Review Article

Epigenetic diagnostic biomarkers for non-small cell lung cancer: present and future perspectives

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Abstract: Non-small cell lung cancer (NSCLC), of which the main histological types are squamous cell carcinoma (SCC), adenocarcinoma, and large cell carcinoma (LCC), continues to be a major health problem worldwide. Despite several crucial breakthroughs in treatment, the 5-year overall survival rate of NSCLC patients (less than 15%) is still far from satisfactory. The poor prognosis is due in part to the lack of early diagnostic biomarkers. However, progress is being made in this area, particularly with respect to epigenetic markers. Epigenetic regulation of gene expression, which is defined as regulation that occurs without altering the DNA sequence, is involved in the pathology of numerous cancers, including NSCLC. Thus, specific aberrant epigenetic changes are potential biomarkers for the early detection and diagnosis of this disease. In this review, we provide insight into the clinical application of two types of epigenetic regulators: DNA methylation and non-coding RNAs (ncRNAs), both of which play crucial roles in NSCLC tumorigenesis and could be useful diagnostic markers and/or therapeutic targets for NSCLC.

Keywords: Non-small cell lung cancer (NSCLC); diagnosis; epigenomics; non-coding RNA (ncRNA)

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Introduction

Lung cancer is the most common cancer in China, accounting for an estimated 17.1% of all cancer diagnoses in 2015, and is the leading cause of cancer-related death in the country (1). Non-small cell lung cancer (NSCLC) accounts for about 85% of lung cancers and mainly presents as three subtypes: squamous cell carcinoma (SCC), adenocarcinoma (AD), and large cell carcinoma (LCC) (2). In 2015, the World Health Organization called for the classification of AD and SCC to include immunohistochemical staining to improve the accuracy of microscopic diagnoses (3,4). Most patients are asymptomatic at the early stages of lung cancer and diagnosis is often delayed until the diseased is at an advanced stage with tumor metastasis, leading to a poor prognosis (5). There are few effective early diagnostic markers for NSCLC, mainly due to its lack of symptoms and heterogeneity of clinical manifestations and pathologic features (6). Therefore, there is an urgent need to identify novel biomarkers that will help to increase the 5-year overall survival rate of NSCLC patients, which is currently less than 15% (7).

Accumulating evidence indicates that genetic and epigenetic changes regulate the development of cancer (8). Epigenetics refers to heritable changes in gene expression that are not due to alterations in the DNA coding sequence (9), such as DNA methylation, histone/nucleosome methylation and acetylation, and direct and indirect regulation by non-coding RNAs (ncRNAs). Interestingly, abnormal epigenetic regulation of gene expression is frequently associated with the development of many cancers, including NSCLC (10). Research on epigenetic modulators may therefore identify

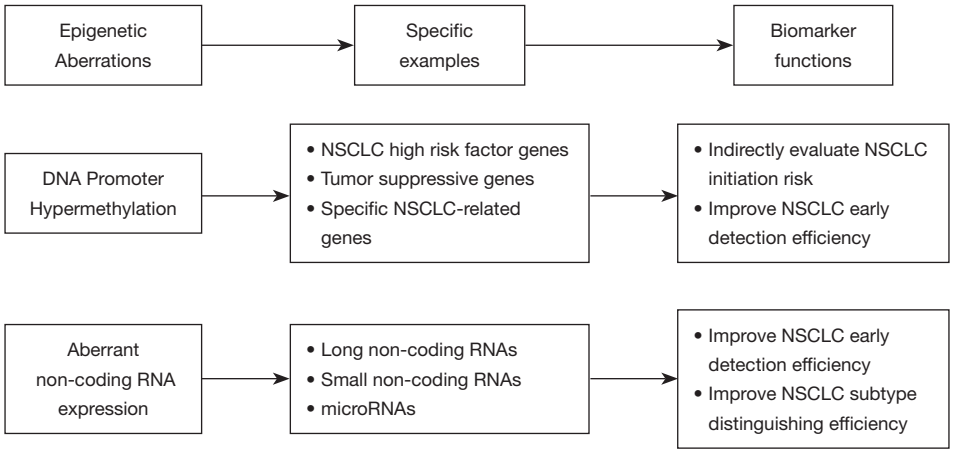

Figure 1 Abstract graph, the specific aberrant epigenetic changes are proposed as potential biomarkers for the early detection and diagnosis of NSCLC. NSCLC, non-small cell lung cancer.

novel potential diagnostic and prognostic biomarkers for this disease.

Here, we review recent work on the potential clinical application of two classes of epigenetic regulators; DNA methylation and ncRNAs, both of which play a role in NSCLC tumorigenesis and may be a source of novel early diagnostic markers and therapeutic targets for NSCLC (*Figure 1*).

DNA methylation

Nuclear transcription factors regulate gene expression by binding to unique sequences in the gene promoter. The activity of transcription factors can be modulated by addition of methyl groups to specific nucleotides by DNA methyltransferases. This mainly occurs at sites enriched in cytosine and guanine dinucleotides (CpG, 5'-C-phosphate-G-3'), which can exist in an unmethylated state or be methylated at cytosine carbon-5. CpGs are scattered throughout the human genome but are often enriched in gene promoters as CpG islands, where they inhibit binding of transcription factors and consequently suppress gene transcription (11). Aberrant methylation of CpGs has been shown to contribute to the initiation and progression of cancer, suggesting that DNA methylation biomarkers could be useful in cancer diagnosis (12). In NSCLC, most DNA methylation occurs as gene-specific hypermethylation and genomic hypomethylation (13). Genes with potential clinical application in NSCLC mainly fall into three groups: NSCLC risk factor-related genes, universal tumor suppressor genes, and NSCLC-specific genes.

NSCLC risk factor-related genes

A number of factors carry a high risk for the development of NSCLC, including aging, cigarette smoking, and chronic inflammation, all of which have been shown to have epigenetic effects (14). Recent studies have identified specific methylated genes as potential NSCLC biomarkers, five of which (AHRR, F2RL3, 2q37.1, 6p21.33, and 12q14.1) have been shown to contain six CpG hypomethylation sites (14). The genes were identified by analysis of the peripheral blood of NSCLC patients, and their hypomethylation could be attributable to smoking. This finding suggests that modification of these genes may increase the risk of NSCLC and could thus be developed as predictive biomarkers (15).

Tumor suppressor genes

Inactivation of several well-known tumor suppressor genes through promoter methylation is an early event in the carcinogenic process of many cancers, including NSCLC (16). In one study, sputum samples collected from patients with SCC up to 3 years before clinical diagnosis showed evidence of aberrant methylation of the $p16^{INK4a}$ and/or O^6-methylguanine-DNA methyltransferase gene (MGMT) promoters in 100% of samples (17). In another study, alterations (including methylation, mutation, or

deletion) of the $p16^{INK4a}$ gene were observed in about 60% of NSCLC patients (18). Notably, methylation of $p16^{INK4a}$ is seen in all SCC patients and has been associated with smoking in AD patients (19). Considering the stability of the modification detected in samples and the accuracy with which it can be detected, DNA methylation of tumor suppressor genes holds great promise as a biomarker for the early diagnosis of NSCLC.

NSCLC-specific genes

In terms of feasibility for use as clinical biomarkers, targeted identification of specific methylated genes is a much preferable approach than genome-wide screening of methylation profiles. The methylation level of numerous genes plays a role in the initiation and progression of lung cancer, and can be easily detected by analysis of circulating tumor DNA (ctDNA) collected from the peripheral blood. ctDNAs are double-stranded DNA fragments ranging in length from 120 to 200 nucleotides (20). They are mostly released from apoptotic or necrotic tumor cells (21), with some originating from circulating tumor cells or released exosomes (22). Since methylated ctDNA is stable and can be collected in a convenient and simple manner, it has potential as an early diagnostic biomarker (23). For instance, analysis of peripheral blood-derived plasma showed aberrant methylation of the DCLK1 promoter in 32 of 65 (49.2%) NSCLC patients and only 8 of 95 (8.4%) of healthy controls (24). In another study, analysis of 330 plasma specimens from three independent case-control studies found that aberrant methylation of the SHOX2 and PTGER4 gene promoters could readily distinguish lung cancer patients from malignancy-free control subjects (25). Analysis of ctDNA from lung cancer patients has detected abnormal methylation levels in numerous genes, including short stature homeobox 2 (SHOX2), Ras association domain family 1 isoform (RASSF1A), retinoic acid receptor beta2 (RARB2), MGMT, and death-associated protein kinase 1 (DAPK) (26).

ncRNAs

Approximately 75% of the human genome is transcribed into RNA, but only a small proportion of that is translated into proteins (27). However, a large number of the remaining ncRNAs are actively involved in regulating cellular behavior through their interaction with RNA-binding proteins and regulation of target gene transcription. Importantly, many ncRNAs regulate the expression of oncogenes and tumor suppressor genes (28). Since ncRNAs can be readily identified and quantified by RT-PCR analysis of peripheral blood cell extracts, there is great interest in their potential clinical application in tumor diagnosis (29) After secreted into circulation, ncRNAs are extracted from blood samples of cancer patients and healthy controls by centrifugation. Consequently, the levels of ncRNAs are capable of being quantified and analyzed in duplicate by quantitative RT-PCR (30).

As a class, ncRNAs include housekeeping (transfer and ribosomal) RNAs and regulatory ncRNAs. The latter group can be further classified as long ncRNAs (lncRNAs >200 nucleotides) and short ncRNAs (<200 nucleotides) (31), which includes microRNAs (miRNAs), small interfering RNAs (siRNAs), small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), and small nuclear RNAs (snRNAs) (32).

Long ncRNAs

Epigenetic reprogramming of cancer cells causes a significant change in the profiles of lncRNAs with functions associated with tumor invasiveness and metastatic potential. Current work suggests that a number of lncRNAs could be used as diagnostic biomarkers for NSCLC.

HOX transcript antisense intergenic RNA (HOTAIR)

HOTAIR was the first lncRNA found to be involved in regulating tumorigenesis, and it has been shown to be dysregulated in several cancers, such as gastric cancer, breast cancer and NSCLC (33). HOTAIR is located within the homeobox C (HOXC) gene cluster on chromosome 12 and is co-expressed with HOXC (34). HOTAIR acts by binding to the polycomb repressive complex 2 (PRC2) and indirectly initiating trimethylation of H3 lysine 27 of the HOXD locus. In turn, this suppresses cell differentiation, enhances cell invasion and migration, and maintains cell stemness (35). HOTAIR-induced increases in cell invasiveness can be attributed to increased protein levels of the matrix metalloproteinases MMP2 and MMP9, which induce degradation of the extracellular matrix. Thus, tumor cells expressing high HOTAIR levels exhibit a high risk for metastasis, which correlates with poor prognosis and poor overall survival rates (36). Notably, HOTAIR levels are markedly higher in plasma and tumor tissues from NSCLC patients compared with normal subjects (37). In one study, HOTAIR levels could distinguish between NSCLC patients

and healthy controls with relatively high sensitivity (76.2%) and specificity (71.9%) (34), confirming the potential utility of HOTAIR as a potential diagnostic biomarker.

Metastasis-associated lung ad transcript 1 (MALAT-1)

MALAT-1, which is located on chromosome 11q13 and is also known as nuclear-enriched abundant transcript 2 (NEAT2), is upregulated in several types of cancer including NSCLC (38). MALAT-1 is 8.7 kb in length and shows high species conservation (39). It regulates the expression of several genes related to cell motility, such as chaperonin containing TCP1 subunit 4 (CCT4), collagen triple helix repeat containing 1 (CTHRC1), hyaluronan mediated motility receptor (HMMR), and Regulator of differentiation 1 (ROD1), which indirectly enhances cell invasiveness. Thus, overexpression of MALAT-1 is significantly associated with the development of metastases (40). Exosome-derived MALAT-1 is significantly upregulated in sera from NSCLC patients compared with healthy controls (41). Interestingly, MALAT-1 levels can discriminate between SCC patients and AD patients and their respective controls with sensitivities of 63% and 48% (42), making it a promising epigenetic biomarker for the early diagnosis of NSCLC.

Colon cancer-associated transcript 2 (CCAT2)

Overexpression of this lncRNAs, which is transcribed from the 8q24 genomic region, is associated with the cell proliferation and invasiveness of various types of cancer (43). In a NSCLC cell line, expression of c-Myc and the miRNAs miR-17-5p and miR-20a were transcriptionally upregulated via CCAT2 and transcription factor 7 like 2 (TCF7L2), which leads to increased tumor growth (44). Moreover, CCAT2 is significantly overexpressed in NSCLC tissue compared with normal tissue (45,46), and its knockdown in NSCLC cell lines inhibited their proliferation and invasion *in vivo* and *in vitro* (45,47). Therefore, CCAT2 may be a promising biomarker for metastatic NSCLC.

NSCLC-specific lncRNAs

In addition to the lncRNAs described in the preceding sections, which regulate the behavior of various kinds of tumors, several lncRNAs have been discovered that are specifically dysregulated in NSCLC. For instance, the lncRNAs inactive X specific transcripts (XIST) and hypoxia inducible factor 1 alpha subunit antisense RNA 1 (HIF1A-AS1) (48) are upregulated in serum samples from NSCLC patients compared with controls. Moreover, aberrant expression of several lncRNAs is observed in different subtypes of NSCLC. Li *et al.* analyzed tissue microarrays from 181 patients with early-stage AD and found that LINC00313 is overexpressed only in patients with T2 and N1 stage AD (49). Zhang *et al.* found that LINC01133 is upregulated in samples of SCC, but not of AD (50), whereas Zhao *et al.* identified 72 aberrantly expressed lncRNAs in both SCC and AD patients (51). Since different NSCLC subtypes are associated with different pathological features and clinical treatment options, identification of lncRNAs capable of distinguishing between the NSCLC subtypes would represent a major advance in NSCLC diagnosis.

MicroRNAs

miRNAs are single-stranded ncRNAs, approximately 20 nucleotides in length, that show high species conservation (31). Since miRNAs are involved in virtually every aspect of cell physiology, it is not surprising that they are also important epigenetic regulators of the development and therapeutic response of NSCLC (52).

Recent studies have identified changes in the levels of several miRNAs in body fluids (e.g., serum, plasma) from cancer patients, highlighting the potential utility of this class of ncRNAs as diagnostic, prognostic, and/or predictive therapeutic response markers (53). Advances in microarray analytical techniques have led to the identification of multiple miRNAs with significant roles in the development of NSCLC. In one meta-analysis of 28 publications that analyzed 2,121 NSCLC patients and 1,582 healthy individuals, blood-derived miRNA levels could diagnose early disease with high accuracy (54). In another study of circulating miRNAs, 39 and 18 miRNAs were discovered to be upregulated and downregulated, respectively, in lung cancer patients compared with healthy controls, and were strongly associated with early or metastatic NSCLC (30). Assessment of panels of miRNA, rather than single miRNAs, will undoubtedly improve the specificity and sensitivity of NSCLC diagnosis (53).

Conclusions

Over the past several years, a broad spectrum of diagnostic biomarkers for NSCLC has been investigated. Here, we reviewed studies on two classes of potential epigenetic biomarkers. DNA promoter hypermethylation is an early event in NSCLC development, and primarily affects genes associated with a high risk of NSCLC, well-known tumor

suppressor genes such as $p16^{INK4a}$, and NSCLC-specific genes such as DCLK1. Most of these epigenetic changes can be detected by analyzing ctDNA in the peripheral circulation. In addition, technological advances in RNA sequencing and microarray analysis have identified several lncRNAs with potential utility for NSCLC diagnosis. Some of these, such as HOTAIR, MALAT-1, and CCAT2, are specifically associated with early-stage and/or metastatic disease, whereas others, such as LINC00313, are specifically overexpressed in a particular NSCLC subtype.

Notably, most epigenetic biomarkers can be readily detected through minimally or non-invasive approaches, which is an important feature for clinical applications. Moreover, combination of these epigenetic biomarkers and other early detection methods could have considerably higher diagnostic value than either method alone. From the data presented here, it seems reasonable to expect increasing focus on discovering epigenetic biomarkers for the diagnosis of NSCLC. Considering the rapid progress of technologies such as cloud computing and analysis of massive datasets, it should be feasible to construct an epigenetics database that includes tumors of many origins. Such a database would not only have clinical utility but also would help to advance our understanding of the initiation, progression, and regulation of cancer.

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Footnote

Conflicts of Interest: TAll authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2018.05.03). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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