Applying circulating tumor DNA methylation in the diagnosis of lung cancer

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Abstract

Lung cancer is the leading cause of cancer-related deaths worldwide. Low dose computed tomography (LDCT) is commonly used for disease screening, with identified candidate cancerous regions further diagnosed using tissue biopsy. However, existing techniques are all invasive and unavoidably cause multiple complications. In contrast, liquid biopsy is a noninvasive, ideal surrogate for tissue biopsy that can identify circulating tumor DNA (ctDNA) containing tumorigenic signatures. It has been successfully implemented to assist treatment decisions and disease outcome prediction. ctDNA methylation, a type of lipid biopsy that profiles critical epigenetic alterations occurring during carcinogenesis, has gained increasing attention. Indeed, aberrant ctDNA methylation occurs at early stages in lung malignancy and therefore can be used as an alternative for the early diagnosis of lung cancer. In this review, we give a brief synopsis of the biological basis and detecting techniques of ctDNA methylation. We then summarize the latest progress in use of ctDNA methylation as a diagnosis biomarker. Lastly, we discuss the major issues that limit application of ctDNA methylation in the clinic, and propose possible solutions to enhance its usage.

Key words: ctDNA; methylation; lung cancer; diagnosis

Introduction

Lung cancer is the most frequent oncogenic malignancy and the leading cause of cancer-related deaths worldwide, with 2.1 million newly diagnosed cases and 1.8 million deaths in 2018.¹ Low-dose computed tomography (LDCT) screening can reduce lung cancer mortality by 20% but causes significant issues, including overdiagnosis.² Although candidate lung cancer regions identified by LDCT can be further diagnosed via tissue biopsies (the most common being bronchoscopy, transthoracic needle aspiration, and surgery), complications may emerge, including hemorrhage, infection, pneumothorax, and even mortality.³–⁵ Moreover, the sensitivity of LDCT may be severely affected by tumor size and location, varying in a substantial range (60–80%).⁶,⁷ Moreover, inevitable intratumoral and intertumoral heterogeneity resulting from selection during carcinogenesis, make cancer treatment and surveillance difficult.⁸,⁹
Considering the aforementioned limitations, in recent years liquid biopsy has been proposed as a favorable alternative. Through noninvasive or minimally invasive approaches, tumor-originating factors in blood and other types of body fluid can be detected for cancer diagnosis and surveillance.\textsuperscript{10} Protein and circulating free DNA (cfDNA) are the most well-studied biomarkers for liquid biopsy, with other markers including circulating tumor cells (CTCs), circulating free RNA, exosomes, and nucleosomes.\textsuperscript{10,11} cfDNA are free DNA fragments circulating in the peripheral blood, ranging from 80 to 200 base pairs (bp),\textsuperscript{12} and those generated from tumor cells are called circulating tumor DNA (ctDNA). ctDNA is hypothesized to enter the peripheral circulation system through both active and passive mechanisms. Tumors may secret DNA fragments into blood spontaneously, whereas apoptosis and necrosis of tumor cells may also generate ctDNA passively. Furthermore, it has been reported that CTCs in circulation are also a possible source for ctDNA, yet the mechanism for their appearance is unclear.\textsuperscript{13–15}

The first study of cfDNA in human blood was published in 1948.\textsuperscript{16} Later, its potential clinical application was advanced by a clinical study that found cfDNA quantity was elevated in participants with diseases.\textsuperscript{17} This phenomenon also occurs in human malignancy,\textsuperscript{18} emphasizing the importance of quantitative changes in ctDNA. Subsequently, other forms of alterations in ctDNA were also detected, including the presence of somatic mutations. The Cobas EGFR mutation test,\textsuperscript{19} the first FDA-approved ctDNA companion diagnostic test, is now used extensively in diagnosis of non-small cell lung cancer. Cancer-specific DNA methylation, another critical aberration in ctDNA, has recently attracted considerable attention, especially in the early detection of malignancy including liver cancer and colon rectal cancer.\textsuperscript{20,21} In this review, we first introduce the underlying biology and commonly used detection techniques of ctDNA methylation, and then discuss the latest developments of its clinical applications in lung cancer diagnosis.

**DNA methylation in cancer**

DNA methylation is a pivotal epigenetic mechanism involved in regulating X chromosome inactivation, genomic imprinting, tissue-specific gene expression, and multiple disorders.\textsuperscript{22,23} DNA methylation usually occurs on cytosine at the 5-carbon position and occurs most frequently around gene promoters in CpG islands, which are CG-enriched regions with a length of approximately 1kb.\textsuperscript{24} Another commonly methylated region is the CpG island shore, which lies close to CpG islands (about 2kb) with a lower C and G density. Methylation in these areas often results in gene silencing, whereas gene body methylation is associated with transcriptional activation. The former is often detected in some tumor suppressor genes in malignant cases, whereas the latter may also result in various diseases.\textsuperscript{25} Notably, DNA methylation has been detected in repetitive elements and CHG/CHH (H stands for C, A, or T) sites as well, and is associated with some interesting biological processes (e.g. stem cell growing). Compared with ctDNA mutations and rearrangements, ctDNA methylation has two main advantages. It may be detectable at an early stage in carcinogenesis,\textsuperscript{26} and helps in determining the origin of malignancy.\textsuperscript{27–29} We exemplify this issue in detail later in this review.

**Techniques for measuring DNA methylation**

DNA methylation was first detected through chromatography in the 1970s, which discriminated methylated from unmethylated deoxynucleosides quantitatively using UV absorbance.\textsuperscript{30} These early technologies proved the
feasibility of charting the DNA methylation landscape in humans. Since then, a variety of techniques have been developed to detect DNA methylation, either at a genome-wide scale or a locus-specific level. Three principle approaches are exploited to detect and/or isolate methylated DNA (Fig. 1), namely immunoprecipitation, methyl-sensitive restriction enzymes, and sodium bisulfite conversion.  

Immunoprecipitation

In the 1980s, specific methylcytosine antibodies were identified. When combined with electron microscopy or confocal fluorescence microscopy, it was possible to detect methylation alterations quantitatively. These anti-SmC (anti-5-methylcytosine) antibodies were then combined with immunological separation techniques (i.e. immunoprecipitation) and the immunocaptured DNA was further examined using microarray hybridization or sequencing-based approaches to explore methylation status at different sites. MeDIP (methylated DNA immunoprecipitation) is representative of immunoprecipitation-based techniques. In this process, immunocaptured methylated DNA fragments are labeled with a Cy5 fluorescence dye, producing green fluorescence; whereas unmethylated DNA fragments are connected to a Cy3 dye and fluoresce red. The affinity of anti-SmC antibodies is key in this assay. Recently, recombinant protein complexes with stronger affinity have been developed and applied in more efficient techniques, such as methyl-CpG immunoprecipitation (MeCIP) and methylated-CpG island recovery assay (MIRA).

Methyl-sensitive restriction enzymes

Several restriction enzymes recognize the same sequence but have differential sensitivity to methylcytosine. For example, HpaII and MspI both recognize and cut DNA at the same sequence CCGG, but Hpa II does not cleave the site when the second C is methylated. Genomic DNA is digested separately with both enzymes, and DNA fragments are PCR amplified and different size fragments are detected via microarray or sequencing. Large fragments will occur when a CpG is methylated in the CCGG sequence. The HELP assay (HpaII tiny fragment enrichment by ligation-mediated PCR) and methyl-sensitive restriction enzyme sequencing (MRE-seq) are both based on methyl-sensitive restriction enzymes, but their applications are limited by the enzyme recognition sites.

Sodium bisulfite conversion

Cytosine residues in genomic DNA can be changed into uracil when treated with sodium bisulfite; this reaction does not occur (or occurs very slowly) at methylcytosine. This difference is exploited to discriminate 5 mC and C, converting genomic chemical modification into an easily detectable DNA residue. Sodium bisulfite conversion has been implemented in various techniques, including bisulfite sequencing (BS), methylation-specific polymerase chain reaction (MS-PCR), and methylation-specific high-resolution melting (MS-HRM). However, some distinct flaws also limit its application, such as reduction in sequence complexity, DNA degradation, and incomplete bisulfite conversion. Based on these principle approaches, sequencing develops into the most widely used technology for detecting methylation. Several platforms are applied recently, including whole-genome bisulfite sequencing (WGBS), SureSelect Methyl-Seq, SeqCap Epi CpGiant, and reduced representation bisulfite sequencing (RRBS). They detect DNA methylation changes at single base resolution, but with diverse genome coverages. Researchers should select proper techniques according to specific research aims.

Representative biomarkers

A number of DNA methylation biomarkers have been associated with lung cancer. In this section, we introduce each biomarker’s biological function and diagnostic performance in lung cancer (Table 1). Generally, sensitivity and specificity are applied to evaluate the diagnostic efficacy. The former measures the proportion of true positives, whereas the latter represents the actual negatives that are correctly identified.

SHOX2

Short stature homebox gene 2 (SHOX2) is located on chromosome 3q (3q25.32). It encodes a homeo-domain transcription factor, which plays pivotal roles in heart, skeletal, and brain development. SHOX2, highly homologous to another short stature homeobox gene SHOX, has mostly been studied in the short stature phenotype of Turner syndrome and idiopathic short stature. Recently, its diagnostic value in lung cancer has also been exemplified by several studies. SHOX2 hypermethylation (mSHOX2) was firstly determined in lung cancer in Schmidt’s study in which 523 tissue samples obtained via bronchial aspirates were detected using differential methylation hybridization (DMH) and real-time PCR. This study yielded a sensitivity of 68% and specificity of 95%. Later, this alteration was confirmed in Katja’s study. He put forward that such alteration might be associated with copy number amplification in the SHOX2 gene. Based on results in lung cancer tissue, further attention was paid to plasma samples. A study with 411 participants yielded a sensitivity of 60% and a specificity of 90%, but diagnostic performance in stage I was poor (sensitivity 27%). In subsequent studies, the overall sensitivity was around 70%, but neither reported the discriminatory power in stage I cancer. Recently, bronchial lavage and pleural effusion were tested and gave similar results. Furthermore, recently multiple genes were combined to enhance sensitivity. A combination of mSHOX2 and mRASSF1A was tested in bronchial lavage and achieved a sensitivity of 85.7% (24/28) in stage I lung cancer, and 50% (2/4) in carcinoma in situ. These studies strongly support a possible SHOX2 application in lung cancer early detection. However, it is noteworthy that
| Author (year) | Sample types | Method | No. of cases | TNM stage (1/2/3/4/unknown) | No. of controls | Sensitivity, % | Specificity, % |
|--------------|--------------|--------|--------------|-----------------------------|----------------|---------------|---------------|
| SHOX2        |              |        |              |                             |                |               |               |
| Kneip (2011) | Plasma       | RT-PCR | 20           | 0/0/0/20/0                  | 20             | 75            | 95            |
| Kneip (2011) | Plasma       | RT-PCR | 188          | 37/29/53/42/27              | 155            | 60            | 90            |
| Dietrich (2012) | Bronchial lavage | RT-PCR | 125         | NA                         | 125            | 78            | 96            |
| Ilse (2013)  | Pleural effusion | RT-PCR | 472         | NA                         | 798            | 39.5          | 96.2          |
| Konecny (2016) | Bronchial lavage | RT-PCR | 38           | 1/4/8/22/3                 | 31             | 83.78         | 84.62         |
| Konecny (2016) | Bronchial lavage | RT-PCR | 38           | 1/4/8/22/3                 | 31             | 64.52         | 78.57         |
| Ren (2017)   | Blood        | RT-PCR | 123          | 51/13/19/25/15             | 130            | 64.2          | 92.3          |
| RASSF1A      |              |        |              |                             |                |               |               |
| Ramirez (2003) | Serum       | MSP    | 50           | 6/11/18/5/10               | 0              | 34            | NA            |
| Rykova (2004) | Plasma       | MSP    | 9            | NA                         | 16             | 44            | 100           |
| Belinsky (2005) | Plasma       | MSP    | 44           | NA                         | 195            | 7             | 98            |
| Wang (2006)  | Plasma       | MSP    | 63           | NA                         | NA             | 22            | 93            |
| Hsu (2007)   | Plasma       | qMSP   | 63           | Stage 1–2: 41              | 36             | 39            | 90            |
| Ponomaryova (2013) | Blood   | qMSP   | 60           | Stage 1–2: 20              | 33             | 66            | 57            |
| Zhang (2011) | Plasma       | MSP    | 110          | Stage 1 & 2                | 50             | 36.36         | 92            |
| Gao (2015)   | Serum        | qMSP   | 40           | 40/0/0/0/0                 | 36             | 52.5          | 97            |
| Gao (2015)   | Plasma       | qMSP   | 58           | 58/0/0/0/0                 | 54             | 43.1          | 96            |
| Ren (2017)   | Bronchial lavage | qMSP   | 123          | 51/13/19/25/15            | 130            | 50.4          | 96.2          |
| p16/CDKN2A   |              |        |              |                             |                |               |               |
| Esteller (1999) | Serum       | MSP    | 22           | 10/3/8/1/0                 | 0              | 13            | NA            |
| An (2002)    | Plasma       | Seminested MSP | 105 | Stage 1: 29 | Stage 2: 54 | Stage 3–4: 22 | 15 | 40 | 100 |
| Bearzatto (2002) | Plasma       | F-MSP  | 35           | 28/4/3/0/0                 | 15             | 40            | 100           |
| Belinsky (2005) | Plasma       | MSP    | 44           | NA                         | 195            | 25            | 88            |
| Belinsky (2005) | Sputum       | MSP    | 44           | NA                         | 112            | 19            | 75            |
| Wang (2006)  | Plasma       | MSP    | 63           | NA                         | 22             | 61            | 92            |
| Wang (2006)  | Sputum       | MSP    | 79           | NA                         | 22             | 65            | 93            |
| Hsu (2007)   | Plasma       | qMSP   | 63           | Stage 1–2: 41              | 36             | 38            | 91            |
| Zhang (2011) | Plasma       | MSP    | 110          | Stage 1 & 2                | 50             | 22.73         | 92            |
| Xiao (2014)  | Plasma       | F-MSP  | 30           | Stage 1–2: 24              | 30             | 50            | 100           |
| Xiao (2014)  | EBC          | F-MSP  | 30           | Stage 1–2: 24              | 30             | 40            | 100           |
| SEPT9        |              |        |              |                             |                |               |               |
| Powrozek (2014) | Plasma       | RT-PCR | 70           | 0/7/23/17/23               | 100            | 44.3          | 96            |
| APC          |              |        |              |                             |                |               |               |
| Usadel (2002) | Serum/plasma | Semiquantitative F-MSP | 89 | NA | 50 | 47 | 100 |

Continued
mSHOX2 is not a lung cancer-specific biomarker. It has been reported in other cancer types, including head and neck squamous cell carcinoma (HNSCC), colorectal cancer (CRC), and ovary cancer. Thus, mSHOX2 may be a marker for many cancer types.

**RASSF1A**

The Ras association domain family 1A (RASSF1A) is a member of the RASS family (RASS), which comprises 10 genes (RASSF1 to RASSF10). These genes play critical roles in regulating cell cycle control, apoptosis, and microtubule stabilization. Hypermethylation in their CpG island promoters has been reported to induce multiple cancers. The RASSF1 gene is located on chromosome 3 (3p21.3). It encodes eight homologous proteins, including RASSF1A. Interacting with tubulin, RASSF1A mainly regulates mitotic progression by stabilizing microtubules in cell.

The first study reporting RASSF1A hypermethylation in peripheral blood was published in 2003. In this study, RASSF1A gene promoter hypermethylation was detected in 17/50 (34%) lung cancer plasma samples. However,
specifivity could not be achieved as no control samples were analyzed. This concern was resolved in another study that found the RASSF1A gene promoter was hypermethylated in 4/9 (44%) lung cancer plasma samples, but hypomethylated in all nonmalignant plasma samples (0/16). The low sensitivity in plasma remained a major issue for RASSF1A in subsequent studies, and it was even reported that sensitivity in sputum (13/44, 25%) came much higher than in plasma (3/44, 7%). Nonetheless, mRASSF1A is useful in multigene panels. A study using a six-gene panel (including BLU, CDH13, FHIT, p16, RARB, and RASSF1A) showed higher sensitivity (68%) in plasma samples in early stage lung cancer (including stages I/II). This rate was even higher (83.64%) in another five-gene panel (including APC, RASSF1A, CDH13, KLK10, and DLEC1). An even higher mRASSF1A specificity (over 98%) was found in a study focused on female lung cancer patients. Therefore, it seems that RASSF1A may work better in combination with other genes rather than as a single diagnostic biomarker, and this may give the best signal in women. RASSF1A methylation was successfully detected in bronchial lavage. Combined with SHOX2, it achieved a sensitivity around 80% and a specificity of 95%. As for mSHOX2, mRASSF1A’s diagnostic potential has also been reported in other cancers.

p16/CDKN2A

p16 (also designated as cyclin-dependent kinase inhibitor 2A, CDKN2A) is located on chromosome 9p21. It encodes a cyclin-dependent kinase inhibitor and regulates the cell cycle through inactivation of the G1 cyclin D-dependent kinase E (CDKE). Hypermethylation in the p16 promoter suppresses gene transcription, resulting in loss of inhibition of cell cycle progression. This epigenetic alteration has been reported in various malignancies. A relationship between p16 hypermethylation and lung cancer was first found in tissue samples. A study validated p16 hypermethylation in both rat models and tissue samples from lung squamous cell carcinoma (SCC) patients. Significantly, they also found that p16 was hypermethylated in some precursor lesions, and this methylation frequency increased with disease progression from basal cell hyperplasia (17%) to carcinoma in situ (50%). Based on these important findings, p16 methylation in plasma samples was studied subsequently, finding that methylation status was in concordance between plasma samples and tissue samples. Moreover, the high sensitivity of p16 methylation was also verified in early stage lung cancer patients. It achieved a sensitivity of 65.5% in stage I lung cancer. Intriguingly, it was more robust in SCC (77.8%) than in adenocarcinoma (ADC) (45.5%). Bearzatto and colleagues also detected p16 methylation in 38% (9/24) of plasma samples for stage I lung cancer patients. Similar sensitivity (54.16%, 13/24) was obtained in another study enrolling stage I and II plasma samples. The high specificity of p16 methylation was also determined in various studies, supporting its diagnostic value in lung cancer early detection. Notably, sputum and exhaled breath condensate may also be alternative samples for lung cancer diagnosis.

SEPT9

Septins are a series of cytoskeleton-related proteins that interact with tubulin and actin. There are 14 members in this family, including SEPT9. SEPT9 regulates cell proliferation, cell motility, and angiogenesis, and can act as both a protooncogene and a tumor suppressor gene, underscoring its role in oncogenesis. Through genomic amplification and overexpression, mSEPT9 has been implicated in breast and ovarian cancer, T cell lymphoma, and some other malignancies. The most important clinical application for SEPT9 methylation is in CRC. Epi proColon, a test examining SEPT9 promoter methylation status, is the first FDA-approved epigenetic tool in cancer diagnosis. SEPT9 hypermethylation has also been reported in other cancers, including biliary cancer and HNSCC.

In recent years, the potential value of SEPT9 methylation in lung cancer has also been put forward. Tomasz and colleagues enrolled 70 lung cancer patients and 100 healthy participants, and detected SEPT9 methylation in ctDNA via real-time PCR. They found that 31/70 (44.3%) lung cancer patients were hypermethylated in blood, whereas only 4/100 were positive in healthy controls (specificity 96%). Intriguingly, detection in pleural effusion and ascites provide further possibilities for SEPT9 sampling in clinical application.

Other biomarkers

In addition to the biomarkers introduced above, other methylation regions have promising potential in lung cancer diagnosis (Table 1). Adenomatous polyposis coli (APC), a Wnt antagonist, has attracted much recent attention as its hypermethylation in plasma and serum proved to be around 50% in lung cancer. Notably, this prevalence was higher in lung ADC than in SCC, making it a possible ADC-specific biomarker. APC hypermethylation is not lung cancer-specific, it has been detected in other cancers. Retinoic acid receptor-β2 (RARB), another tumor suppressor gene, is hypermethylated both in lung cancer tissue and plasma and is significantly associated with pathological types and grades. Similar finding have also been reported for the SRY-box containing gene 17 (SOX17), cadherin13 (CDH13), and Death-associated protein kinase (DAPK).

Methylation panels

There is a recent trend towards combining analysis of different methylation regions into a single panel to increase sensitivity (Table 2). For example, a combination of SHOX2 and PTGER4 achieved a sensitivity of 67% when fixing the specificity at 90%, while yielding a
Table 2. Summary of multigene panels from liquid biopsy in lung cancer diagnosis.

| Author (year) | Panel | Sample types | Method | No. of cases | TNM stage (1/2/3/4/unknown) | No. of controls | Sensitivity, % | Specificity, % |
|---------------|-------|--------------|--------|--------------|----------------------------|----------------|---------------|----------------|
| Belinsky (2005)<sup>75</sup> | p16/MGMT/RASSF1A | Plasma MSP | 44 | NA | 195 | 36 | 81 |
| Belinsky (2005)<sup>75</sup> | p16/MGMT/RASSF1A | Plasma MSP | 44 | NA | 112 | 38 | 89 |
| Hsu (2007)<sup>71</sup> | BLU/CDH13/FHIT/ p16/RARB/RASSF1A | Plasma qMSP | 63 | Stage 1–2: 41 Stage 3–4: 21 Unknown: 1 | 36 | 73 | 82 |
| Ostrow (2010)<sup>109</sup> | KIF1A/DCC/RARB/ NISCH APC/RASSF1A/ CDH13/KLK10/ DLEC1 | Plasma qMSP | 70 | Stage 1 & 2 | 80 | 73 | 71 |
| Zhang (2011)<sup>72</sup> | APC/CDH1/ DCC/RASSF1A/AIM1 SHOX2/SEPT9 | Serum F-MSP | 76 | 41/17/11/5/2 | 30 | 84.2 | 56.7 |
| Dietrich (2013)<sup>76</sup> | SHOX2/SEPT9 | Pleural effusion qPCR | 8 | NA | 56 | 14 | 100 |
| Ponomaryova (2013)<sup>127</sup> | RARB2/RASSF1A | Blood qMSP | 60 | Stage 1–2: 20 Stage 3: 40 Stage 1–2: 8 Stage 3–4: 12 Unknown: 3 | 33 | 87 | 75 |
| Wielcher (2015)<sup>79</sup> | HOXD10/PAX9/ PTPRN2/STAG3 | Serum/ plasma MSRE+qPCR | 23 | | 23 | 97 | 73 |
| Hulbert (2016)<sup>111</sup> | CDO1/TAC1/SOX17 | Plasma qMSP | 150 | 136/14/0/0/0 | 60 | 93 | 62 |
| Hulbert (2016)<sup>111</sup> | HOXA7/TAC1/SOX17 | Sputum qMSP | 150 | 136/14/0/0/0 | 60 | 98 | 71 |
| Weiss (2016)<sup>52</sup> (specificity fixed) | SHOX2/PTGER4 | Plasma RT-PCR | 50 | 12/11/16/11/0 | 122 | 67 | 90 |
| Weiss (2016)<sup>52</sup> (sensitivity fixed) | SHOX2/PTGER4 | Plasma RT-PCR | 50 | 12/11/16/11/0 | 122 | 90 | 73 |
| Zhang (2017)<sup>54</sup> | SHOX2/RASSF1A | Bronchial lavage Sanger sequencing | 284 | 28/30/133/93/0 | 38 | 81 | 97.4 |
| Ren (2017)<sup>55</sup> | SHOX2/RASSF1A | Bronchial lavage Sanger sequencing/ RT-PCR | 123 | 51/13/19/25/15 | 130 | 71.5 | 70 |
| Ooki (2017)<sup>115</sup> | CDO1/HOXA9/AJAP1/ PTGDR/UNCX/ MARCH11 | Serum qMSP | 43 | 43/0/0/0/0 | 42 | 72.1 | 71.4 |
| Nunes (2018)<sup>76</sup> | SCGB3A1/SEPT9/ SOX17 | Plasma qMSP | 73 | NA | 103 | 39.4 | 85.1 |
| Nunes (2018)<sup>76</sup> | APC/FOXA1/RASSF1A | Plasma qMSP | 73 | NA | 103 | 85.7 | 75.7 |

AIM1, absent in melanoma 1; AJAP1, adherens junction associated protein 1; APC, adenomatous polyposis coli; BLU, a candidate tumor suppressor gene; CDH1, cadherin 1; CDH13, cadherin13; CDO1, cysteine dioxygenase 1; DCC, DCC netrin 1 receptor; DLEC1, deleted in lung and esophageal cancer 1; FHIT, fragile histidine triad gene; F-MSP, fluorescent MSP; FOXA1, forkhead box A1; HOXA7, homeobox A7; HOX9, homeobox A9; HOXD10, homeobox D10; KIF1A, kinesin family member 1A; KLK10, kallikrein-related peptidase 10; MARCH11, membrane-associated ring-CH-type finger 11; MGMT, O6-methylguanine-DNA methyltransferase; MSP, methylation specific PCR; MSRE, methylation-sensitive restriction enzymes; NA, not available; NISCH, Nischarin; p16, p16 gene; PAX9, paired box 9; PCR, polymerase chain reaction; PTGDR, prostaglandin D2 receptor, PTGER4, prostaglandin E receptor 4; PTPRN2, protein tyrosine phosphatase receptor type N2; qMSP, quantitative MSP; RARB, retinoic acid receptor-β2; RASSF1A, ras association domain family 1A; RT-PCR, real-time PCR; SCGB3A1, secretoglobin family 3A member 1; SEPT9, septin 9; SHOX2, short stature homeobox gene 2; SOX17, SRY-box containing gene 17; STAG3, stromal antigen 3; TAC1, tachykinin precursor 1; UNCX, UNC homeobox.
specificity of 73% when fixing the sensitivity at 90% (AUC = 0.88).33 In another study, SOX17 combined with CDO1 and TAC1 showed a sensitivity of 86% and a specificity of 78% (AUC = 0.77) in lung cancer plasma.113 Favorable results were also achieved in stage 1A lung cancer: a six-gene panel (CDO1, HOXA9, AJAP1, PTGDR, UNCX, and MARCH1) was sensitive in 72.1% (31/41) of stage 1A lung cancer plasma, with a specificity of 71.4% (30/42).115 However, larger panels often necessitate higher budgets, a feature that must be considered for clinical application. In addition, with developments in high-throughput techniques (such as microarray and sequencing), it may be possible to expand the number of biomarkers analyzed. In recent studies, thousands of methylation alterations have been detected simultaneously, illustrating the pathogenesis of several diseases and facilitating diagnosis of multiple malignancies.20,21,116,117

Current limitations and potential solutions

Despite the multiple aforementioned advantages, none of the methylation biomarkers has yet been applied to lung cancer diagnosis in a clinical setting. The most important factor contributing to this situation is low sensitivity. As illustrated above, methylation often occurs at an early stage in carcinogenesis, and is sometimes even detectable in precursor lesions of lung cancer.94 However, these tumor-derived DNA fragments exist at very low levels in plasma. It was reported that 2121-8787 copies of cfDNA (= 7-29 ng) could be extracted from 1 ml plasma.118,119 Hematopoietic cells, such as leukocytes, are the major source of cfDNA—ctDNA is a small portion of this material.120 In fact, it has been estimated that only two copies of ctDNA can be purified in 1 ml plasma from stage I cancer.121 Furthermore, after treatment with sodium bisulfate conversion, the ctDNA quantity will decrease, sometimes with <50% remaining.122 This amount is far beyond the discriminatory threshold of quantitative methylation-specific PCR (qMSP), a technology applied in most previous studies,123 making it difficult to find a robust biomarker. Today, techniques with remarkably higher discriminatory abilities, including digital PCR and next-generation sequencing, are applied.

Another crucial shortcoming of methylation biomarker is insufficient specificity. cfDNA methylation levels are affected by various factors, including age, gender, and smoking history.124,125 Notably, although significantly related to lung cancer, smoking itself strongly changes gene methylation status. Some biomarkers believed to be cancer-specific (such as SHOX2,126 RASSF1A,71 p16,75 and NISCH120) have proven to be impacted by smoking. Choosing proper control groups with matched demographic characteristics is crucial for further studies. Additionally, the application of bioinformatics to detect these signals may be helpful to eliminate these confounding factors. These approaches, along with combining multiple biomarkers, may enable a highly reliable test for lung cancer early diagnosis.

Conclusions

In this review, we focused on ctDNA methylation as a promising diagnostic approach for lung cancer. It could serve as a surrogate for tissue biopsy in patients with candidate cancerous regions identified via LDCT, making this diagnostic process less uncomfortable and expensive. Besides plasma and serum, more attention should be paid to other samples in future studies, including pleural effusion, sputum, and bronchoalveolar lavage fluid. Although unlikely to replace the existing gold standard within a short time frame, ctDNA methylation does provide a possible alternative for cancer diagnosis and surveillance in the future, particularly in the context of ctDNA.

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Conflict of interest statement

None declared.

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