Precision Clinical Medicine, 2(1), 2019, 45–56

doi: 10.1093/pcmedi/pbz003
Advance Access Publication Date: 15 March 2019
Review

REVIEW

Applying circulating tumor DNA methylation in the diagnosis of lung cancer

Lei Li[1,2], Kai Fu[1], Wenyu Zhou[1] and Michael Snyder[1,*]

[1]Department of Genetics, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, CA 94305, USA, and [2]Department of Pulmonary and Critical Care Medicine, West China Hospital, Sichuan University, 37 Guoxuexiang, Chengdu 610041, China

*Correspondence: Michael Snyder, mpsnyder@stanford.edu

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]

Received: 17 December 2018; **Revised:** 17 January 2019; **Accepted:** 14 March 2019

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.[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.[10,11] cfDNA are free DNA fragments circulating in the peripheral blood, ranging from 80 to 200 base pairs (bp),[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.[13–15]

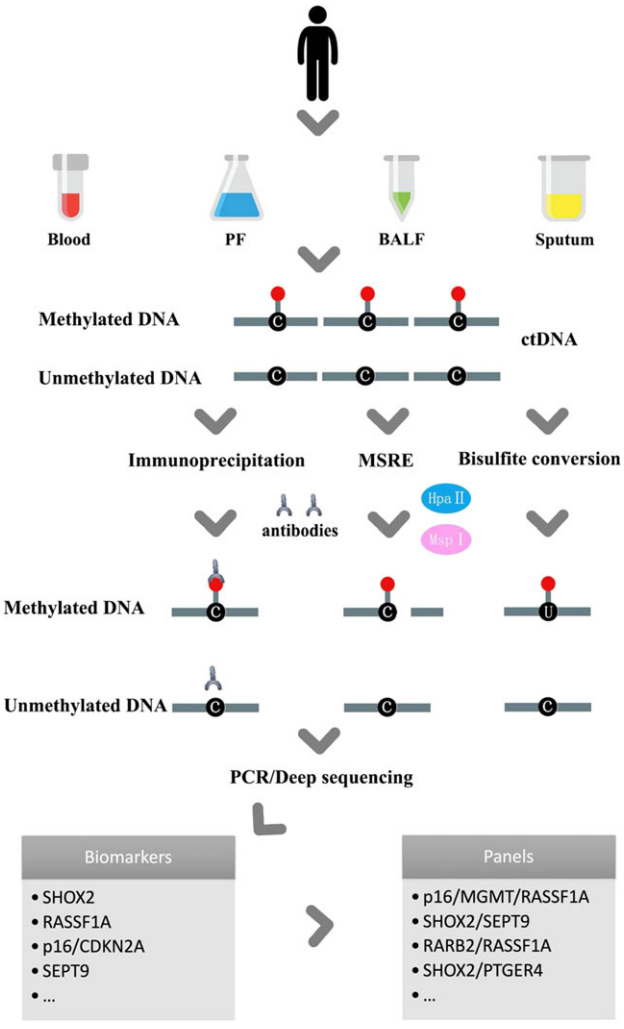

Figure 1. Discovery and detection of methylated biomarkers and panels. PF, pleural fluid; BALF, bronchoalveolar lavage fluid; MSRE, methyl-sensitive restriction enzyme; MGMT, O6-methylguanine-DNA methyltransferase; PTGER4, prostaglandin E receptor 4.

The first study of cfDNA in human blood was published in 1948.[16] Later, its potential clinical application was advanced by a clinical study that found cfDNA quantity was elevated in participants with diseases.[17] This phenomenon also occurs in human malignancy,[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,[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.[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.[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.[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.[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,[26] and helps in determining the origin of malignancy.[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.[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.[31]

Immunoprecipitation

In the 1980s, specific methylcytosine antibodies were identified. When combined with electron microscopy[32] or confocal fluorescence microscopy,[33] it was possible to detect methylation alterations quantitatively. These anti-5mC (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.[34] The affinity of anti-5mC 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)[35] and methylated-CpG island recovery assay (MIRA).[36]

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[37] (HpaII tiny fragment enrichment by ligation-mediated PCR) and methyl-sensitive restriction enzyme sequencing (MRE-seq)[38] 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.[39] 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.[37,40]

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).[41] 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 homeobox 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.[42–44] 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.[45] 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[46] in which 523 tissue samples obtained via bronchial aspirates were detected using differential methylation hybridization (DMH) and real-time PCR. In this study, mSHOX2 achieved a sensitivity of 68% and specificity of 95%. Later, this alteration was confirmed in Katja's study.[47] He put forward that such alteration might be associated with copy number amplification in the SHOX2 gene. Based on results in lung cancer tissue,[48,49] 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%).[50] In subsequent studies, the overall sensitivity was around 70%, but neither reported the discriminatory power in stage I cancer.[51,52] Recently, bronchial lavage[51,53–55] and pleural effusion[56,57] 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,[54] and 50% (2/4) in carcinoma *in situ*.[55] These studies strongly support a possible SHOX2 application in lung cancer early detection. However, it is noteworthy that

Table 1. Overview of primary methylation biomarkers from liquid biopsy in lung cancer diagnosis.

Author (year)	Sample types	Method	No. of cases	TNM stage (1/2/3/4/unknown)	No. of controls	Sensitivity, %	Specificity, %
SHOX2							
Kneip (2011)[50] (training group)	Plasma	RT-PCR	20	0/0/0/20/0	20	75	95
Kneip (2011)[50] (validation group)	Plasma	RT-PCR	188	37/29/53/42/27	155	60	90
Dietrich (2012)[53]	Bronchial lavage	RT-PCR	125	NA	125	78	96
Ilse (2013)[57]	Pleural effusion	RT-PCR	472	NA	798	39.5	96.2
Konecny (2016)[51]	Bronchial lavage	RT-PCR	38	1/4/8/22/3	31	83.78	84.62
Konecny (2016)[51]	Blood	RT-PCR	38	1/4/8/22/3	31	64.52	78.57
Ren (2017)[55]	Bronchial lavage	Sanger sequencing/RT-PCR	123	51/13/19/25/15	130	64.2	92.3
RASSF1A							
Ramirez (2003)[68]	Serum	MSP	50	6/11/18/5/10	0	34	NA
Rykova (2004)[69]	Plasma	MSP	9	NA	16	44	100
Belinsky (2005)[75]	Plasma	MSP	44	NA	195	7	98
Wang (2006)[70]	Plasma	MSP	63	NA	22	82	93
Hsu (2007)[71]	Plasma	qMSP	63	Stage 1–2: 41 Stage 3–4: 21 Unknown: 1	36	39	90
Ponomaryova (2013)[127]	Blood	qMSP	60	Stage 1–2: 20 Stage 3: 40	33	66	57
Zhang (2011)[72]	Plasma	MSP	110	Stage 1 & 2	50	36.36	92
Gao (2015)[73]	Serum	qMSP	40	40/0/0/0/0	36	52.5	97
Gao (2015)[73]	Plasma	qMSP	58	58/0/0/0/0	54	43.1	96
Ren (2017)[55]	Bronchial lavage	Sanger sequencing/RT-PCR	123	51/13/19/25/15	130	50.4	96.2
p16/CDKN2A							
Esteller (1999)[82]	Serum	MSP	22	10/3/8/1/0	0	13	NA
An (2002)[85]	Plasma	Seminested MSP	105	Stage 1: 29 Stage 2: 54 Stage 3–4: 22	0	73.3	NA
Bearzatto (2002)[83]	Plasma	F-MSP	35	28/4/3/0/0	15	40	100
Belinsky (2005)[75]	Plasma	MSP	44	NA	195	25	88
Belinsky (2005)[75]	Sputum	MSP	44	NA	112	19	75
Wang (2006)[70]	Plasma	MSP	63	NA	22	61	92
Wang (2006)[70]	Sputum	MSP	79	NA	22	65	83
Hsu (2007)[71]	Plasma	qMSP	63	Stage 1–2: 41 Stage 3–4: 21 Unknown: 1	36	38	91
Zhang (2011)[72]	Plasma	MSP	110	Stage 1 & 2	50	22.73	92
Xiao (2014)[86]	Plasma	F-MSP	30	Stage 1–2: 24 Stage 3: 6	30	50	100
Xiao (2014)[86]	EBC	F-MSP	30	Stage 1–2: 24 Stage 3: 6	30	40	100
SEPT9							
Powrozek (2014)[100]	Plasma	RT-PCR	70	0/7/23/17/23	100	44.3	96
APC							
Usadel (2002)[103]	Serum/plasma	Semiquantitative F-MSP	89	NA	50	47	100

Continued

Table 1. Continued

Author (year)	Sample types	Method	No. of cases	TNM stage (1/2/3/4/unknown)	No. of controls	Sensitivity, %	Specificity, %
Rykova (2004)[69]	Plasma	MSP	9	NA	16	30	100
Zhang (2011)[72]	Plasma	MSP	110	Stage 1 & 2	50	47.27	90
Zhai (2014)[74]	Plasma	MSP	42	Stage 1–2: 6 Stage 3–4: 36	40	52.4	100
Gao (2015)[73]	Serum	qMSP	40	40/0/0/0/0	36	42.5	94
Gao (2015)[73]	Plasma	qMSP	58	58/0/0/0/0	54	24.1	98
Ali (2017)[102]	Serum	MSP	160	0/0/74/86/0	70	52.5	85.7
RARB							
Wang (2006)[70]	Plasma	MSP	63	NA	22	62	93
Wang (2006)[70]	Sputum	MSP	79	NA	22	66	88
Hsu (2007)[71]	Plasma	qMSP	63	Stage 1–2: 41 Stage 3–4: 21 Unknown: 1	36	37	83
Ostrow (2010)[109] (evaluation set)	Plasma	F-MSP	13	NA	24	38	96
Ostrow (2010)[109] (independent set)	Plasma	F-MSP	70	49/2/20/4/5	80	16	96.3
Zhang (2011)[72]	Plasma	MSP	110	Stage 1 & 2	50	20	94
Ponomaryova (2011)[107]	Plasma	qMSP	52	Stage 1–2: 25 Stage 3: 27	26	63	51
SOX17							
Hulbert (2016)[111]	Plasma	qMSP	150	136/14/0/0/0	60	73	84
Hulbert (2016)[111]	Sputum	qMSP	150	136/14/0/0/0	60	84	88
Balgkouranidou (2016)[112] (operable NSCLC)	Plasma	MSP	49	Stage 1: 14 Stage 2–3: 29 Unknown: 6	49	56.2	98
Balgkouranidou (2016)[112] (advanced NSCLC)	Plasma	MSP	74	0/0/0/74/0	49	36.4	98
CDH13							
Wang (2006)[70]	Plasma	MSP	63	NA	22	74	97
Hsu (2007)[71]	Plasma	qMSP	63	Stage 1–2: 41 Stage 3–4: 21 Unknown: 1	36	34	84
Zhai (2014)[74]	Plasma	MSP	42	Stage 1–2: 6 Stage 3–4: 36	40	54.8	100
DAPK							
Esteller (1999)[82]	Serum	MSP	22	10/3/8/1/0	0	18	NA
Ali (2017)[102]	Serum	MSP	160	0/0/74/86/0	70	52.5	85.7
Yang (2018)[113]	Serum	MSP	117	NA	115	27.4	100

APC, adenomatous polyposis coli; CDH13, cadherin13; CDKN2A, cyclin-dependent kinase inhibitor 2A; DAPK, Death-associated protein kinase; EBC, exhaled breath condensate; F-MSP, fluorescent MSP; MSP, methylation-specific PCR; NA, not available; p16, p16 gene; PCR, polymerase chain reaction; qMSP, quantitative MSP; RARB, retinoic acid receptor-β2; RASSF1A, ras association domain family 1A; RT-PCR, real-time PCR; SEPT9, septin 9; SHOX2, short stature homeobox gene 2; SOX17, SRY-box containing gene 17.

mSHOX2 is not a lung cancer-specific biomarker. It has been reported in other cancer types, including head and neck squamous cell carcinoma (HNSCC),[58,59] colorectal cancer (CRC),[60] and ovary cancer.[61] 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 (RASSF), which comprises 10 genes (RASSF1 to RASSF10). These genes play critical roles in regulating cell cycle control, apoptosis, and microtubule stabilization.[62–64] Hypermethylation in their CpG island promoters has been reported to induce multiple cancers.[62] The RASSF1 gene is located on chromosome 3 (3p21.3). It encodes eight homologous proteins, including RASSF1A.[65] Interacting with tubulin, RASSF1A mainly regulates mitotic progression by stabilizing microtubules in cell.[66,67]

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,

specificity could not be achieved as no control samples were analyzed.[68] 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).[69] The low sensitivity in plasma remained a major issue for RASSF1A in subsequent studies,[70–74] and it was even reported that sensitivity in sputum (13/44, 25%) came much higher than in plasma (3/44, 7%).[75] 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.[76] 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%.[54,55] As for mSHOX2, mRASSF1A's diagnostic potential has also been reported in other cancers.[69,76]

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).[77] Hypermethylation in the p16 promoter suppresses gene transcription, resulting in loss of inhibition of cell cycle progression.[78–80] This epigenetic alteration has been reported in various malignancies.[81]

A relationship between p16 hypermethylation and lung cancer was first found in tissue samples.[48,49,72,82–85] 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%).[84] 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.[71,72,82] 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.[85] Intriguingly, it was more robust in SCC (77.8%) than in adenocarcinoma (ADC) (45.5%). Bearzatto and colleagues[83] 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.[86] The high specificity of p16 methylation was also determined in various studies,[70–72] further supporting its diagnostic value in lung cancer early detection. Notably, sputum[70,75] and exhaled breath condensate[86] 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,[87–89] 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,[90–92] T cell lymphoma,[93] and some other malignancies.[94] The most important clinical application for SEPT9 methylation is in CRC.[60,61,76,95,96] Epi proColon,[97,98] 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[99] and HNSCC.[58,59]

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%).[100] Intriguingly, detection in pleural effusion[56] and ascites[61] 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,[101] has attracted much recent attention as its hypermethylation in plasma and serum proved to be around 50% in lung cancer.[69,72,73,102,103] Notably, this prevalence was higher in lung ADC than in SCC,[104] making it a possible ADC-specific biomarker. APC hypermethylation is not lung cancer-specific, it has been detected in other cancers.[76,105] Retinoic acid receptor-β2 (RARB), another tumor suppressor gene,[106] is hypermethylated both in lung cancer tissue and plasma,[70–72,107–109] and is significantly associated with pathological types and grades.[110] Similar finding have also been reported for the SRY-box containing gene 17 (SOX17),[76,111,112] cadherin13 (CDH13),[70,71,74] and Death-associated protein kinase (DAPK).[82,102,113,114]

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)[75]	p16/MGMT/RASSF1A	Plasma	MSP	44	NA	195	36	81
Belinsky (2005)[75]	p16/MGMT/RASSF1A	Plasma	MSP	44	NA	112	38	89
Hsu (2007)[71]	BLU/CDH13/FHIT/p16/RARB/RASSF1A	Plasma	qMSP	63	Stage 1–2: 41 Stage 3–4: 21 Unknown: 1	36	73	82
Ostrow (2010)[109]	KIF1A/DCC/RARB/NISCH	Plasma	F-MSP	70	49//2/20/4/5	80	73	71
Zhang (2011)[72]	APC/RASSF1A/CDH13/KLK10/DLEC1	Plasma	MSP	110	Stage 1 & 2	50	83.64	74
Begum (2011)[128]	APC/CDH1/MGMT/DCC/RASSF1A/AIM1	Serum	F-MSP	76	41/17/11/5/2	30	84.2	56.7
Dietrich (2013)[56]	SHOX2/SEPT9	Pleural effusion	qPCR	8	NA	56	14	100
Ponomaryova (2013)[127]	RARB2/RASSF1A	Blood	qMSP	60	Stage 1–2: 20 Stage 3: 40	33	87	75
Wielscher (2015)[129]	HOXD10/PAX9/PTPRN2/STAG3	Serum/plasma	MSRE+qPCR	23	Stage 1–2: 8 Stage 3–4: 12 Unknown: 3	23	97	73
Hulbert (2016)[111]	CDO1/TAC1/SOX17	Plasma	qMSP	150	136/14/0/0/0	60	93	62
Hulbert (2016)[111]	HOXA7/TAC1/SOX17	Sputum	qMSP	150	136/14/0/0/0	60	98	71
Weiss (2016)[52] (specificity fixed)	SHOX2/PTGER4	Plasma	RT-PCR	50	12/11/16/11/0	122	67	90
Weiss (2016)[52] (sensitivity fixed)	SHOX2/PTGER4	Plasma	RT-PCR	50	12/11/16/11/0	122	90	73
Zhang (2017)[54]	SHOX2/RASSF1A	Bronchial lavage	Sanger sequencing	284	28/30/133/93/0	38	81	97.4
Ren (2017)[55]	SHOX2/RASSF1A	Bronchial lavage	Sanger sequencing/RT-PCR	123	51/13/19/25/15	130	71.5	70
Ooki (2017)[115]	CDO1/HOXA9/AJAP1/PTGDR/UNCX/MARCH11	Serum	qMSP	43	43/0/0/0/0	42	72.1	71.4
Nunes (2018)[76]	SCGB3A1/SEPT9/SOX17	Plasma	qMSP	73	NA	103	39.4	85.1
Nunes (2018)[76]	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; HOXA9, homeobox A9; HOXD10, homeobox D10; KIF1A, kinesin family member 1A; KLK10, kallikrein-related peptidase 10; MARCH11, membrane-associated ring-CH-type finger 11; MGMT, O^6-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).[52] 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.[111] Favorable results were also achieved in stage 1A lung cancer: a six-gene panel (CDO1, HOXA9, AJAP1, PTGDR, UNCX, and MARCH11) 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.[84] 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,[48] RASSF1A,[71] p16,[75] and NISCH[126]) 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.

Acknowledgement

We thank John Vaughen from the Stanford Technical Communication Program for comments on the manuscript.

Conflict of interest statement

None declared.

References

1. Bray F, Ferlay J, Soerjomataram I, *et al.* Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018;**68**:394–424.
2. National Lung Screening Trial Research Team. Reduced lung-cancer mortality with low-dose computed tomographic screening. *N Engl J Med* 2011;**365**:395–409.
3. Ost D, Fein AM, Feinsilver SH. Clinical practice. The solitary pulmonary nodule. *N Engl J Med* 2003;**348**:2535–42.
4. Wiener RS, Wiener DC, Gould MK. Risks of Transthoracic Needle Biopsy: How High? *Clin Pulm Med* 2013;**20**:29–35.
5. Silvestri GA, Vachani A, Whitney D, *et al.* A Bronchial Genomic Classifier for the Diagnostic Evaluation of Lung Cancer. *N Engl J Med* 2015;**373**:243–51.
6. Rivera MP, Mehta AC, Wahidi MM. Establishing the diagnosis of lung cancer: Diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines. *Chest* 2013;**143**:e142S–65S.
7. Wang Memoli JS, Nietert PJ, Silvestri GA. Meta-analysis of guided bronchoscopy for the evaluation of the pulmonary nodule. *Chest* 2012;**142**:385–93.
8. Yates LR, Gerstung M, Knappskog S, *et al.* Subclonal diversification of primary breast cancer revealed by multiregion sequencing. *Nat Med* 2015;**21**:751–9.
9. Perakis S, Speicher MR. Emerging concepts in liquid biopsies. *BMC Med* 2017;**15**:75.
10. Wan JCM, Massie C, Garcia-Corbacho J, *et al.* Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat Rev Cancer* 2017;**17**:223–38.

11. Yoruker EE, Holdenrieder S, Gezer U. Blood-based biomarkers for diagnosis, prognosis and treatment of colorectal cancer. *Clin Chim Acta* 2016;**455**:26–32.
12. Pos O, Biro O, Szemes T, *et al*. Circulating cell-free nucleic acids: characteristics and applications. *Eur J Hum Genet* 2018;**26**:937–45.
13. Crowley E, Di Nicolantonio F, Loupakis F, *et al*. Liquid biopsy: monitoring cancer-genetics in the blood. *Nat Rev Clin Oncol* 2013;**10**:472–84.
14. Jung K, Fleischhacker M, Rabien A. Cell-free DNA in the blood as a solid tumor biomarker—a critical appraisal of the literature. *Clin Chim Acta* 2010;**411**:1611–24.
15. Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer* 2011;**11**:426–37.
16. Mandel P, Metais P. Les acides nucléiques du plasma sanguin chez l'homme. *C R Seances Soc Biol Fil* 1948;**142**:241–3.
17. Koffler D, Agnello V, Winchester R, *et al*. The occurrence of single-stranded DNA in the serum of patients with systemic lupus erythematosus and other diseases. *J Clin Invest* 1973;**52**:198–204.
18. Shapiro B, Chakrabarty M, Cohn EM, *et al*. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. *Cancer* 1983;**51**:2116–20.
19. Kwapisz D. The first liquid biopsy test approved. Is it a new era of mutation testing for non-small cell lung cancer? *Ann Transl Med* 2017;**5**:46.
20. Guo S, Diep D, Plongthongkum N, *et al*. Identification of methylation haplotype blocks aids in deconvolution of heterogeneous tissue samples and tumor tissue-of-origin mapping from plasma DNA. *Nat Genet* 2017;**49**:635–42.
21. Xu RH, Wei W, Krawczyk M, *et al*. Circulating tumour DNA methylation markers for diagnosis and prognosis of hepatocellular carcinoma. *Nat Mater* 2017;**16**:1155–61.
22. Straussman R, Nejman D, Roberts D, *et al*. Developmental programming of CpG island methylation profiles in the human genome. *Nat Struct Mol Biol* 2009;**16**:564–71.
23. Riggs AD. X inactivation, differentiation, and DNA methylation. *Cytogenet Cell Genet* 1975;**14**:9–25.
24. Bird A, Taggart M, Frommer M, *et al*. A fraction of the mouse genome that is derived from islands of nonmethylated, CpG-rich DNA. *Cell* 1985;**40**:91–9.
25. Portela A, Esteller M. Epigenetic modifications and human disease. *Nat Biotechnol* 2010;**28**:1057–68.
26. Robertson KD. DNA methylation and human disease. *Nat Rev Genet* 2005;**6**:597–610.
27. Sun K, Jiang P, Chan KC, *et al*. Plasma DNA tissue mapping by genome-wide methylation sequencing for noninvasive prenatal, cancer, and transplantation assessments. *Proc Natl Acad Sci USA* 2015;**112**:E5503–12.
28. Lehmann-Werman R, Neiman D, Zemmour H, *et al*. Identification of tissue-specific cell death using methylation patterns of circulating DNA. *Proc Natl Acad Sci USA* 2016;**113**:E1826–34.
29. Moran S, Martinez-Cardus A, Sayols S, *et al*. Epigenetic profiling to classify cancer of unknown primary: a multicentre, retrospective analysis. *Lancet Oncol* 2016;**17**:1386–95.
30. Kuo KC, McCune RA, Gehrke CW, *et al*. Quantitative reversed-phase high performance liquid chromatographic determination of major and modified deoxyribonucleosides in DNA. *Nucleic Acids Res* 1980;**8**:4763–76.
31. Harrison A, Parle-McDermott A. DNA methylation: a timeline of methods and applications. *Front Genet* 2011;**2**:74.
32. Adouard V, Dante R, Niveleau A, *et al*. The accessibility of 5-methylcytosine to specific antibodies in double-stranded DNA of Xanthomonas phage XP12. *Eur J Biochem* 1985;**152**:115–21.
33. Oakeley EJ, Podesta A, Jost JP. Developmental changes in DNA methylation of the two tobacco pollen nuclei during maturation. *Proc Natl Acad Sci USA* 1997;**94**:11721–5.
34. Weber M, Davies JJ, Wittig D, *et al*. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet* 2005;**37**:853–62.
35. Gebhard C, Schwarzfischer L, Pham TH, *et al*. Genome-wide profiling of CpG methylation identifies novel targets of aberrant hypermethylation in myeloid leukemia. *Cancer Res* 2006;**66**:6118–28.
36. Rauch T, Li H, Wu X, *et al*. MIRA-assisted microarray analysis, a new technology for the determination of DNA methylation patterns, identifies frequent methylation of homeodomain-containing genes in lung cancer cells. *Cancer Res* 2006;**66**:7939–47.
37. Thu KL, Pikor LA, Kennett JY, *et al*. Methylation analysis by DNA immunoprecipitation. *J Cell Physiol* 2010;**222**:522–31.
38. Maunakea AK, Nagarajan RP, Bilenky M, *et al*. Conserved role of intragenic DNA methylation in regulating alternative promoters. *Nature* 2010;**466**:253–7.
39. Frommer M, McDonald LE, Millar DS, *et al*. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci USA* 1992;**89**:1827–31.
40. Beck S, Rakyan VK. The methylome: approaches for global DNA methylation profiling. *Trends Genet* 2008;**24**:231–7.
41. Sun Z, Cunningham J, Slager S, *et al*. Base resolution methylome profiling: considerations in platform selection, data preprocessing and analysis. *Epigenomics* 2015;**7**:813–28.
42. Blaschke RJ, Hahurij ND, Kuijper S, *et al*. Targeted mutation reveals essential functions of the homeodomain transcription factor Shox2 in sinoatrial and pacemaking development. *Circulation* 2007;**115**:1830–8.
43. Blaschke RJ, Monaghan AP, Schiller S, *et al*. SHOT, a SHOX-related homeobox gene, is implicated in craniofacial, brain, heart, and limb development. *Proc Natl Acad Sci USA* 1998;**95**:2406–11.
44. Clement-Jones M, Schiller S, Rao E, *et al*. The short stature homeobox gene SHOX is involved in skeletal abnormalities in Turner syndrome. *Hum Mol Genet* 2000;**9**:695–702.
45. Marchini A, Rappold G, Schneider KU. SHOX at a glance: from gene to protein. *Arch Physiol Biochem* 2007;**113**:116–23.
46. Schmidt B, Liebenberg V, Dietrich D, *et al*. SHOX2 DNA methylation is a biomarker for the diagnosis of lung cancer based on bronchial aspirates. *BMC Cancer* 2010;**10**:600.
47. Schneider KU, Dietrich D, Fleischhacker M, *et al*. Correlation of SHOX2 Gene Amplification and DNA Methylation in Lung Cancer Tumors. *BMC Cancer* 2011;**11**:102.
48. Ilse P, Biesterfeld S, Pomjanski N, *et al*. Analysis of SHOX2 methylation as an aid to cytology in lung cancer diagnosis. *Cancer Genomics Proteomics* 2014;**11**:251–8.
49. Ni S, Ye M, Huang T. Short stature homeobox 2 methylation as a potential noninvasive biomarker in bronchial aspirates for lung cancer diagnosis. *Oncotarget* 2017;**8**:61253–63.
50. Kneip C, Schmidt B, Seegebarth A, *et al*. SHOX2 DNA Methylation Is a Biomarker for the Diagnosis of Lung Cancer in Plasma. *J Thorac Oncol* 2011;**6**:1632–8.

51. Konecny M, Markus J, Waczulikova I, *et al*. The value of SHOX2 methylation test in peripheral blood samples used for the differential diagnosis of lung cancer and other lung disorders. *Neoplasma* 2016;**63**:246–53.
52. Weiss G, Schlegel A, Kottwitz D, *et al*. Validation of the SHOX2/PTGER4 DNA Methylation Marker Panel for Plasma-Based Discrimination between Patients with Malignant and Nonmalignant Lung Disease. *J Thorac Oncol* 2016;**12**:77–84.
53. Dietrich D, Kneip C, Raji O, *et al*. Performance evaluation of the DNA methylation biomarker SHOX2 for the aid in diagnosis of lung cancer based on the analysis of bronchial aspirates. *Int J Oncol* 2012;**40**:825–32.
54. Zhang C, Yu W, Wang L, *et al*. DNA Methylation Analysis of the SHOX2 and RASSF1A Panel in Bronchoalveolar Lavage Fluid for Lung Cancer Diagnosis. *J Cancer* 2017;**8**:3585–91.
55. Ren M, Wang C, Sheng D, *et al*. Methylation analysis of SHOX2 and RASSF1A in bronchoalveolar lavage fluid for early lung cancer diagnosis. *Ann Diagn Pathol* 2017;**27**:57–61.
56. Dietrich D, Jung M, Puetzer S, *et al*. Diagnostic and prognostic value of SHOX2 and SEPT9 DNA methylation and cytology in benign, paramalignant and malignant pleural effusions. *PLoS One* 2013;**8**:e84225.
57. Ilse P, Biesterfeld S, Pomjanski N, *et al*. SHOX2 DNA methylation is a tumour marker in pleural effusions. *Cancer Genomics Proteomics* 2013;**10**:217–23.
58. de Vos L, Gevensleben H, Schröck A, *et al*. Comparison of quantification algorithms for circulating cell-free DNA methylation biomarkers in blood plasma from cancer patients. *Clin Epigenetics* 2017;**9**:125.
59. Schrock A, Leisse A, de Vos L, *et al*. Free-Circulating Methylated DNA in Blood for Diagnosis, Staging, Prognosis, and Monitoring of Head and Neck Squamous Cell Carcinoma Patients: An Observational Prospective Cohort Study. *Clin Chem* 2017;**63**:1288–96.
60. Bergheim J, Semaan A, Gevensleben H, *et al*. Potential of quantitative SEPT9 and SHOX2 methylation in plasmatic circulating cell-free DNA as auxiliary staging parameter in colorectal cancer: a prospective observational cohort study. *Br J Cancer* 2018;**118**:1217–28.
61. Jung M, Putzer S, Gevensleben H, *et al*. Diagnostic and prognostic value of SHOX2 and SEPT9 DNA methylation and cytology in benign, paramalignant, and malignant ascites. *Clin Epigenetics* 2016;**8**:24.
62. van der Weyden L, Adams DJ. The Ras-association domain family (RASSF) members and their role in human tumourigenesis. *Biochim Biophys Acta* 2007;**1776**:58–85.
63. Dammann R, Li C, Yoon JH, *et al*. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet* 2000;**25**:315–9.
64. Ponting CP, Benjamin DR. A novel family of Ras-binding domains. *Trends Biochem Sci* 1996;**21**:422–5.
65. Dammann R, Schagdarsurengin U, Strunnikova M, *et al*. Epigenetic inactivation of the Ras-association domain family 1 (RASSF1A) gene and its function in human carcinogenesis. *Histol Histopathol* 2003;**18**:665–77.
66. Liu L, Tommasi S, Lee DH, *et al*. Control of microtubule stability by the RASSF1A tumor suppressor. *Oncogene* 2003;**22**:8125–36.
67. Vos MD, Martinez A, Elam C, *et al*. A role for the RASSF1A tumor suppressor in the regulation of tubulin polymerization and genomic stability. *Cancer Res* 2004;**64**:4244–50.
68. Ramirez JL, Sarries C, de Castro PL, *et al*. Methylation patterns and K-ras mutations in tumor and paired serum of resected non-small-cell lung cancer patients. *Cancer Lett* 2003;**193**:207–16.
69. Rykova EY, Skvortsova TE, Laktionov PP, *et al*. Investigation of tumor-derived extracellular DNA in blood of cancer patients by methylation-specific PCR. *Nucleosides Nucleotides Nucleic Acids* 2004;**23**:855–9.
70. Wang YC, Hsu HS, Chen TP, *et al*. Molecular diagnostic markers for lung cancer in sputum and plasma. *Ann N Y Acad Sci* 2006;**1075**:179–84.
71. Hsu HS, Chen TP, Hung CH, *et al*. Characterization of a multiple epigenetic marker panel for lung cancer detection and risk assessment in plasma. *Cancer* 2007;**110**:2019–26.
72. Zhang Y, Wang R, Song H, *et al*. Methylation of multiple genes as a candidate biomarker in non-small cell lung cancer. *Cancer Lett* 2011;**303**:21–8.
73. Gao L, Xie E, Yu T, *et al*. Methylated APC and RASSF1A in multiple specimens contribute to the differential diagnosis of patients with undetermined solitary pulmonary nodules. *J Thorac Dis* 2015;**7**:422–32.
74. Zhai X, Li SJ. Methylation of RASSF1A and CDH13 genes in individualized chemotherapy for patients with non-small cell lung cancer. *Asian Pac J Cancer Prev* 2014;**15**:4925–8.
75. Belinsky SA, Klinge DM, Dekker JD, *et al*. Gene promoter methylation in plasma and sputum increases with lung cancer risk. *Clin Cancer Res* 2005;**11**:6505–11.
76. Nunes SP, Moreira-Barbosa C, Salta S, *et al*. Cell-Free DNA Methylation of Selected Genes Allows for Early Detection of the Major Cancers in Women. *Cancers (Basel)* 2018;**10**:E357.
77. Baylin SB, Herman JG, Graff JR, *et al*. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res* 1998;**72**:141–96.
78. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993;**366**:704–7.
79. Strauss M, Lukas J, Bartek J. Unrestricted cell cycling and cancer. *Nat Med* 1995;**1**:1245–6.
80. Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell* 1995;**81**:323–30.
81. Park JW, Baek IH, Kim YT. Preliminary study analyzing the methylated genes in the plasma of patients with pancreatic cancer. *Scand J Surg* 2012;**101**:38–44.
82. Esteller M, Sanchez-Cespedes M, Rosell R, *et al*. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res* 1999;**59**:67–70.
83. Bearzatto A, Conte D, Frattini M, *et al*. p16(INK4A) Hypermethylation detected by fluorescent methylation-specific PCR in plasmas from non-small cell lung cancer. *Clin Cancer Res* 2002;**8**:3782–7.
84. Belinsky SA, Nikula KJ, Palmisano WA, *et al*. Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. *Proc Natl Acad Sci USA* 1998;**95**:11891–6.
85. An Q, Liu Y, Gao Y, *et al*. Detection of p16 hypermethylation in circulating plasma DNA of non-small cell lung cancer patients. *Cancer Lett* 2002;**188**:109–14.
86. Xiao P, Chen JR, Zhou F, *et al*. Methylation of P16 in exhaled breath condensate for diagnosis of non-small cell lung cancer. *Lung Cancer* 2014;**83**:56–60.
87. Hall PA, Russell SE. The pathobiology of the septin gene family. *J Pathol* 2004;**204**:489–505.
88. Connolly D, Abdesselam I, Verdier-Pinard P, *et al*. Septin roles in tumorigenesis. *Biol Chem* 2011;**392**:725–38.

89. Estey MP, Di Ciano-Oliveira C, Froese CD, *et al*. Mitotic regulation of SEPT9 protein by cyclin-dependent kinase 1 (Cdk1) and Pin1 protein is important for the completion of cytokinesis. *J Biol Chem* 2013;**288**:30075–86.
90. Kalikin LM, Qu X, Frank TS, *et al*. Detailed deletion analysis of sporadic breast tumors defines an interstitial region of allelic loss on 17q25. *Genes Chromosomes Cancer* 1996;**17**: 64–8.
91. Kalikin LM, Frank TS, Svoboda-Newman SM, *et al*. A region of interstitial 17q25 allelic loss in ovarian tumors coincides with a defined region of loss in breast tumors. *Oncogene* 1997;**14**:1991–4.
92. Kalikin LM, Sims HL, Petty EM. Genomic and expression analyses of alternatively spliced transcripts of the MLL septin-like fusion gene (MSF) that map to a 17q25 region of loss in breast and ovarian tumors. *Genomics* 2000;**63**:165–72.
93. Sorensen AB, Warming S, Fuchtbauer EM, *et al*. Alternative splicing, expression, and gene structure of the septin-like putative proto-oncogene Sint1. *Gene* 2002;**285**:79–89.
94. Montagna C, Lyu MS, Hunter K, *et al*. The Septin 9 (MSF) gene is amplified and overexpressed in mouse mammary gland adenocarcinomas and human breast cancer cell lines. *Cancer Res* 2003;**63**:2179–87.
95. Semaan A, van Ellen A, Meller S, *et al*. SEPT9 and SHOX2 DNA methylation status and its utility in the diagnosis of colonic adenomas and colorectal adenocarcinomas. *Clin Epigenetics* 2016;**8**:100.
96. Behrouz Sharif S, Hashemzadeh S, Mousavi Ardehaie R, *et al*. Detection of aberrant methylated SEPT9 and NTRK3 genes in sporadic colorectal cancer patients as a potential diagnostic biomarker. *Oncol Lett* 2016;**12**:5335–43.
97. Lamb YN, Dhillon S. Epi proColon((R)) 2.0 CE: A Blood-Based Screening Test for Colorectal Cancer. *Mol Diagn Ther* 2017; **21**:225–32.
98. Potter NT, Hurban P, White MN, *et al*. Validation of a real-time PCR-based qualitative assay for the detection of methylated SEPT9 DNA in human plasma. *Clin Chem* 2014; **60**:1183–91.
99. Branchi V, Schaefer P, Semaan A, *et al*. Promoter hypermethylation of SHOX2 and SEPT9 is a potential biomarker for minimally invasive diagnosis in adenocarcinomas of the biliary tract. *Clin Epigenetics* 2016;**8**:133.
100. Powrozek T, Krawczyk P, Kucharczyk T, *et al*. Septin 9 promoter region methylation in free circulating DNA-potential role in noninvasive diagnosis of lung cancer: preliminary report. *Med Oncol* 2014;**31**:917.
101. Fodde R, Kuipers J, Rosenberg C, *et al*. Mutations in the APC tumour suppressor gene cause chromosomal instability. *Nat Cell Biol* 2001;**3**:433–8.
102. Ali A, Kumar S, Kakaria VK, *et al*. Detection of Promoter DNA Methylation of APC, DAPK, and GSTP1 Genes in tissue Biopsy and Matched Serum of Advanced-Stage Lung Cancer Patients. *Cancer Invest* 2017;**35**:423–30.
103. Usadel H, Brabender J, Danenberg KD, *et al*. Quantitative adenomatous polyposis coli promoter methylation analysis in tumor tissue, serum, and plasma DNA of patients with lung cancer. *Cancer Res* 2002;**62**:371–5.
104. Huang T, Li J, Zhang C, *et al*. Distinguishing Lung Adenocarcinoma from Lung Squamous Cell Carcinoma by Two Hypomethylated and Three Hypermethylated Genes: A Meta-Analysis. *PLoS One* 2016;**11**:e0149088.
105. Radpour R, Barekati Z, Kohler C, *et al*. Hypermethylation of tumor suppressor genes involved in critical regulatory pathways for developing a blood-based test in breast cancer. *PLoS One* 2011;**6**:e16080.
106. Fan J, Eastham L, Varney ME, *et al*. Silencing and re-expression of retinoic acid receptor beta2 in human melanoma. *Pigment Cell Melanoma Res* 2010;**23**:419–29.
107. Ponomaryova AA, Rykova EY, Cherdyntseva NV, *et al*. RARbeta2 gene methylation level in the circulating DNA from blood of patients with lung cancer. *Eur J Cancer Prev* 2011;**20**:453–5.
108. Feng H, Zhang Z, Qing X, *et al*. Promoter methylation of APC and RAR-beta genes as prognostic markers in non-small cell lung cancer (NSCLC). *Exp Mol Pathol* 2016;**100**:109–13.
109. Ostrow KL, Hoque MO, Loyo M, *et al*. Molecular analysis of plasma DNA for the early detection of lung cancer by quantitative methylation-specific PCR. *Clin Cancer Res* 2010; **16**:3463–72.
110. Walter RFH, Rozynek P, Casjens S, *et al*. Methylation of L1RE1, RARB, and RASSF1 function as possible biomarkers for the differential diagnosis of lung cancer. *PLoS One* 2018; **13**:e0195716.
111. Hulbert A, Jusue-Torres I, Stark A, *et al*. Early Detection of Lung Cancer using DNA Promoter Hypermethylation in Plasma and Sputum. *Clin Cancer Res* 2016;**23**:1998–2005.
112. Balgkouranidou I, Chimonidou M, Milaki G, *et al*. SOX17 promoter methylation in plasma circulating tumor DNA of patients with non-small cell lung cancer. *Clin Chem Lab Med* 2016;**54**:1385–93.
113. Yang XY, Zhang J, Yu XL, *et al*. Death-associated protein kinase promoter methylation correlates with clinicopathological and prognostic features in nonsmall cell lung cancer patients: A cohort study. *J Cancer Res Ther* 2018;**14**:S65–s71.
114. Zhang J, Yu XL, Zheng GF, *et al*. DAPK promoter methylation status correlates with tumor metastasis and poor prognosis in patients with non-small cell lung cancer. *Cancer Biomark* 2015;**15**:609–17.
115. Ooki A, Maleki Z, Tsay JJ, *et al*. A Panel of Novel Detection and Prognostic Methylated DNA Markers in Primary Non-Small Cell Lung Cancer and Serum DNA. *Clin Cancer Res* 2017;**23**:7141–52.
116. Chen R, Xia L, Tu K, *et al*. Longitudinal personal DNA methylome dynamics in a human with a chronic condition. *Nat Med* 2018;**24**:1930–9.
117. Hao X, Luo H, Krawczyk M, *et al*. DNA methylation markers for diagnosis and prognosis of common cancers. *Proc Natl Acad Sci USA* 2017;**114**:7414–9.
118. Phallen J, Sausen M, Adleff V, *et al*. Direct detection of early-stage cancers using circulating tumor DNA. *Sci Transl Med* 2017;**9**:eaan2415.
119. Worm Ørntoft MB. Review of Blood-Based Colorectal Cancer Screening: How Far Are Circulating Cell-Free DNA Methylation Markers From Clinical Implementation? *Clin Colorectal Cancer* 2018;**17**:e415–e33.
120. Lui YY, Chik KW, Chiu RW, *et al*. Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. *Clin Chem* 2002;**48**:421–7.
121. Bettegowda C, Sausen M, Leary RJ, *et al*. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* 2014;**6**:224ra24.
122. Worm Ørntoft MB, Jensen SO, Hansen TB, *et al*. Comparative analysis of 12 different kits for bisulfite conversion of circulating cell-free DNA. *Epigenetics* 2017;**12**:626–36.
123. Chaudhuri AA, Binkley MS, Osmundson EC, *et al*. Predicting Radiotherapy Responses and Treatment Outcomes Through

Analysis of Circulating Tumor DNA. *Semin Radiat Oncol* 2015; **25**:305–12.
124. Liu F, Killian JK, Yang M, *et al*. Epigenomic alterations and gene expression profiles in respiratory epithelia exposed to cigarette smoke condensate. *Oncogene* 2010;**29**:3650–64.
125. Hannum G, Guinney J, Zhao L, *et al*. Genome-wide methylation profiles reveal quantitative views of human aging rates. *Mol Cell* 2013;**49**:359–67.
126. Ostrow KL, Michailidi C, Guerrero-Preston R, *et al*. Cigarette smoke induces methylation of the tumor suppressor gene NISCH. *Epigenetics* 2013;**8**:383–8.
127. Ponomaryova AA, Rykova EY, Cherdyntseva NV, *et al*. Potentialities of aberrantly methylated circulating DNA for diagnostics and post-treatment follow-up of lung cancer patients. *Lung Cancer* 2013;**81**:397–403.
128. Begum S, Brait M, Dasgupta S, *et al*. An epigenetic marker panel for detection of lung cancer using cell-free serum DNA. *Clin Cancer Res* 2011;**17**:4494–503.
129. Wielscher M, Vierlinger K, Kegler U, Ziesche R, Gsur A, Weinhausel A. Diagnostic Performance of Plasma DNA Methylation Profiles in Lung Cancer, Pulmonary Fibrosis and COPD. *EBioMedicine* 2015;**2**:929–36.