

# DNA methylation: its role in transcriptional regulation and association with lung cancer

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**Abstract:** DNA methylation is a fundamental biochemical modification that in conjunction with noncoding RNAs, histone modifications, and chromatin remodeling institutes the epigenetic machinery of mammalian cells. As a result of the second decade of intense epigenetic research and its role in human disease, substantial new mechanisms have been uncovered. However, it is well acknowledged that we have just scratched the tip of the iceberg. Epigenetic deregulation appears to be one of the foundations of major human diseases, including lung cancer, which is the most frequent cause of cancer-related deaths. Currently, significant effort is made to dissect the role of epigenetic deregulation in the development of lung cancer and utilize this knowledge in diagnostic and therapeutic applications. Taking advantage of the recent technologies in genomic research, many studies have been conducted to discover and validate abnormal DNA methylation patterns that may shed light on cancer development pathways and open new areas of potential clinical exploitation. In this article, we provide basic information of the DNA methylation involved in gene regulation and review the latest literature on potential relevant translational applications in lung cancer. Particular emphasis is given to the development and validation of DNA methylation biomarkers that may assist in the clinical management of lung cancer.

**Keywords:** epigenetics, biomarkers, body fluids

## DNA methylation in transcriptional regulation

The term “Epigenetics” is used to define the heritable changes in phenotype or gene expression caused by mechanisms not involving primary DNA sequence alterations.<sup>1</sup> These mechanisms include DNA methylation, histone tail modifications, nucleosomal rearrangements/remodeling, and noncoding RNAs (ncRNAs). These mechanisms interact with each other and follow a precise molecular orchestration in order to maintain or change epigenetic marks throughout the genome; this crosstalk seems to be pivotal for the maintenance of cell differentiation, growth, and homeostasis.<sup>2,3</sup> There are many enzymes mediating this crosstalk. For instance, MeCP2, a methyl-binding domain-containing protein, decks on methylated DNA and recruits co-repressor complexes such as histone methyltransferases and histone deacetylases; thus, establishing an inactive chromatin state that hinders transcription factor accessibility and leads to loss of gene expression.<sup>4,5</sup> It has been shown that DNA methylation status can determine nucleosome occupancy and further influence gene expression.<sup>6</sup> Recently, it has been shown that long ncRNAs also regulate the interaction between chromatin and remodeling complexes, such as PRC2,<sup>7</sup> by bringing them to specific genomic loci and shifting their position when necessary.<sup>8</sup>

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DNA methylation is the most studied epigenetic modification in humans. It designates the addition of a methyl group (CH<sub>3</sub>) at position 5' of the amino base cytosine within CpG dinucleotides,<sup>9</sup> leading to the formation of 5-methylcytosine. This reaction is catalyzed by DNA methyltransferases (DNMTs), which can act *de novo* (mainly DNMT3a and DNMT3b) or maintain the existing methylation patterns (mainly DNMT1)<sup>10–12</sup> assisted by accessory proteins such as UHRF1. Demethylation can be passive when the maintenance mechanism fails to copy the pattern to the newly synthesized strand following DNA replication, or an active process that occurs via a 5-hydroxymethyl cytosine intermediate catalyzed by TET proteins and encompasses the action of a number of accessory enzymes.<sup>11–17</sup> It seems that the first oxidation of 5-methylcytosine to 5-hydroxymethyl cytosine is carried out by TET1<sup>13</sup> while several ways of thereafter transforming 5-hydroxymethyl cytosine to cytosine have been suggested and involve both nuclear and base excision repair pathways.<sup>15,16</sup>

DNA methylation is implicated in various physiological processes, such as cell differentiation, X-chromosome inactivation, gene imprinting, and genomic stability. DNA methylation alterations in human disease are well established to date,<sup>18</sup> although the list of epigenetic-driven diseases is growing fast. Global hypomethylation<sup>19</sup> and loci-specific hypermethylation<sup>20</sup> have been frequently reported in cancer.<sup>21</sup> Originally, changes in DNA methylation were studied in gene promoters, where hypermethylation was associated with downregulation of tumor suppressor genes and hypomethylation triggers upregulation of oncogenes.<sup>22</sup> More recently, taking advantage of the development of genome-wide analysis techniques, intra- and intergenic methylation changes have also been described. Alterations of DNA methylation within the gene body can affect alternative intragenic promoters and enhancers, ncRNA expression, transposable elements mobility, alternative splicing, or polyadenylation.<sup>23,24</sup> Intergenic methylation changes can also affect enhancers or insulators; therefore, leading to gene silencing or activation, respectively,<sup>25</sup> or alter the expression of intergenic ncRNAs.<sup>23,24</sup> The involvement of DNA methylation in transcriptional regulation is based on the chromatin conformation changes that are associated with it. As mentioned DNA methylation occurs in combination with other epigenetic mechanisms and determines whether a particular stretch of DNA is loose and accessible to the transcriptional machinery or is tightly wrapped around nucleosomes in a dense chromatin form prohibitive of transcription factor binding.<sup>23</sup> The most exciting point is probably that this relationship

is bidirectional; it is now known that certain transcription factors can place epigenetic marks upon binding to DNA and can therefore alter DNA methylation.<sup>26</sup>

In biomarker studies, both DNA methylation and messenger RNA (mRNA) expression compete for a place in molecular diagnostics. The main disadvantage of detecting DNA methylation only is that it does not indicate the level of expression deregulation of the affected gene(s). One would argue that expression deregulation ultimate proof is only demonstrated by protein assays; however, determining mRNA expression is providing good preliminary evidence. In the case of DNA methylation, there is no such evidence and one can hypothesize the outcome on RNA.

On the other hand, DNA methylation analysis has significant analytical and biological advantages. DNA methylation changes require at least one cell cycle, which makes this modification robust following sampling; RNA expression profiles deteriorate quickly upon removal of the sample from the body. In addition, DNA is a much more stable chemical entity than RNA; therefore, DNA methylation assays do not need special sampling requirements and are applicable to archival material, where mRNA analysis can be particularly challenging.

## Lung cancer biomarkers

Lung cancer is one of the most frequently diagnosed cancers in Europe<sup>27</sup> and the US,<sup>28</sup> accounting for 13% of all new cases of cancer in the world.<sup>29</sup> Lung cancer is divided into two main types: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). NSCLC represents approximately 85% of lung tumors with adenocarcinoma, squamous cell carcinoma, and large cell carcinoma representing the main histological subtypes. The precise histomolecular classification of lung tumors plays a critical role in personalized medicine and effective treatment of patients.<sup>30</sup> Lung cancer still remains the leading cause of cancer-related deaths worldwide.<sup>29</sup> This is mainly due to very late diagnosis,<sup>31</sup> and there are worldwide efforts from the lung cancer research community to introduce national screening strategies.<sup>32</sup> There is a consensus that implementation of molecular markers can assist clinical management of lung cancer. Multiple studies focused on the development of biomarkers for early detection,<sup>33–36</sup> metastatic risk assessment,<sup>37,38</sup> and therapeutic stratification of patients.<sup>39–41</sup>

According to the Biomarkers Definitions Working Group, the term “biomarker” is used to describe a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic

responses to a therapeutic intervention. Biomarker qualification involves four major steps: 1) discovery phase, 2) experimental validation, 3) preclinical validation, and 4) clinical validation.<sup>31,42</sup> Numerous studies describe new biomarkers and their potential use in the clinic;<sup>43,44</sup> however, very few of them manage to reach clinical validation.<sup>35</sup> The main obstacles are the poor reproducibility of the studies, sample heterogeneity, methodological biases, quality control implementation, and significant interlaboratory variability in experimental performance and data analysis.<sup>45</sup> To date, biomarkers for lung cancer are mainly used to define and monitor therapy (eg, *EGFR*, *KRAS*, or *ALK* mutations),<sup>46</sup> DNA-based biomarkers present practical advantages,<sup>47</sup> and the established evidence regarding the involvement of epigenetic deregulation in human cancer, including lung,<sup>48</sup> sets research of DNA methylation biomarkers at very high priority in lung cancer diagnostics.<sup>49</sup>

## DNA methylation detection techniques and methodologies

The need for detection of abnormally methylated DNA in tissue samples and body fluids inevitably leads to discussing methodology issues and the appropriateness of different approaches. A large number of DNA methylation detection methods and modifications exist to date, each having certain advantages and disadvantages.

There are three major approaches regarding the means of discrimination between 5'-methyl cytosine and cytosine:

1. Chromatin immunoprecipitation,<sup>50</sup> which utilizes anti-5'-methyl cytosine<sup>34</sup> or methyl-binding domain proteins.<sup>50</sup>
2. Methylation-specific restriction enzyme<sup>51</sup>-based methods, which take advantage of the differential recognition of methylated DNA sequences over their unmethylated counterparts.<sup>52</sup>
3. Bisulfite-based methods, which utilize the fact that bisulfite deaminates cytosine to uracil but does not affect 5'-methyl cytosine, effectively transforming this epigenetic modification into a sequence difference.<sup>53</sup>

Regarding throughput, genome-wide or gene/locus-specific approaches are employed according to the needs. Microarrays and deep sequencing are mainly employed in the first type while dideoxy sequencing,<sup>54</sup> pyrosequencing,<sup>55</sup> and MALDI-TOF<sup>56</sup> are normally utilized for medium-throughput, locus-specific applications. Historically, a few other techniques have been used, such as single-strand conformation polymorphism analysis<sup>57</sup> and high-resolution melting.<sup>58</sup> A very frequent method also used for locus-specific applications is methylation-specific

polymerase chain reaction (MSP)<sup>53,59</sup> and its quantitative real-time derivative qMSP<sup>60</sup> or MethyLight.<sup>61</sup>

Various particularities can be recognized in detecting DNA methylation in body fluids. These samples are known to carry a tiny load of abnormal nucleic acids in the presence of high amounts of "contaminating" normal DNA. Exception to this is the detection of DNA methylation in the peripheral leukocytes in leukemias or for monitoring therapeutic schemes with epigenetic drugs<sup>62</sup> where the target DNA is ample.

The reliable detection of nucleic acids associated with minimal residual disease in samples such as plasma/serum, urine, sputum, bronchial washings (BWs), saliva, is demanding and devious. The most widely used approach has been bisulfite conversion of DNA followed by MSP or qMSP. The concept here is that methylation-specific primers bearing cytosines (forward) and guanines (reverse) preferably at the 3' end of both primers will generate amplicons only from the methylated DNA copies of the target sequence under optimal conditions. The vast experience of the research community in polymerase chain reaction amplification techniques allows, in combination with the availability of new engineered hot-start *Taq* (Thermus Aquaticus) polymerases and reliable thermocycling hardware, to reach high levels of fidelity. Endpoint MSP is a method that revolutionized epigenetic research.<sup>53</sup> Its main disadvantage is the lack of quantitation efficiency as well as problems in sensitivity and specificity. The latter can be overcome by using methylation enrichment pyrosequencing, which employs pyrosequencing to confirm the status of an MSP amplicon.<sup>63</sup> The real-time version of MSP, referred to as qMSP<sup>60</sup> or MethyLight,<sup>61</sup> overcomes many of the endpoint assay problems. It is highly sensitive (especially when using fluorescent probes) in visualizing minute amounts that would never been seen on gels. The use of fluorescent probes adds one more level of sequence specificity and allows for multicolor detection of internal controls to normalize for DNA input. What is currently missing from qMSP/MethyLight is an internal control of bisulfite conversion.

As mentioned, an important problem in body fluids is the low availability of DNA. To make the problem more challenging, bisulfite conversion reduces DNA quality while the subsequent cleanup limits significantly recovery of DNA quantities. It is widely accepted today in the epigenetic biomarker research community that it is unlikely to find the perfect single marker. Most studies point to the discovery of panels of biomarkers, and thus multiple assays. Therefore, the tiny amount of DNA recovered has to be split in different reactions. One way to overcome this problem is to multiplex four targets per reaction using probes with different

fluorescent dyes.<sup>64</sup> Of course the level of optimization required to prove equal amplification factors over five concentration logs between the different amplicons is significant. The abundance of each target is different; thus, in the absence of such calibration, the high-abundance target ( $10^{-1}$ – $10^{-2}$  per genome) will probably consume resources impairing amplification of the rare ( $\leq 10^{-3}$ ) copies. A different approach is the post-bisulfite whole-genome (bisulfiteome) amplification,<sup>65</sup> which demonstrates promising findings, but further research is required to prove the potential extent of its use.

A last important point, frequently bypassed, regarding methodology is the amount of DNA added in a qMSP reaction. qMSP users claim detection of targets in dilution with normal DNA as low as  $10^{-4}$ . In this case, one needs to ensure  $10^4$  genome equivalents (32 ng) are added in the reaction to have one abnormal copy keeping in mind that MSP targets only one bisulfite converted strand. This is also an important issue for consideration in whole bisulfite amplification.

Overall, massive progress has been demonstrated in the last decade in methodological approaches for DNA methylation detection in body fluids. Many academic groups have involved themselves in this research, and the contribution of the biotechnical industry must also be acknowledged. Although there are still issues regarding the quality control of the used techniques, there should be no doubt that these will be overcome and a clinically useful method will be very soon available.

## DNA methylation aberrations in lung cancer

The high frequency of DNA methylation aberrations in lung cancer has been shown in a large number of studies. Development of epigenetic biomarkers, which could reach clinically validated applications, would assist in personalized management of patients with lung cancer. Identification of markers for early detection (diagnosis), risk assessment (prognosis), and therapeutic stratification of patients is of utmost importance and has been the subject of numerous studies during the last decade.<sup>66</sup> Investigating the tumorous, dysplastic, or normal lung tissue is a more direct way to search for biomarkers when compared with surrogate tissues. The relevant literature is vast, and older studies have been previously reviewed.<sup>49</sup> In the present review, we are summarizing the most recent (post-2012) information and attempt to evaluate the progress of DNA methylation biomarkers in the clinical management of lung cancer. We searched for reports in PubMed using combinations of keywords such as “lung cancer”, “DNA methylation”, “biomarker”, “diagnosis”, “early

detection”, “prognosis”, “body fluids”, “plasma”, “serum”, “sputum”, “bronchial lavage”, and “bronchial washings”.

The findings of recent DNA methylation studies in lung cancer tissue are summarized in Table 1. This table contains 131 genes that can be categorized according to the biological processes they are involved in (Figure 1). While the vast majority of the studies analyzed in Table 1 are mainly focused on the aberrant promoter DNA methylation, studies investigating whole genome as well as gene body methylation patterns are also included. For instance, Nelson et al<sup>67</sup> tried to identify new methylation key markers utilizing whole-genome and exonic regions of various lung cancer-related genes. Among the most thoroughly studied genes for DNA methylation in lung cancer are *RASSF1*, *RAR $\beta$* , *p16 (CDKN2A)*, *MGMT*, *SHOX2*, *APC*, *BRCA1*, and *DAPK*, providing a fair list of candidates with biomarker potential. However, Do et al,<sup>68</sup> in an attempt to confirm previous studies showing tumor-specific methylation of DNA repair genes, suggest that methylation frequency of lung cancer-associated genes like *ATM*, *BRCA1*, *MLH1*, and *XPC* is likely to be overestimated in the literature. This raises the importance of diligent validation of previous results, especially if obtained using different methodologies.

### Diagnostic markers

As mentioned earlier, despite the advances in medicine and cancer biology, lung cancer is largely diagnosed at late stages, leading to poor prognosis and high mortality rates.<sup>31</sup> Still the only screening method is low-dose computerized tomography scan of the lungs that is applied to high-risk individuals but includes exposure to radiation, a high rate of potential false positives, and high cost.<sup>69</sup> Development of a biomarker-based test could boost early or more accurate diagnosis that is able to distinguish among the different types of lung cancers. Zhao et al<sup>70</sup> proposed a panel of four genes (*MYF6*, *SIX6*, *BCL2*, and *RAR $\beta$* ) that could offer a sensitivity of 93.07% and a specificity of 83.33% in the diagnosis of stage I NSCLC. A similar study shows that the methylation status of *NEUROG2*, *NID2*, *RASSF1A*, *APC*, and *HOXC9* can also serve in early diagnosis of NSCLC (sensitivity 91.26%, specificity 84.62%); in particular, *NEUROG2* seems to be able to discriminate between adenocarcinomas and squamous cell carcinomas.<sup>71</sup> The DNA methylation status of miRNAs could also serve as a diagnostic marker in NSCLC. Hypermethylation in NSCLC tissue was observed for hsa-miR-34a, hsa-miR-9-2, and hsa-miR-9-3<sup>33</sup> as well as miR-193.<sup>72</sup> Furthermore, in order to empower the precision of the diagnostic outcome of each study, several meta-analyses

**Table 1** List of recent (post-2012) studies identifying abnormal DNA methylation patterns in human lung cancer tissue

Publication	Gene (genomic region)	Methylation	Methodology	Sample size	Comments
Bediaga et al <sup>33</sup>	hsa-miR-43a	Hyper 83%	Pyrosequencing	39 NSCLC patients (T/N)	DNA methylation abnormalities contribute little to the deregulation of the miRNAs tested
	hsa-miR-92	Hyper 45%			
	hsa-miR-93	Hyper 46%			
	hsa-miR-27b	Hypo 51%			
	hsa-miR-182	Hypo 31%			
	hsa-miR-24.1	Hypo 39%			
	hsa-miR-23b	Hypo 28%			
	hsa-miR-200b	Hypo 23%			
	hsa-miR-486	Hypo 44%			
	hsa-miR-338	Hypo 74%			
	LINE-1	Hypo 77%			
Bradly et al <sup>77</sup>	MGMT, DAPK, RASSF1, CDH1, LET7-3-a, NORE1, PTEN	No age-related differences	Pyrosequencing	198 NSCLC patients	Patients $\geq 60$ years with p16 hypermethylation had shortened time to recurrence and a shortened survival time ACs present greater WTI methylation than SCCs As the smoking habit increases, methylation decreases
Bruno et al <sup>10</sup>	p16 WT1	Hyper Mean percentage of methylation tumors: $16.2 \pm 3.4$ normal adjacent tissue: $5.6 \pm 1.7$	Pyrosequencing	16 NSCLC patients	Statistically significant differences were observed for each of the five loci/genes between lung cancer samples and controls
Carvalho et al <sup>11</sup>	CDKN2A	Hyper	MethylCap-seq Validation: bisulfite sequencing, MSP	7 NSCLC patients (T/N) validation: 96 NSCLC patients (T/N)	The methylation status of the chromosomal region chr2:119,331,343 to 119,331,692 could distinguish between AC and SCC
	chr2:119,331,343 to 119,331,692	Hyper			
	chr2:119,328,097 to 119,328,400	Hyper			
Cortés-Sempere et al <sup>72</sup>	RASSF1 ZIC4 IGFBP3	Hyper Hyper Methylated in resistant to cisplatin patients	Bisulfite, MSP	61 NSCLC patients (T)	The authors propose a test that could predict resistance to cisplatin therapy, with an accuracy and precision of 0.84 and 0.9, respectively RASSF1A methylation is related to poor prognosis of early-stage NSCLC and longer disease-free survival after paclitaxel-based neoadjuvant chemotherapy RASSF1 and DAPK1 methylation, along with tumor stage, can define three subgroups with strikingly different prognosis
de Fraipont et al <sup>88</sup>	RASSF1, DAPK1		MSP, pyrosequencing	208 NSCLC samples (T)	High methylation of SHOX2 and PITX2 was a predictor of progression-free survival
Dietrich et al <sup>84</sup>	PITX2, SHOX2	Mean percent of methylation for SHOX was 41.8% and for PITX2 was 13.1%	qMSP	465 (SHOX2) and 445 (PITX2) NSCLC patients (T)	

(Continued)

Table 1 (Continued)

Publication	Gene (genomic region)	Methylation	Methodology	Sample size	Comments
Do et al <sup>68</sup>		Frequency of methylation: No methylation detected No methylation detected No methylation detected No methylation detected 2% 13% 42% 2%	MS-HRM. External validation using the TCGA database. Validation: Big Dye terminator v3.1	56 NSCLC samples (T)	Promoter methylation frequency of ATM, BRCA1, MLH1, and XPC is likely to be overestimated in the literature
Geng et al <sup>71</sup>	ATM BRCA1 MLH1 XPC ERCC1 MGMT NEIL1 RAD23B NEUROG2, NID2, RASSF1A, APC and HOXC9		MSP	15 healthy volunteers, 103 stage I NSCLC tumors (T), and 26 noncancerous controls (N)	This five-gene panel with sensitivity of 91.26% with specificity of 84.62% could detect stage I NSCLC
Zhao et al <sup>70</sup>	MGMT	Median percent methylation in tumors was 26.1% and in normal adjacent tissue 2.4%	MSP, qMSP	1,160 NSCLC tissues and 970 controls	The methylation status of NEUROG2 could distinguish AC from SCC This meta-analysis identified a strong association between methylation of MGMT gene and NSCLC
Gu et al <sup>74</sup>	PI6 <sup>INK4A</sup>	Median percent methylation in tumors was 44% and in normal adjacent tissue 15% Hypermethylated in cancer	MSP, qMSP	2,652 NSCLC patients with 5,175 samples (T/N)	Strong and significant correlation between tumor tissue and autologous samples of PI6 <sup>INK4A</sup> promoter methylation
Guo et al <sup>75</sup>	APC	Hypermethylation in cancer	MSP, qMSP	2,259 NSCLC tumor samples and 1,039 controls	This meta-analysis showed that APC methylation was strongly associated with NSCLC, especially AC
Harada et al <sup>78</sup>	BRCA1	Methylation in 13 of 70 patients (18.6%)	MSP	70 patients with curatively resected stage I NSCLC (T/N)	Recurrence-free survival rate in patients with BRCA1 methylation was lower than in those without BRCA1 methylation
Heller et al <sup>72</sup>	miR-193a miR-9-3	Mean percent methylation in tumors was 33% and in normal adjacent tissue 21% Mean percent methylation in tumors was 25% and in normal adjacent tissue 11% Frequency of methylation	MS-HRM, BGS	101 patients (T/N) with stage I-III NSCLC	76% of the patients were methylated for miR-9-3 and/or miR-193a Poor survival of patients with miR-9-3-methylated compared to miR-9-3-unmethylated SCC
Heller et al <sup>79</sup>	HOXA2 PRDM14	78% 72%	Methylated DNA immunoprecipitation, microarray, MS-HRM. Validation: BGS	101 stage I, II, or III NSCLC patients (T/N)	HOXA2 and HOXA10 methylation was associated with better survival in SCC



Table 1 (Continued)

Publication	Gene (genomic region)	Methylation	Methodology	Sample size	Comments
	<i>MGMT</i>	0.12 (-0.27, 0.95)			Stage was associated with methylation of <i>MGMT</i>
	<i>ESR1</i>	0.28 (-0.67, 1.46)			
	<i>DAPK</i>	0.87 (-0.97, 2.26)			
Lee et al <sup>14</sup>	<i>TMEFF2</i>	Methylation found in 52.5% of tumor samples	MSP	Tumor and corresponding nonmalignant lung tissue specimens (n=139)	<i>TMEFF2</i> methylation was more frequent in ACs without <i>EGFR</i> mutation
Lee et al <sup>20</sup>	<i>RASSF1A</i>	Methylation found in 44.7% of tumor and 2.6% of normal samples	Pyrosequencing, MSP	206 NSCLC tumor and 40 adjacent normal samples	<i>RASSF1A</i> methylation was more frequent in ever-smokers and tumors with <i>TP53</i> mutation
Lee et al <sup>15</sup>	<i>Wif1</i>	Methylation found in 47.5% of tumor and 20.9% of normal tissues	MSP	139 NSCLC patients (T/N)	Strong methylation was an unfavorable prognostic factor for stage I and SCC patients <i>Wif1</i> is hypermethylated in tumors compared to normal adjacent tissue
Li et al <sup>16</sup>					<i>Wif1</i> methylation is associated with unfavorable prognosis in ACs patients with <i>EGFR</i> mutation Identification of 108 differentially methylated genes in tumor compared to normal tissues, involved in cell development, immune response, and apoptosis pathways
Li et al <sup>24</sup>	<i>miR-503</i>	Methylation found in 80% of tumor and 20% of normal samples	Bisulfite Restriction Analysis (COBRA), Bisulfite-treated DNA sequencing assays (BSP)	65 NSCLC patients (T/N)	<i>miR-503</i> is methylated in tumors compared to normal samples
Liu et al <sup>17</sup>	<i>ANKRD18A</i>	Methylation found in 68.4% of tumor and 0% of normal samples	Methylation-specific PCR, bisulfite-sequencing PCR	38 NSCLC tumors and 20 normal samples	<i>ANKRD18A</i> hypermethylation was associated with lung cancer
Liu et al <sup>18</sup>	<i>ANKRD18B</i>	Methylation found in 53.1% of tumor and 0% of normal samples	Methylation-specific PCR and BGS	98 NSCLC patients (10 adjacent normal samples)	<i>ANKD18B</i> exhibited higher methylation in tumor compared to normal samples
Liu et al <sup>19</sup>	<i>ALX4</i>	Methylation found in 55% of tumor and 0% of normal samples	MSP and BGS	98 NSCLC patients (20 adjacent normal samples)	<i>ANKD18B</i> hypermethylation was associated with poor differentiation and earlier stage tumors Tumor suppressor <i>ALX4</i> is downregulated in NSCLC due to DNA methylation
Liu et al <sup>20</sup>	<i>LHX6</i>	Methylation found in 56% of tumor and 0% of normal samples	MSP and BGS	93 primary NSCLC tumor and 20 normal samples	Tumor suppressor <i>LHX6</i> is downregulated in NSCLC due to DNA methylation
Lokk et al <sup>27</sup>	Whole genome	Differential methylation of clustered genes	InfiniumH Human-Methylation27 RevB BeadChips (Illumina Inc.); Validation: Sanger Sequencing	48 patients with stage I NSCLC and 18 matching cancer-free lung samples	<i>DXS9879E</i> ( <i>LAGE3</i> ), <i>RTEL1</i> and <i>MTM1</i> hypermethylation, and <i>SCUBE3</i> , <i>SYT2</i> , <i>KCNK3</i> , <i>KCNK4</i> , <i>GRIK3</i> , <i>CRB1</i> , <i>SOCS2</i> , <i>ACTA1</i> , <i>ZNF660</i> , <i>MDF1</i> , <i>ALDH1A3</i> , and <i>SRD5A2</i> hypomethylation were associated with poor survival



Lu et al <sup>121</sup>	lncRNA MEG3	Methylation found in 68% of normal and 96% of tumor samples	Bisulfite sequencing	44 patients (T/N)	Downregulation of lncRNA MEG3 observed in NSCLC is partly due to promoter hypermethylation DNA promoter methylation leads to repression of miR-31 expression
Meng et al <sup>122</sup>	miR-31		TCGA data set (Illumina Infinium Human DNA Methylation 450 beadchip data)	177 lung AC samples containing both methylation and miR-31 expression from the 233-patient set of the databank 46 NSCLC samples (T/N)	
Morán et al <sup>91</sup>	CALCA, MMP-2	Hyper	Illumina GoldenGate Methylation bead array, validation with qMSP (only for RASSF1 and p16)		Hypermethylation of CALCA and MMP-2 was associated with a poor clinical outcome
	RASSF1	Hyper			Hypermethylation of RASSF1 was a protective variable SCC was associated with hypermethylation of these 12 genes
	CALCA, HOXB2, IRF7, MOS, MT1A, MYCL2, p16, RARA, RASSF1, SEPT5, SOX17 and SPARC	Hyper			Global hypermethylation was associated with bad prognosis in stage IIIA NSCLC patients Well-differentiated tumors showed hypermethylation of IRF5 and NPR2
	SFN (14-3-3 sigma)	Hypo			
	ZMYND10	Hypo			
	IRF5, NRP2	Hyper			
Na et al <sup>85</sup>	DKK1	Methylation found in 48.9% of tumors and 22.3% of normal samples	MSP	139 NSCLC patients (T/N)	DKK1 methylation was more frequent in patients with stage I NSCLC
	MicroRNA-34b/c	More than 5% methylation found in 42% of tumor and 10% of normal samples Methylation fold change in cancer:	Melting-curve analysis. Validation: BGS	140 early-stage lung ACs and 10 nonmalignant samples	DKK1 methylation was associated with better survival of males, ever-smokers and AC patients without EGFR mutation Methylation of microRNA-34b/c predicts poor disease-free survival in early-stage ACs
Nadal et al <sup>82</sup>					
Nelson et al <sup>67</sup>	HOXA9 SOX1 DDR1 SLC5A8	Hyper (10.3 fold) Hyper (5.9-fold) Hypo (8.1-fold) Hypermethylated in cancer	Illumina GoldenGate arrays, validation: bisulfite pyrosequencing	47 NSCLC patients. Validation set: 99 NSCLC patients (T/N)	The identified genes consistently have altered methylation in lung tumors (no difference between SCC and ACs)
Ko et al <sup>89</sup>			Restriction landmark genomic scanning. Validation: bisulfite-modified sequencing and qMSP	23 NSCLC patients (T/N)	Hypermethylation of SLC5A8 promoter, which is mainly observed in tumor samples, seems to affect the expression of SLC5A8
Rammath et al <sup>86</sup>	CYP24A1	More than 10% methylation found in 35.5% of tumor samples	Bisulfite DNA pyrosequencing	90 surgically resected lung ACs	Patients with unmethylated CYP24A1 had worse disease-free and overall survival

(Continued)

Table 1 (Continued)

Publication	Gene (genomic region)	Methylation	Methodology	Sample size	Comments
Sandoval et al <sup>123</sup>	HIST1H4F, PCDHGB6, NPBWR1, ALX1, and HOXA9		DNA methylation microarray. Validation: pyrosequencing	Array: 444 stage I NSCLC patients. Validation: 143 stage I NSCLC patients	A signature based on the number of hypermethylated events distinguished stage I NSCLC patients with high and low risk of relapse
Sato et al <sup>124</sup>	ADCY5, EVX1, GFRA1, PDE9A, and TBX20	Hyper	Single-CpG resolution Infinium array. Validation: pyrosequencing	145 AC patients (T/N) and 36 normal tissue from patients without any primary lung tumor	Methylation of these five genes in tumor samples was correlated with less mRNA production and tumor aggressiveness
Sato et al <sup>125</sup>	Genome wide		Single-CpG resolution Infinium array. Validation: pyrosequencing	139 NSCLC paired samples and 36 normal samples from patients without any cancer previously tested. Validation: 50 paired samples	DNA methylation profiles related to carcinogenic factors such as smoking and chronic obstructive pulmonary disease appear to be established in noncancerous lung tissue from patients with ACs and may determine the aggressiveness of the tumors
Scesnaite et al <sup>126</sup>	<i>p16</i> , RARB, RASSF1	Frequency of methylation 20%–30%	MSP	212 NSCLC patients	Hypermethylation of <i>p16</i> was predominant in men and SCCs RARB hypermethylation was more frequent in females and ACs
Shi et al <sup>127</sup>	MGMT, DAPK1 Cos-trans-methylation quantitative trait loci in the genome	15%–20%	Illumina Infinium HumanMethylation450 BeadChip, methylation quantitative trait loci analyses	244 stage I–IIIA NSCLC patients (T/N)	Identification of 34,304 cis- and 585 trans-methylation quantitative trait loci
Shinjo et al <sup>128</sup>	CCNA1, ACAN, GFRA1, EDARADD, MGC45800, and <i>p16</i>		Genome-wide DNA methylation microarray analysis. Validation: pyrosequencing	41 AC patients. Validation: 128 AC patients (T/N)	The study reveals six newly identified CIMP (CpG island methylator phenotype) markers that may be useful in the accurate and practical epigenomic classifications of lung cancer
Selamat et al <sup>129</sup>	ABCA3	Hyper	Illumina Infinium HumanMethylation27 platform	59 AC patients (T/N)	The hypermethylated cluster was associated with smoking status and KRAS mutations in AC
	SOX17 TMEIM204	Hyper Hyper			
	FAM83A AGR2, KRT8, SFN LGALS4	Hypo Hypo Hyper in smokers compared to nonsmokers			
Suzuki et al <sup>130</sup>	LINE-1 SLIT2 MAL IGFBP7	Frequency of methylation 55% (hypo) 64% (hyper) 46% (hyper) 54% (hyper)	Pyrosequencing	56 NSCLC patients (T/N)	LINE-1 methylation was lower in SCC than ACs SLIT2 methylation was higher in COPD when compared with non-COPD cases MAL hypermethylation was higher in men, smokers and patients without EGFR mutations

Tan et al <sup>131</sup>	PRDM5	Hypermethylated in cancer	MSP	30 SCC patients (T/N)	PRDM5 methylation was correlated with tumor differentiation and lymph node metastasis
Tang et al <sup>132</sup>	PCDH10	Methylation found in 50% of tumors and 0% of normal samples	MSP	40 NSCLC patients (T/N)	PCDH10 promoter methylation was related to smoking
Tekpli et al <sup>133</sup>	CYP1A1	High methylation in never-smokers	Pyrosequencing	120 early-stage NSCLC patients (T/N)	Low CYP1A1 enhancer methylation in histologically normal lung was associated with high CYP1A1 mRNA levels and smoking-induced genetic alterations
Tennis et al <sup>134</sup>	Wnt7a	Hypermethylated in cancer	Pyrosequencing	82 NSCLC patients (T/N)	The Wnt7a promoter is hypermethylated in cancer resulting in decreased activity
Tessema et al <sup>135</sup>	TOX2	Found methylated in 28% (TOX2), 5% (TOX) and 58% (TOX3) of tumor and in 0% of normal samples	MSP, bisulfite sequencing	190 primary NSCLC tumors (and some adjacent normal samples)	Prevalence for TOX3 methylation among lung cancer patients was greater in SCC (79%) compared to ACs (56%)
	TOX				
	TOX3				
	TOX4				
Walter et al <sup>136</sup>	ERBB2	No methylation detected	qMSP (with pre-amp) and pyrosequencing	42 late-stage NSCLC (T)	ERBB2 and ZEB2 methylation status was strongly associated with an epithelial phenotype in patients who fail frontline chemotherapy
	ZEB2	Hypermethylated in cancer		60 late-stage NSCLC (T)	
Wilkerson et al <sup>137</sup>	Genome wide	Hypermethylated in cancer	Methylation-SNP analysis (DNA digested by methylation-sensitive <i>HpaII</i> to <i>HpaII</i> -undigested DNA by Affymetrix 250K Sty microarrays)	Published cohorts (n=504) Validation cohort (n=116)	ACs are classified in Magnoid, Squamoid, and Bronchioid. The main methylation alteration detected was genome-wide hypermethylation in Magnoid
Wu et al <sup>81</sup>	TFPI-2	Found methylated in 27.1% of the samples	MSP	133 non-metastatic NSCLC patients (T)	Methylated TFPI-2 was associated with poor survival
Yin et al <sup>138</sup>	SOX17	Methylated in 60.2% of the tumor samples	MSP	88 primary NSCLC samples	SOX17 methylation was associated with sex and poor tumor differentiation
Zhao et al <sup>139</sup>	RAR $\beta$	Methylation found in 57.5% of tumor and 17.5% of normal samples	MSP	80 NSCLC patients (T/N)	RAR $\beta$ methylation was more frequently found in tumors (more closely related to SCC) compared to normal adjacent tissue
Zhao et al <sup>70</sup>	MYF6, SIX6, BCL2, and RARB	Hyper	MSP	101 stage I NSCLC patients and 30 patients with noncancerous lung diseases	A panel of four genes (MYF6, SIX6, BCL2, and RARB) has a sensitivity of 93.07% and a specificity of 83.33% in the diagnosis of stage I NSCLC
					Co-methylation of SIX6 and SOX1 correlated with SCC
					Co-methylation of BCL2, RARB, and SIX6, but not the methylation of either single gene, was associated with smoking

(Continued)

Table 1 (Continued)

Publication	Gene (genomic region)	Methylation	Methodology	Sample size	Comments
Zhu et al <sup>83</sup>	ALX1, HPP1, OC2, PDGFRA, and SMPD3		MSP	155 patients with stage IIIB-IV NSCLC who received EGFR-TKI therapy (T)	No difference in methylation of ALX1, HPP1, OC2, PDGFRA, and SMPD3 between tumor and normal samples
Cao et al <sup>83</sup>	SFRP5			33 SCLC patients	Patients with unmethylated SFRP5 are more likely to benefit from EGFR-TKI therapy ( $P=0.002$ ), independent of EGFR genotype
Kalani et al <sup>40</sup>	miR-886-3p	Hypermethylated in cancer	Bisulfite sequencing (BSP) and methylation-specific PCR (MSP)	18 SCLC tumor and 5 normal samples	miR-886 promoter methylation was correlated with shorter overall survival of SCLC patients
Tanaka et al <sup>41</sup>	Genome wide		MIRA technique Validation: COBRA and bisulfite genome sequencing	12 SCLC, 47 NSCLC resected tumors, and 15 malignant pleural effusions in SCLC patients	This study identified 73 gene targets that are methylated in >77% of primary SCLC tumors
	miR-34a	Methylation found in 15% of SCLC and 28% of NSCLC	MSP		The frequency of miR-34b/c methylation was higher in SCLC than in NSCLC
	miR-34b/c	Methylation found in 67% of SCLC and 26% of NSCLC			

**Abbreviations:** AC, adenocarcinoma; BGS, bisulfite genomic sequencing; BSP, bisulfite sequencing PCR; COBRA, combined bisulfite restriction analysis; COPD, chronic obstruction pulmonary disease; FFPE, formalin fixed paraffin embedded; GEO, Gene Expression Omnibus; IQR, interquartile range; lncRNA, long non coding RNA; mRNA, messenger RNA; MS-HRM, methylation-sensitive high-resolution melting; MSP, methylation specific PCR; NCBI, National Center for Biotechnology Information; NSCLC, non-small-cell lung cancer; PCR, polymerase chain reaction; qMSP, quantitative methylation-specific PCR; SCC, squamous cell carcinoma; SCLC, small-cell lung cancer; T, tumor-only samples; T/N, tumor-normal pairs.

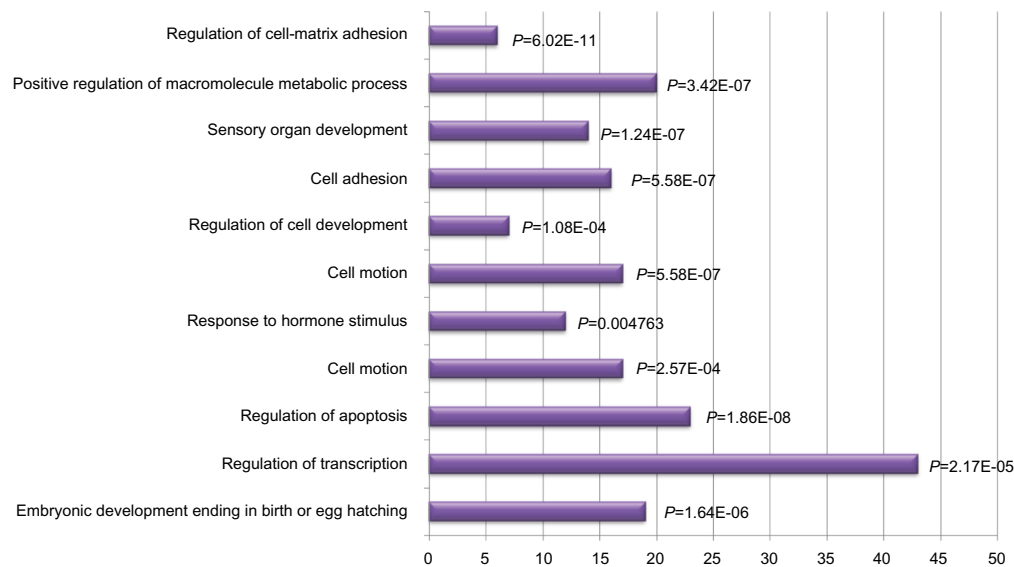
have been conducted.<sup>70,73-75</sup> Taking into account the results of many individual studies, these meta-analyses confirmed that the methylation status of *MGMT*,<sup>70</sup> *CDKN2A*,<sup>74</sup> *APC*,<sup>75</sup> and *RARβ*<sup>73</sup> promoters is strongly related to lung cancer development.

### Prognostic markers

Lung cancer is a highly heterogeneous malignancy. Subpopulations of patients exhibit different risk of metastatic relapse.<sup>76</sup> Therefore, markers of prognostic value enabling prediction of the disease aggression and potential course present a long-standing clinically unmet need. The methylation status of numerous genes has been associated with patients' survival. Recent studies have associated methylation of *CDKN2A*,<sup>77</sup> *BRCA1*,<sup>78</sup> *HOXA-2/10*,<sup>79</sup> *CHFR*,<sup>80</sup> *TFPI-2*,<sup>81</sup> miR-9-3,<sup>72</sup> and miR-34b/c<sup>82</sup> with poor survival of patients with NSCLC and miR-886-3p<sup>83</sup> in SCLC patients. On the other hand, hypermethylation of *SHOX2*, *PITX2*,<sup>84</sup> *DKK1*,<sup>85</sup> and *CYP24A1*<sup>86</sup> was related to better prognosis in NSCLC. In addition to hypermethylation, cancer-specific hypomethylated loci have been proposed as prognostic factors. More specifically, low methylation levels of *SCUBE3*, *SYT2*, *KCNC3*, *KCNC4*, *GRIK3*, *CRB1*, *SOCS2*, *ACTA1*, *ZNF660*, *MDFI*, *ALDH1A3*, and *SRD5A2* in lung tumors was an indicator of poor survival in a recent study by Lokk et al.<sup>87</sup> Surprisingly, although *RASSF1* hypermethylation was mainly associated with poor outcome,<sup>88-90</sup> a study by Morán et al,<sup>91</sup> proposed its protective value.

### Therapeutic stratification markers

Directly linked to prognostication is the need for biomarkers that can predict response to particular therapeutic regimens in order to facilitate personalized therapy. Currently, chemotherapy and/or radiotherapy are used based on classical histopathological criteria with very little to no input from molecular constitution of the tumor. However, as personalized medicine rapidly gains ground with the use of targeted therapies (*EGFR*, *ALK*, etc) it becomes profound that a more global genetic and epigenetic profiling of individual tumors is required. Many studies have shown that specific DNA methylation signatures can divide patients into subgroups depending on their response to specific anticancer therapy.<sup>76,92,93</sup> Hypermethylation of *IGFBP3* has been associated with lower response of NSCLC patients to cisplatin therapy,<sup>92</sup> while methylation of miR-503 seems to also play a role in this pathway.<sup>94</sup> Another recent study reports that unmethylated *SFRP5* is a predictor of response to EGFR tyrosine kinase inhibitors therapy, independently of *EGFR* genotype.<sup>93</sup>



**Figure 1** Bar chart demonstrating the distribution of the most important groups describing the function of the genes with abnormal DNA methylation in lung cancer tissue. **Notes:** Gene ontology was performed based on biological function using The Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (<http://david.abcc.ncifcrf.gov/>).

Also, a phase 2 clinical trial investigated whether *MGMT* promoter methylation can predict response to Temozolomide treatment in advanced aerodigestive tract (including lung cancer) and colorectal cancers. Unfortunately, the number of lung cancer patients exhibiting *MGMT* methylation was too small to support the power of the study, leading to uninterpretable results.<sup>95</sup>

## DNA methylation detection in body fluids

The high frequency of DNA methylation detected in primary tumors led researchers to explore the feasibility of detecting these alterations in body fluids for diagnostic purposes. Body fluids represent an extremely attractive clinical material for the management of disease because they can be collected using noninvasive or minimally invasive procedures. Assays target cancer-related DNA methylation abnormalities that may be detected in circulating tumor cells or in cell-free DNA or both. However, the detection of cancer-specific DNA methylation in body fluids presents major challenges, mainly associated with low target abundance in an excess of normal DNA. Similarly to the “Lung cancer biomarkers” section, older reports have been adequately reviewed previously;<sup>49</sup> therefore, we present the most recent updates (post-2012) in this review (Table 2).

### Bronchial washings

Although bronchoscopy does not qualify as a noninvasive process, it is a standard part of the clinical workup of

patients suspected for lung cancer, and currently, diagnosis on this sample type is confined to cytological examination. Unfortunately, the diagnostic efficiency of this technique is relatively poor and nearly half of the cases are missed.<sup>96,97</sup> Therefore, it is imperative to develop molecular biomarkers capable of improving the diagnostic efficiency of lung cytology.

The feasibility detecting promoter hypermethylation in bronchial lavage was demonstrated 15 years ago,<sup>98</sup> and this was followed by a number of relevant publications (reviewed by Liloglou et al<sup>49</sup>). More recently, the combined detection of *RASSF1* methylation and *KRAS* mutation in BWs has been reported to aid in the detection of malignancy in false-negative or ambiguous cytological outcomes by correctly diagnosing as cancer 29% of the false-negative or doubtful cytological outcomes.<sup>99</sup> Furthermore, a panel of DNA methylation biomarkers has been described to show a substantial gain in sensitivity of detection over standalone cytology.<sup>60</sup> In this large retrospective case-control study, encompassing BWs from 655 individuals, a diagnostic algorithm based on the methylation profile of *CDKN2A*, *TERT*, *WT1*, and *RASSF1* demonstrated 82% sensitivity, 92% specificity, and a diagnostic accuracy of 85.9%. Finally, DNA methylation of *SHOX2* alone demonstrated a very high sensitivity in BWs (96%) but the specificity was less than optimal (78%).<sup>100</sup> However, a significant improvement in specificity has been shown for *SHOX2* methylation when endobronchial ultrasound with transbronchial needle aspiration (EBUS-TBNA) samples were used; specificity reached 99% in this study

**Table 2** List of recent (post-2012) studies involving detection of DNA methylation in body fluids from patients with lung cancer

Publication	Gene (genomic region)	Sample size	Methodology	Tissue/body fluid	Comments
van der Drift et al <sup>99</sup>	RASSF1A	129 lung cancer patients and 28 controls	qMSP	Bronchial washings	RASSF1A hypermethylation in 50% of central tumors and 31% of peripheral tumors RASSF1A methylation detected in 4/17 patients with nondiagnostic bronchoscopy When combined with KRAS mutation, methylation RASSF1A provided an additional diagnostic value of 29% Evaluation of SHOX2 methylation in a case-control study conferred 96% specificity and 78% sensitivity Methylation panel showed 82% sensitivity, 91% specificity, and 85.9% diagnostic accuracy 94% sensitivity and 98% specificity for detection of malignant lymph nodes by SHOX2 methylation in EBUS-TBNA Negative predictive value increases from 80% to 99% when SHOX2 assessment is used in combination with pathological examination
Dietrich et al <sup>100</sup>	SHOX2	125 lung cancer patients and 125 controls	HeavyMethyl qPCR	Bronchial aspirates	
Nikolaïdis et al <sup>101</sup>	p16, RASSF1, TERT, WT1	Training set: 194 cases/213 controls Validation set: 139 cases/109 controls	qMSP	Bronchial lavage	
Darwiche et al <sup>101</sup>	SHOX2	154 lung cancer patients (lymph node aspirates)	qMSP	EBUS-TBNA	
Shin et al <sup>142</sup>	MAGE A3, p16	65 lung cancer cases, 68 benign lung diseases, 30 healthy individuals	Nested-MSP	Sputum (induced)	MAGE A3 and p16 methylation was more frequent in the sputum of lung cancer than in benign lung disease and healthy controls Methylation of MAGE A3 and p16 was higher in MAGE A1-A6-positive group when compared to the negative group RASSF1A best of the three in discriminating lung cancer cases in both sets (sensitivity 41%–52% and specificity 94%–96%) Assessing RASSF1A improved the sensitivity of cytological evaluation, from 22% to 52% without diminishing the specificity (94%) Seven-gene panels improved the classification accuracy obtained with covariates alone (CO cohort: 62%–71% and NM cohort: 58%–77%) NM cohort: methylation of five or more genes in the seven-gene panel conferred a 22-fold increase in risk for lung cancer
Hubers et al <sup>102</sup>	RASSF1A, APC, CYGB	Training set: 98 cases/90 controls Validation set: 60 cases/445 controls	qMSP	Sputum (spontaneous)	
Leng et al <sup>103</sup>	Overall, 31 candidate genes evaluated CO panel: MGMT, DAPK, PAX5b, Dal1, PCDH20, Jph3, Kif1a NM panel: DAPK, PAX5b, PAX5a, Dal1, GATA5, SULF2, CXCL14 ANKRD18B	CO cohort: 64 lung cancer cases/64 controls NM cohort: 40 stage I asymptomatic lung cancer cases/90 controls	MSP	Sputum (spontaneous)	
Liu et al <sup>118</sup>	ANKRD18B	Sputum: 85 lung cancer cases Plasma: 65 lung cancer cases 10 controls (tissue)	MSP and BGS	Sputum Plasma	ANKRD18B aberrant methylation detected in the sputum of 69.7% (23/33) of the patients who showed tissue methylation ANKRD18B aberrant methylation detected in the plasma of 72.7% (24/33) of the patients who showed tissue methylation ANKRD18B hypermethylation is cancer-specific (not found in control samples) BRMS1 promoter was methylated both in NSCLC primary tissues (59.6%) and in corresponding cell-free DNA (47.9%) but not in cell-free DNA from healthy individuals (0%) Stage I–III NSCLC patients with BRMS1 methylation in cell-free DNA had lower disease-free interval and overall survival In stage IV patients, methylation of BRMS1 in cell-free DNA was associated with a lower progression-free and overall survival
Balgkouranidou et al <sup>105</sup>	BRMS1	Training set: 57 stage I–III NSCLC patients (tumor tissue, adjacent normal and corresponding plasma – for 48 patients) Validation set: 74 stage IV NSCLC patients cell-free DNA Control group: 24 healthy donors cell-free DNA	MSP tissue samples qMSP – cell-free DNA samples	Tissue (fresh frozen) Plasma	

Kontic et al <sup>144</sup>	RASSF1A, CDH13, MGMT, ESRI, DAPK	Primary tumor tissue and nonmalignant lung tissue from 65 NSCLC patients and corresponding blood samples (from 51 cases)	Pyrosequencing	Tissue (fresh frozen)	DAPK, RASSF1A, and CDH13 were significantly hypermethylated in lung tumors compared with matched normal adjacent tissue. CDH13 hypermethylation was observed more frequently in women and adenocarcinomas. Stage IV patients were more likely to have MGMT hypermethylation. ESR1 showed higher methylation levels in blood from patients with hypermethylation in their tumor samples. TMEFF2 hypermethylation was found in 52.5% (73/139) of tumors and 15.8% (22/139) of adjacent normal adjacent tissue. TMEFF2 methylation was significantly lower in adenocarcinomas with EGFR mutations. Detection of TMEFF2 methylation in serum showed 9.2% sensitivity and 100% specificity. RASSF1 methylation was observed in 85.71% (48/56) of lung cancer patients' blood. RARβ methylation was found in 80.36% (45/56) of lung cancer patients' blood. Methylation of both genes was observed in 75% (42/56) of lung cancer patients' blood. Control samples did not show methylation neither in RASSF1 nor in RARβ.
Lee et al <sup>114</sup>	TMEFF2	139 NSCLC T/N	Nested – MSP	Tissue (fresh frozen)	TMEFF2 hypermethylation was found in 52.5% (73/139) of tumors and 15.8% (22/139) of adjacent normal adjacent tissue. TMEFF2 methylation was significantly lower in adenocarcinomas with EGFR mutations. Detection of TMEFF2 methylation in serum showed 9.2% sensitivity and 100% specificity. RASSF1 methylation was observed in 85.71% (48/56) of lung cancer patients' blood. RARβ methylation was found in 80.36% (45/56) of lung cancer patients' blood. Methylation of both genes was observed in 75% (42/56) of lung cancer patients' blood. Control samples did not show methylation neither in RASSF1 nor in RARβ.
Li et al <sup>106</sup>	RASSF1, RARβ	316 NSCLC patients and 50 healthy donors 56 lung cancer patients and 52 controls	MSP	Serum Peripheral blood	RASSF1 methylation was observed in 85.71% (48/56) of lung cancer patients' blood. RARβ methylation was found in 80.36% (45/56) of lung cancer patients' blood. Methylation of both genes was observed in 75% (42/56) of lung cancer patients' blood. Control samples did not show methylation neither in RASSF1 nor in RARβ.
Ponomyayova et al <sup>107</sup>	RASSF1, RARβ	60 untreated NSCLC patients (blood samples prior to treatment, after neoadjuvant treatment and after surgery) 32 healthy volunteers (age comparable)	qMSP	Blood (plasma and cellular fraction)	RASSF1 and RARβ methylation showed 85% sensitivity and 75% specificity (81% accuracy) in circulating DNA (free and cell surface-bound combined analysis). RASSF1 and RARβ methylation levels in cirDNA decreased after chemotherapy, reaching healthy subject levels after tumor resection.
Powrozek et al <sup>104</sup>	SEPTIN9	70 lung cancer patients 100 healthy individuals	Abbott mSEPT9 detection Kit (real-time PCR)	Serum	Tumor relapses were observed in all NSCLC patients showing increased methylation levels of either gene 9 months after surgery. Aberrant promoter methylation of SEPTIN9 detected in 44.3% of lung cancer patients, providing 92.3% specificity. SEPTIN9 methylation more frequently observed in NSCLC than SCLC patients (53% vs 26%).
Tan et al <sup>143</sup>	CDKN2A, RASSF1A, FHIT	200 primary lung cancer patients 200 healthy individuals	qMSP (SYBR green)	Peripheral blood	Methylation levels of CDKN2A, RASSF1A, and FHIT were significantly higher in lung cancer cases when compared to controls. SHOX2 methylation detected not only malignant pleural effusions from lung cancer patients but also metastases of other malignant tumors.
Ilse et al <sup>109</sup>	SHOX2	Pleural effusions from 1617 patients	qMSP	Pleural effusion	Best diagnostic accuracy was achieved when cytological evaluation was combined with SHOX2 methylation (58% sensitivity and 96.2% specificity).

**Abbreviations:** BGS, bisulfite genomic sequencing; CO, Colorado; EBUS-TNA, endobronchial ultrasound with transbronchial needle aspiration; NM, New Mexico; NSCLC, non-small-cell lung cancer; PCR, polymerase chain reaction; qMSP, quantitative methylation-specific PCR; qPCR, quantitative PCR; T/N, tumor -normal pairs.

while sensitivity was improved (82%–99%).<sup>101</sup> Moreover, the negative predictive value also increased from 80% to 99%, which from a clinical point of view, might help in avoiding further unnecessary invasive procedures.

### Sputum

Sputum is another type of body fluid that has been widely tested for lung cancer biomarker qualification. Its major advantage over BWs is that it is noninvasive and can be practiced in three consecutive day collections at home and sent by post. Its major disadvantage though is the high heterogeneity of the sample in lung cell content (a large percentage of samples are inadequate and contain mainly oral epithelial cells),<sup>49</sup> especially when it is noninduced. The methylation status of numerous genes has been recently studied (Table 2) in both spontaneous and induced sputum specimens; some of them alone<sup>102</sup> and some of them grouped in panels.<sup>103</sup> DNA methylation in sputum has also been shown to improve the classification accuracy. Leng et al,<sup>103</sup> in a large case–control study using two different cohorts of patients, reported that seven-gene panels improved the prediction accuracy of standalone clinicopathological data models. More recently, *RASSF1* methylation seemed to significantly aid cytological evaluation by improving sensitivity (from 22% to 52%) without diminishing specificity (94%).<sup>102</sup>

### Plasma/serum

The detection of cancer-indicative biomarkers in blood has been the holy grail of cancer molecular diagnostics. Blood is not only an easily accessible and minimally invasive specimen but also highly standardized in the clinical setting across the globe. The two main problems lie with the high dilution of the potential target but also the lack of localized origin. In other words, abnormal DNA in the bloodstream could have originated anywhere in the body. Nevertheless both plasma and serum specimens from lung cancer patients have been used to detect DNA methylation aberrations in numerous genes;<sup>49</sup> the most recent studies are listed in Table 2. Although most of these alterations seem to be highly specific, they do not reach the sensitivity levels demanded in clinical diagnostics. For instance, *SEPT9* promoter hypermethylation demonstrated 44.3% sensitivity and 92.3% specificity,<sup>104</sup> while *BRMS1* exhibited 47.9% sensitivity and 100% specificity.<sup>105</sup> So far, *RASSF1A* and *RARβ* seem to be the most promising candidates since different studies have shown that both markers combined can achieve 75%–85% sensitivity and 75%–100% specificity.<sup>106,107</sup> Beyond diagnosis, blood has also been recently used to evaluate significance of gene promoter

hypermethylation in plasma of NSCLC patients for prognosis and therapeutic monitoring.<sup>105,107</sup> Ponomaryova et al<sup>107</sup> report that the methylation index for *RASSF1A* and *RARβ* in plasma from lung cancer patients decreased after neoadjuvant chemotherapy and the methylation levels were comparable to those observed in healthy individuals after surgery. Furthermore, all five patients who had a relapse showed an increase in the methylation level of either gene within 9 months after surgery. More recently, the presence of promoter hypermethylation of *BRMS1* in NSCLC patients' plasma was associated with a lower overall survival for stage I–III and stage IV tumors.<sup>105</sup>

Despite the increasing number of relevant studies addressing DNA promoter methylation as a promising biomarker in body fluids, the preclinical research context and lack of clinical validation in follow-up studies in different cohorts has hampered the development of clinically useful diagnostic markers. In this regard, so far the EpiProLung BL Reflex Assay (Epigenomics, Berlin, Germany) is the only clinically validated and Conformité Européenne (CE)-labeled DNA methylation biomarker for diagnosing lung cancer. The assay, which determines the methylation level of *SHOX2*,<sup>35</sup> has been thoroughly validated in bronchial aspirates showing 78% sensitivity and 96% specificity.<sup>100</sup> In addition to bronchial aspirates, *SHOX2* methylation has also been analyzed in other body fluids such as plasma, pleural effusions, and transbronchial aspirates.<sup>101,108,109</sup> Ilse et al<sup>109</sup> noted that the detection of malignancy in pleural effusions improved when cytological examination was combined with *SHOX2* assessment and that *SHOX2* methylation was not only found in malignant effusions from lung cancer patients but also in effusions from malignant tumors of other origins.

## Concluding summary

DNA methylation is a critical epigenetic modification affecting chromatin conformation and transcriptional activity, subsequently influencing globally the biology of the mammalian cell. Inability to maintain correct DNA methylation patterns inevitably leads to disease. The list of relevant human diseases is growing and ranges from developmental disorders to autoimmune conditions and cancer. Lung cancer is the most lethal form of human neoplasia, and epigenetic deregulation has been extensively shown in lung neoplastic tissues utilizing a wide range of methodological approaches. The feasibility of identifying abnormally methylated DNA in body fluids has been established. The focus now is moving to identifying the correct targets and improving the methodologies to reach clinical standards. Despite the significant volume of relevant literature, currently there is not enough



published work to include sufficient clinical validation to allow moving into a randomized clinical trial. The latter will be the ultimate test proving the patient benefit, since early detection of lung cancer will certainly lead to a stage shift and thereafter improvement of survival of lung cancer patients.

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## Disclosure

The authors report no conflicts of interest in this work.

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