,54,81-85,102-104). Each of these epigenetic changes has specific testing methods and different degrees of clinical applicability. Presently most lung cancer epigenetic biomarkers are in development and will probably not have clinical application for several years (104).

#### ***DNA hypermethylation***

DNA 5'-cytosine hypermethylation is an early lung carcinogenesis (6,8,9). Many genes are hypermethylated in lung cancer including p16, PAK3, NISCH, KIF1A, OGDHL, BRMS1, FHIT, CTSZ, CCNA1, NRCAM, LOX, MGMT, DOK1, SOX15, TCF21, DAPK, RAR, RASSF1, CYGB, MSX1, BNC1, CTSZ, and CDKN2A (105-118). The percent of hypermethylation for each gene varies, with some like p16 and MGMT hypermethylated in 100% of patients with pulmonary SqCC up the 3 years before cancer diagnosis (119). p16 inhibits cyclin-dependent kinases 4 and 6, which after binding cyclin D1, phosphorylate and inactivate the retinoblastoma tumor suppressor gene, blocking cell cycle

**Table 2** Selected ongoing clinical trials involving hypomethylating agents and HDAC inhibitors for the treatment of lung cancer

Clinical trial	Phase	Protocol#	Intervention and endpoint
Chemotherapy with or without epigenetic priming in NSCLC	II	NCT01846897	This is a randomized trial evaluating the efficacy of epigenetic priming by 5-AZA (either po or SC) and entinostat prior to standard second line cytotoxic chemotherapy
Epigenetic priming prior to nivolumab	II	NCT01928576	Determine and compare the response rate to nivolumab with or without priming with 5-AZA SC and entinostat in metastatic NSCLC that progressed on previous chemotherapy
Oral 5-AZA (CC-486) combined with romidepsin (expansion cohort for NSCLC)	I	NCT01537744	This phase I trial is studying the safety and efficacy of the combination po 5-AZA with IV romidepsin in advanced solid tumors including NSCLC
5-fluoro-2-deoxycytidine (FdCyd) with tetrahydrouridine (THU)	II	NCT00978250	Evaluate the safety and efficacy of the hypomethylating agent FdCyd combined with THU in patients with previously treated breast cancer, head and neck cancer, urothelial transitional carcinoma and NSCLC
CC-486 with MK-3475	I	NCT02546986	Randomized, double blind study to assess the safety and efficacy of oral 5-AZA with pembrolizumab vs. pembrolizumab with placebo in previously treated advanced NSCLC
Entinostat with pembrolizumab	IB/2	NCT02437136	Dose-escalation study evaluating the safety and tolerability of entinostat in combination with pembrolizumab in patients with pre-treated NSCLC or melanoma. Cohort 2 will include patients with NSCLC who previously responded then failed PD-1 or PD-L1 blocking antibody
CC-486 with nab-paclitaxel	II	NCT02250326	Assess and compare the safety and efficacy of nab-paclitaxel with CC-486 vs. nab-paclitaxel monotherapy as second line treatment in advanced non-squamous NSCLC
Azacitidine and entinostat before (priming) chemotherapy in treating patients with advanced NSCLC	II	NCT01846897	Percentage of patients progression-free at 6 months from the time of randomization
HDAC inhibitor vorinostat with chemotherapy and radiation therapy for treatment of locally advanced NSCLC	I	NCT01059552	To assess that safety and maximally tolerated dose of vorinostat in combination with chemoradiation for unresectable locally advanced NSCLC
Vorinostat-iressa combined therapy on resistance by BIM polymorphysim in EGFR mutant lung cancer	I	NCT02151721	This is a phase I study assessing the MTD of vorinostat-gefitinib combined therapy in EGFR mutant lung cancer

HDAC, histone deacetylase; NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; BIM, B-cell chronic lymphocytic leukemia-lymphoma like 11 gene; AZA, azacytidine; SC, sub-cutaneous; MTD, maximum tolerated dose.

progression (119). p16 is lost in ~70% of lung cancer cases, often by promoter methylation, promoting the G<sub>1</sub> to S phase transition (119-121). Interestingly, p16 methylation occurs in normal-appearing epithelium from smokers and precursors lesions, and increases in frequency with the progression of the carcinogenic process (120). The specific mechanism(s) by which each gene hypermethylation event promotes cancer vary, but most involve repression of tumor suppressor genes with concomitant activation of genes promoting cell growth and cell cycle progression (105-122). Some of the genes hypermethylated in lung cancer and their functions are given in *Table 3*.

DNA hypermethylation in lung cancer patients can be detected in bronchoscopic washings/brushings, sputum

samples, and blood (plasma and serum), all of which are less invasive and easier on the patient than a tumor biopsy. These techniques may also eventually be useful in the detection of very early lung tumors or newly recurring tumors not detectable by other methods (120). 5'-cytosine methylation is quantified predominantly by three different molecular methods:

- Methylation-sensitive restriction enzymes: most restriction endonucleases do not cut methylated DNA, while others only cut methylated DNA (137,138). There are many variations of this analysis, but they generally compare the activities of endonucleases that cut or will not cut methylated DNA, often using the isoschizomers *MspI* and *HpaII* which recognize



**Table 3** Selected genes subjects to hypermethylation in lung cancer

Mechanism	Gene	Encoded protein	Gene function	References
DNA repair	<i>MGMT</i>	O-6-methylguanine DNA methyltransferase	Removes alkyl from the O6 position of guanine	(123)
	<i>hMLH1</i>	DNA mismatch repair protein MLH1	Involved in DNA repair	(124)
	<i>MSH2</i>	DNA mismatch repair protein MSH2	Involved in DNA repair	(124)
Apoptosis	<i>DAPK</i>	Death associated protein kinase	Pro-apoptotic	(125,126)
	<i>CASP8</i>	Caspase-8	Effector of extrinsic apoptosis. Occurs selectively in small cell lung cancer	(127,128)
	<i>TNFRSF6</i>	FAS receptor	Part of TNF-receptor superfamily, mediates extrinsic apoptosis. Silenced in 40% of SCLC	(127)
	<i>DR4, DR5</i>	Death receptor 4 and 5	Part of TNF-receptor superfamily, mediates extrinsic apoptosis. Silenced in 40% of SCLC	(127)
Cell cycle	<i>P16</i>	Cyclin-dependent kinase 4 inhibitor A	CDK4/6 inhibitor involved in cell cycle arrest at G1/S checkpoint	(15,35,41,120)
	<i>PTEN</i>	Phosphatase and tensin homolog	Negative regulator of AKT/MTOR pathway, and cell cycle	(129)
	<i>RASSF1A</i>	Ras association domain family 1	Involved in cell cycle regulation, and ras-induced apoptosis	(130)
Cell adhesion and invasion	<i>CDH1</i>	E-cadherin	Promotes cell-cell adhesion, inhibits cell motility, invasion and metastasis	(131)
	<i>CDH13</i>	H-cadherin	Involved in regulation of cell proliferation	(131)
	<i>TSLC1</i>	Tumor suppressor in lung cancer 1	Involved in cell-cell adhesion	(132)
Transcription regulation	<i>APC</i>	Adenomatosis polyposis coli	Negative regulator of WNT pathway, and $\beta$ -catenin	(133)
	<i>RAR<math>\beta</math>-2</i>	Retinoic acid receptor $\beta$	Involved in cell growth and differentiation	(134)
	<i>SHOX2</i>	Homeobox family gene	Transcriptional regulator involved in cell growth and differentiation	(135)
	<i>RUNX3</i>	Runt-related transcription factor 3	Transcription factor that acts as tumor suppressor gene and is pro-apoptotic	(130,136)

SCLC, small cell lung cancer.

CCGG with *HpaII* cutting blocked by either cytosine methylated and *MspI* activity blocked with the outer cytosine being methylated (137,138). Following endonuclease treatment, methylated or un-methylated DNA sequences are enriched and analyzed by PCR or DNA sequencing (120,137-139);

- Bisulfite conversion: under the correct conditions treatment of DNA with sodium bisulfite causes deamination of unmethylated cytosine to uracil, while leaving methylated cytosine intact. With PCR amplification the deaminated cytosine (a uracil) is copied into a thymine. The PCR products can be then analyzed by sequencing or mass spectrometry. Comparison of identical DNA samples with and without bisulfite allows analysis of the methylcytosine content and the specific methyl-cytosine moieties (140-142);

- Affinity purification methods: this technique uses either a methylcytosine-specific antibody or a tagged *E. coli* methyl-binding domain protein to immunoprecipitate methylated DNA (143-145). The resulting immunoprecipitates are commonly analyzed by next generation DNA sequencing (146).

A large number of studies have demonstrated that alteration in cytosine hypermethylation has diagnostic and prognostic value in lung cancer (82,102,105-122), and in some cases appears to predict treatment responses (137-139,141-143,145-147). For example, Zhang *et al.* (147) examined the methylation of 20 TSGs in 78 NSCLCs compared to 50 matched plasma samples from individuals without cancer. A five-gene set (APC, RASSF1A, CHD13, KLK10, and DLEC1) showed significantly higher methylation in lung cancer patients and had a sensitivity of 83.64% and a specificity of 74.0% for cancer diagnosis in

the Chinese population. The same study revealed that in a 64 lung cancer patient sample, patients with four or more concurrently methylated genes in a 15 gene panel (APC, CHD13, KLK13, DLEC1, RASSF1A, EFEMP1, SFRP1, RAR, p16INK4A, RUNX3, Hmlh1, DAPK, BRAC1, p14ARF) had a poorer progression-free two year survival of 13.8 months with four or more genes methylated compared to 17.8 months with less than four gene methylated. Last a study by Salazar *et al.* (148) revealed that lung cancer patients with an unmethylated plasma CHFR gene responded significantly better to EGFR tyrosine kinase inhibitors than those with a methylated CHFR gene, demonstrating that gene methylation might be useful in predicting therapy responses.

### *miR as biomarkers in lung cancer*

miRs taken from sputum and blood may be useful lung cancer biomarkers (82-85,120). They are very stable in human plasma and have value in initial lung cancer detection (149-151). Bianchi *et al.* (150,151) developed a miR-34 group based test that detects lung cancer in 80% of asymptomatic high-risk individuals who are otherwise healthy smokers. Interestingly, this class of miRs has shown value in predicting lung cancer relapse, where low expression of these miRs were highly predictive of relapse (152). Similarly low miR-30a, miR-107, miR-138, miR-204, miR-32, miR-148b, miR-145, miR-224, miR-200c, miR-125b, and miR-375 predict a poor clinical outcome, and events such as increased lymph node metastasis and larger tumor size, while high miR-126, miR-21, miR-197, mi-150, and miR-141 levels predict also predict a poor outcome (153-167).

miRs have also been shown to have value in predicting therapeutic responses. For example, Zhao *et al.* (167) found that circulating miRs had value in predicting EGFR mutation, gefitinib sensitivity, and the patient's prognosis (167). Last, miRs-33a and miR-124 may have prognostic value in lung cancer, with higher levels inhibiting the EMT transition and tumor metastasis, respectively (168,169). miRs are typically quantified by PCR amplification (149-169). Presently changes in miRs are seldom used clinically and developing reliable miR panels for clinical use in lung cancer diagnosis, prognostication, and treatment will take several more years (120).

### *Epigenetic changes in histones as lung cancer biomarkers*

Histone modifications and changes in the expression

patterns of HATs and HDACs may have value in early tumor detection, prognostication, and the guiding of epigenetic targeted therapies (55-61). Presently histone modifications and HATs and HDACs are used in the treatment of lung cancer (59-74), but their clinical use as lung cancer biomarkers and use in guiding therapy is several years away (75-88,118).

## Conclusion and future directions

Epigenetics plays an important role in early lung cancer development and progression. Recent studies have shown that methylation of TSGs correlates with the prognosis of resected early stage NSCLC, and this can be exploited to recognize which patient may benefit from adjuvant epigenetic therapy in order to reduce the risk of relapse after surgery. Since it can affect multiple pathways (21-24,28-32,46,54-61,67-69,82-85) that regulate all major properties of the cancer cell (105-122), targeting the epigenome may hold promise (137-148) in lung cancer therapy (153-169).

In spite of some disappointing clinical outcomes in earlier studies employing only epigenetic therapy, the field continues to evolve. Indeed, the remarkable responses to subsequent chemotherapy after epigenetic therapy with AZA and entinostat constitutes a paradigm shift in the management of metastatic NSCLC (76). There is a current trend to explore epigenetic priming agents to render lung cancer more susceptible to cytotoxic chemotherapy and immunotherapy. Finding predictive biomarkers to select patients who may derive benefits from epigenetic modulation and defining pharmacodynamic markers to gauge the efficacy of these agents, optimizing their effects, and their delivery sequence in conjunction with other antineoplastic agents, all constitute major challenges that need to be explored to move this promising field ahead.

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## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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