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Review

DNA methylation in lung cancer patients: Opening a "window of life" under precision medicine

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ABSTRACT

DNA methylation is a work of adding a methyl group to the 5th carbon atom of cytosine in DNA sequence under the catalysis of DNA methyltransferase (DNMT) to produce 5-methyl cytosine. Some current studies have elucidated the mechanism of lung cancer occurrence and causes of lung cancer progression and metastasis from the perspective of DNA methylation. Moreover, many studies have shown that smoking can change the methylation status of some gene loci, leading to the occurrence of lung cancer, especially central lung cancer. This review mainly introduces the role of DNA methylation in the pathogenesis, early diagnosis and screening, progression and metastasis, treatment, and prognosis of lung cancer, as well as the latest progress. We point out that methylation markers, sample tests, and methylation detection limit the clinical application of DNA methylation. If the liquid biopsy is to become the main force in lung cancer diagnosis, it must make efficient use of limited samples and improve the sensitivity and specificity of the tests. In addition, we also put forward our views on the future development direction of DNA methylation.

1. Introduction

As one of the most common malignant tumors [1], lung cancer kills 1.8 million people in 2020, accounting for 18% of all cancer deaths [2]. Surprisingly, the morbidity and mortality rates are nearly the same. Therefore, how to detect early lung cancer effectively and accurately has become a difficult problem for doctors. Additionally, identifying the subtypes and stages of lung cancer is also a major problem for doctors. In the age of molecular diagnosis, DNA methylation is very essential to solving these two problems.

The occurrence, development, and prognosis of lung cancer are closely related to genetic information. In recent years, epigenetics has been developing rapidly, and new achievements in this field are of great significance in the diagnosis, treatment, and prognosis of lung cancer. The core content of epigenetics is that the gene sequence doesn't change but the expression of the gene changes, and the changes are reversible and heritable [3]. Epigenetics has several aspects, including DNA methylation, histone modification, and the aberrant expression of non-coding RNA (ncRNA) [4–8]. Among which DNA methylation is the most well studied epigenetic modification.

Abbreviations: AC, adenocarcinoma; C/EBPα, CCAAT/enhancer-binding protein-α; cfDNA, cell-free DNA; cfMeDIP–seq, cell-free methylated DNA immunoprecipitation and high-throughput sequencing; CGIs, CpG islands; CIMP, CpG island methylation phenotype; CpG, cytosine-phosphate-guanine; ctDNA, circulating tumor DNA; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; DNMT, DNA methyltransferase; DNMTis, DNA methyltransferase inhibitors; GADD45A, growth arrest and DNA damage protein 45 A; HDACis, histone deacetylase inhibitors; LDCT, low-dose computed tomography; LUAD, lung adenocarcinoma; MBP, methyl binding protein; MeDIP-seq, Methylated DNA Immunoprecipitation Sequencing; MeGDP, methylation of gene body difference to promoter; MS-HRM, methylation-specific high-resolution melting; ncRNA, non-coding RNA; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; ORR, objective response rate; OS, overall survival; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; PFS, progression-free survival; SCC, squamous cell carcinoma; SCLC, small cell lung cancer; TET1, Ten-eleven translocation protein 1; TFs, transcription factors; TMB, tumor mutation burden; 5-AzaC, , 5-Azacytidine; 5-Aza-CdR, 5-Aza-2'-deoxycytidine.

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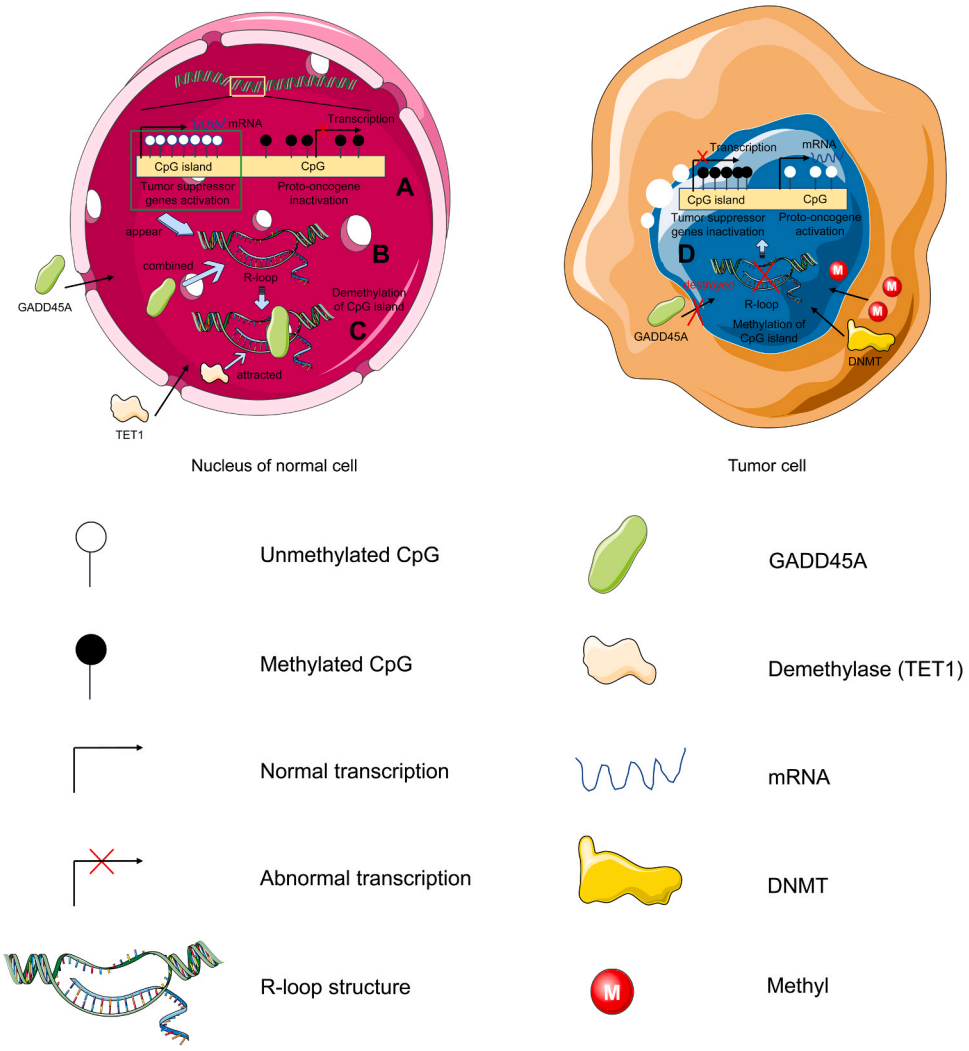

Fig. 1. Comparison of methylation regulation mechanisms between normal and tumor cells. (A) Under normal conditions, most CpG is methylated, and a small part of the areas that are not methylated will form CpG islands. CpG island is rich in double nucleotide "CG" sequence and contains tumor suppressor genes. The proto-oncogene is located outside of CpG island. Thus, proto-oncogenes are low or no expressed and tumor suppressor genes are activated normally. (B) The R-loop structure appears in the CpG islands, and the growth arrest and DNA damage protein 45 A (GADD45A) in cells will enter the nucleus to bind to the R-loop. (C) After GADD45A binds to the R-loop, the demethylase ten-eleven translocation protein 1 (TET1) is recruited to carry out the demethylation process of CpG island, so as to maintain the unmethylated state of CpG island. (D) After R-loop is destroyed, GADD45A could not bind to R-loop, and the demethylase TET1 will no longer enter the nucleus. At the same time, DNMT enters the nucleus to promote CpG island methylation. And then tumor suppressor genes are silenced and proto-oncogenes are overactivated, further leading to tumorigenesis.

Then what is DNA methylation? DNA methylation is a work of adding a methyl group to the 5th carbon atom of cytosine in DNA sequence under the catalysis of DNMT to produce 5-methyl cytosine. Scientists discovered this phenomenon as early as 1948 [9]. DNA methylation does not change the DNA sequence, but it silences genes to which the methyl group is attached. DNA methylation is a programmed process in cells and it can cause methylation or demethylation of various genes at different times and under different circumstances [10].

DNA methylation detection has gained increasing popularity among doctors in recent years. Abnormal methylation occurs in patients with different diseases [11]. Widschwendter et al. [12] pointed out that epigenetic-based DNA methylation detection can meet the requirements of tumor risk prediction and is of great significance for tumor risk screening and prevention. Grote et al. [13] also proposed to use methylation detection for the early diagnosis of lung cancer. By detecting the methylation of specific genes, patients with early lung cancer can be effectively screened. Among them, SHOX2 and RASSF1A genes are common DNA methylation markers in lung cancer [14–16]. With the development of methylation detection technology, DNA methylation detection has been used in staging diagnosis of lung cancer patients, individualized selection of chemotherapeutic drugs, and prognosis judgment.

In conclusion, DNA methylation is a very important epigenetic marker. And it has great potential to improve patient survival and is the key to precision medicine. This review mainly introduces the role of DNA methylation in pathogenesis, early diagnosis and screening, progression and metastasis, treatment, and prognosis of lung cancer, as well as the latest progress. Besides, we also expound some existing problems in this field and the future development direction.

2. DNA methylation and the mechanism of tumorigenesis

2.1. DNA methylation and oncogenesis

DNA methylation is not only closely related to normal life activities of humans, but also plays an important role in the process of tumorigenesis [17]. Yu et al. [18] methylated the tumor suppressor gene p16 in mouse embryonic stem cells. The results showed that 27% of the mice with p16 methylation showed symptoms such as lung cancer, leukemia, sarcoma, while the wild-type control mice showed no tumorigenesis. The results suggested that DNA methylation is the cause of cancer. Under normal conditions, the majority of cytosine-phosphate-guanine (CpG) in the human body is methylated, and a small part of the regions that are not methylated form CpG islands (CGIs) (Fig. 1(A)). CpG island is mainly located in the promoter region of the gene and partly in the exon region, which also contains numerous housekeeping genes, tumor suppressor genes, and DNA repair genes (Fig. 1(A)) [10]. CpG island is usually in a non-methylated state, and the mechanism of maintaining this state needs further study. At present, Ginno et al. [19] found that the CpG island would have R-loop structures, which could

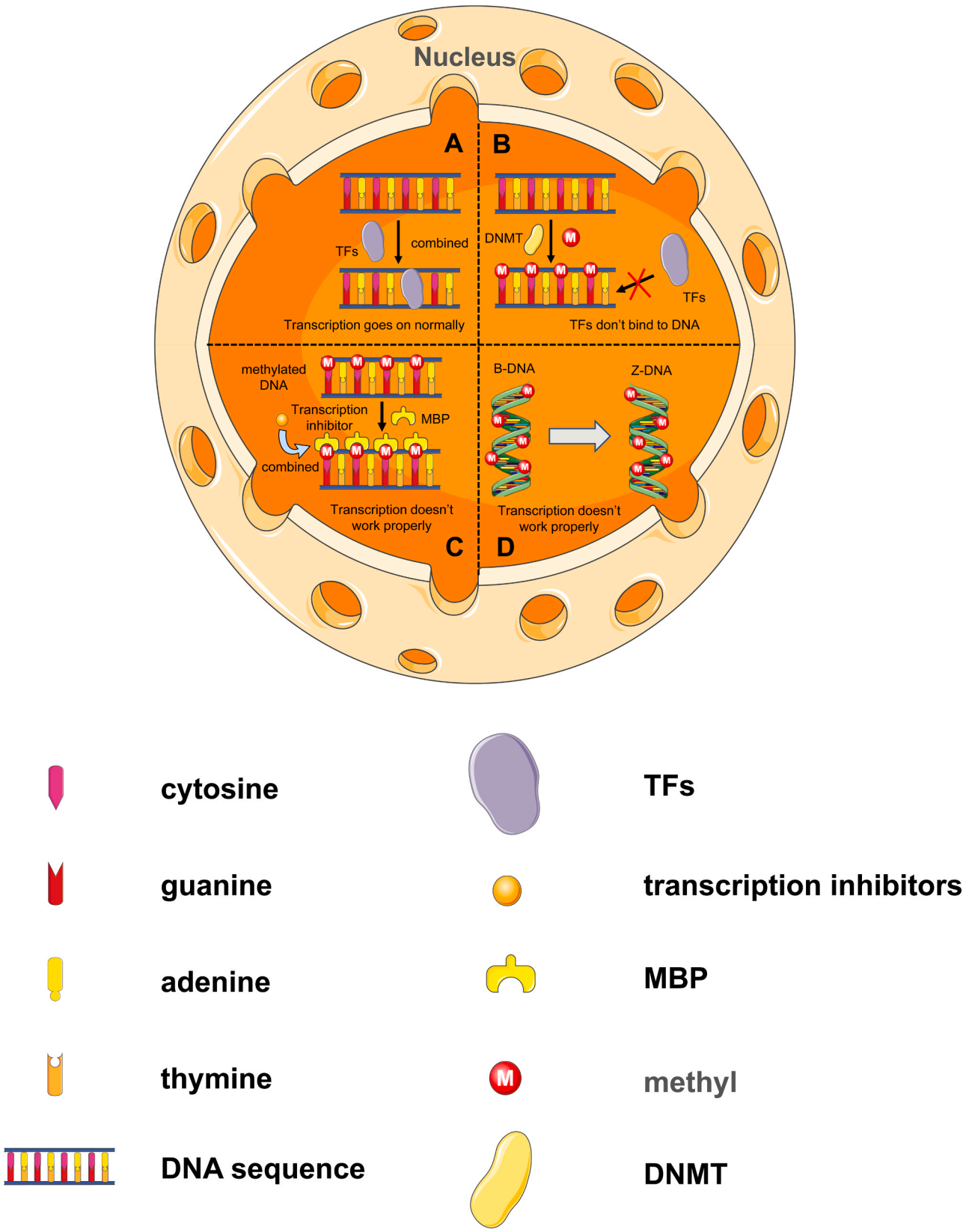

Fig. 2. The diagram of abnormal transcription caused by DNA methylation and normal transcription. (A) Transcription factors (TFs) combine with DNA to complete the transcription process. (B) DNA methylation occurs in DNA strands under the catalysis of DNMT. This inhibits the binding of TFs to DNA sequences and prevents transcription from working properly. (C) After the DNA strand is methylated, the methylated binding protein (MBP) binds to the methyl group on the DNA strand, and then the transcription inhibitor binds to the MBP to inhibit gene transcription. (D) DNA methylation converts the structure of the DNA strand from B-DNA to Z-DNA, thereby inhibiting gene transcription.

Table 1
Summary of methylation markers in the pathogenesis, progression and metastasis, and prognosis of lung cancer.

Methylation marker	Application	Methylation level	Outcome	References
AHRR (cg05575921), 6p21.33 (cg06126421), F2RL3 (cg03636183)	Pathogenesis	Decrease[a]	Increased risk of lung cancer	[31]
2q37.1 (cg21566642)	Pathogenesis	Decrease	Increased risk of lung cancer	[32]
EPHB6	Progression and metastasis	Increase[a]	NSCLC is more likely to metastasize	[74]
HS3ST2	Progression and metastasis	Increase	NSCLC is more likely to metastasize	[75]
TMEM88	Progression and metastasis	Increase	NSCLC is more likely to metastasize	[76]
DAL-1	Progression and metastasis	Increase	NSCLC is more likely to metastasize	[77]
ELMO3	Progression and metastasis	Decrease	NSCLC is more likely to metastasize	[78]
MGMT	Progression and metastasis	Increase	NSCLC appears metastasis or local recurrence	[81]
HMLH1	Prognosis	Increase	NSCLC cells develop resistance	[89]
IGFBP-3	Prognosis	Increase	NSCLC cells develop resistance	[90]
RASSF1A	Prognosis	Increase	Gemcitabine could be more effective for NSCLC patients	[91]
MGMT	Prognosis	Increase	Temozolomide could be more effective for SCLC patients	[92]
TMEM196	Prognosis	Increase	The prognosis is poor and the survival rate is low	[40]
HERC5	Prognosis	Increase	The prognosis is poor and the survival rate is low	[94]
GRK6	Prognosis	Increase	LUAD cells metastasize	[95]
FAM83A	Prognosis	Decrease	The prognosis is poor for LUAD patients	[96]

[a] Both increase and decrease refer to the changes of methylation levels of methylation markers in the experimental group.

protect it from binding to methyltransferase (Fig. 1(B)). Arab et al. [20] found that growth arrest and DNA damage protein 45 A (GADD45A) recruited demethylase ten-eleven translocation protein 1 (TET1) to demethylate CGIs by binding to the R-loop (Fig. 1(C)). Once these protective mechanisms are destroyed, tumor suppressor genes and repair genes will be silenced, and proto-oncogenes will activate and express tumor-related proteins, further leading to tumorigenesis (Fig. 1(D)).

2.2. DNA methylation and gene expression regulation

In terms of gene expression regulation, transcription factors (TFs) normally combine with DNA to complete the transcription process (Fig. 2(A)). However, Harbers et al. [21] found that DNA transfected cells could not be transcribed after methylation (Fig. 2(B), (C), (D)), but could be restored after DNA demethylation. Therefore, they put forward the viewpoint of DNA methylation that might regulate gene transcription in eukaryotes. Similar studies have shown that DNA methylation may be involved in regulatory networks during gene transcription as a regulatory factor [22]. That is, DNA methylation can regulate gene expression. It has been found that DNA methylation affects gene transcription mainly by inhibiting the binding of TFs to the methylated DNA sequence (Fig. 2(B)), chromatin structure changes (Fig. 2(D)), and methyl binding protein (MBP) bind to transcription inhibitors (Fig. 2(C)). Temiz et al. [23] found that B-DNA would be converted to Z-DNA during DNA methylation (Fig. 2(D)), but the mechanism of the conversion was unknown. Li et al. [24] found that methylation of gene body difference to promoter (MeGDP) can act as a predictor to predict gene expression.

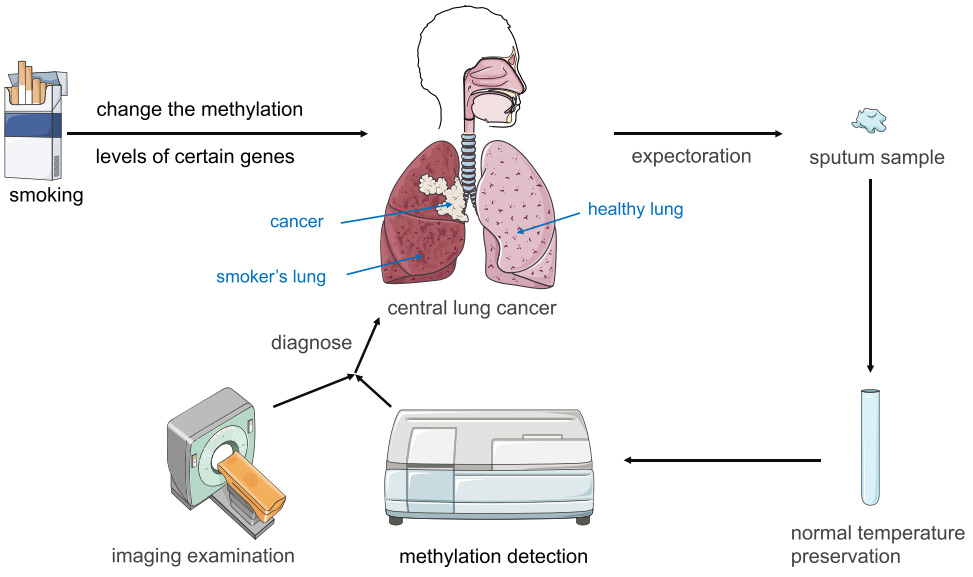

Fig. 3. Sputum methylation detection and causes of central lung cancer. Long-term smoking can change the methylation status of certain gene loci and lead to the development of central lung cancer. The sputum methylation test is very suitable for the screening of central lung cancer. People are required to collect sputum through coughing for three consecutive days and keep it in a collection bottle at room temperature. Sputum samples are then sent to the laboratory for methylation testing. For high-risk groups such as long-term smokers, sputum methylation detection combined with imaging examination is usually adopted for diagnosis and screening of central lung cancer.

2.3. DNA methylation is the bridge between smoking and lung cancer

As is known to all, smoking is the main cause of lung cancer. Doll et al. [25] confirmed that smoking causes lung cancer through 50 years of epidemiological investigation. According to epidemiological statistics, small cell lung cancer (SCLC) is closely related to smoking and second-hand smoke exposure, and most patients with SCLC have a history of smoking [26]. And the markers of methylation caused by smoking persist years after quitting smoking. So how does smoking cause lung cancer? Several scientists have found that DNA methylation is a "bridge" between smoking and lung cancer.

Xu et al. [27] compared smokers with non-smokers and found that the methylation level of genes related to lung cancer in smokers decreased. Ma et al. [28] found that smoking carcinogenesis was closely related to DNA methylation in the study of the epigenetic mechanism of tobacco carcinogenesis. In a long-term study, Vaz et al. [29] found that long-term exposure of human bronchial epithelial cells to cigarette smoke would cause high methylation of gene promoters and activate key signaling pathways that drive tumorigenesis. They also found that these methylation abnormalities were highly consistent with the methylation abnormalities in non-small cell lung cancer (NSCLC). Obviously, hypermethylation of the gene promoter is very likely to cause NSCLC. Clark et al. [30] also found that long-term exposure of cells to cigarette smoke leads to progressive epigenetic changes. This change is a key factor in lung cancer. They concluded that environment-induced epigenetic changes could replace gene drive changes in cancer, laying the groundwork for cancer. Zhang et al. [31] found that the lower the methylation level of AHRR (cg05575921), 6p21.33 (cg06126421), and F2RL3 (cg03636183) in smokers, the higher the risk of lung cancer (Table 1). Moreover, they believe that smokers should be preliminarily screened by judging the methylation levels of characteristic sites, and the high-risk population should be further examined. Besides Baglietto et al. [32] found that the methylation level of 2q37.1 (cg21566642) site was the lowest among smokers (Table 1), and the methylation level increased gradually with the increase of smoking cessation time. They concluded that methylation levels at this site were closely related to the risk of lung cancer (Table 1). These findings suggest that smoking can change the methylation status of certain gene loci and lead to the development of lung cancer, especially central lung cancer (Fig. 3). Possibly, in the future, doctors could warn smokers to reduce or quit smoking by determining their risk of lung cancer based on their methylation status at specific sites in the body.

DNA methylation provides a new understanding of the pathogenesis of lung cancer, but more discoveries are needed to clarify how abnormal methylation causes lung cancer. Additionally, we still need to further explore which genes are closely related to the process of smoking leading to lung cancer. Only in this way can we judge the risk of lung cancer in smokers from the level of DNA methylation, and timely let smokers quit smoking, to prevent the occurrence of lung cancer.

3. DNA methylation is a warning sign of lung cancer

According to statistics, the five-year survival rate of patients diagnosed with lung cancer early is 92%. Those diagnosed with advanced lung cancer had a five-year survival rate of only 5%. So early diagnosis is very vital for patients. In the past two decades, early screening and timely treatment have led to significant reductions in lung cancer mortality rates in both men and women in the United States [33]. They achieved this by using a wide range of low-dose computed tomography (LDCT) tests, which predict the probability of lung cancer by imaging features of pulmonary nodules in patients [34]. However, this method has many drawbacks, including exposing patients to more radiation, low diagnostic accuracy, and low diagnostic efficiency [35]. Nowadays, DNA methylation, with its more sensitive and more stable advantages, can effectively compensate for the defects of imaging examination, and improve the early diagnosis rate of lung cancer.

Table 2
Summary of specificity and sensitivity of some methylation markers in early diagnosis.

Methylation marker	Sample type	Sensitivity	Specificity	References
RASSF1A	Carcinoma tissues bronchial brushing sample	64% 60%	100% 90%	[45]
RASSF1A	Plasma	SCLC: 52%[a]	SCLC: 96.2%[a]	[44]
RASSF1A, PCDHGB6, HOXA9	Carcinoma tissues bronchial brushing sample	92% 80%	100% 100%	[46]
RASSF1A, RARB2	Plasma	87%	75%	[48]
SHOX2	Plasma	60%	90%	[15]
SHOX2	Alveolar lavage fluid	68%	95%	[37]
SHOX2	Plasma	65.5%	90%	[49]
PTGER4	Plasma	56.3%	90%	[49]
SHOX2, PTGER4	Plasma	75.6%	84.8%	[49]
SHOX2, PTGER4	Plasma	67% 90%	90% 73%	[50]
SHOX2, RASSF1A	Bronchoalveolar lavage fluid	81%	97.4%	[47]
HOXA9	Plasma	SCC: 55.2%[b] SCLC: 63.9%[a]	SCC: 74.3%[b] SCLC:84.2%[a]	[44]
p16(INK4a), RARB2	bronchial aspirates	69%	87%	[13]

[a] This data refers to the sensitivity and specificity of methylation markers for screening small cell lung cancer (SCLC).
[b] This data refers to the sensitivity and specificity of methylation markers for screening squamous cell carcinoma (SCC).

3.1. Detection of single-gene methylation and early diagnosis of lung cancer

Studies have found that methylation of PTGER4, SHOX2, and RASSF1A genes in the body is more likely to cause lung cancer [14,16,36]. SHOX2 gene methylation showed a specificity of 90% and sensitivity of 60% in differentiating between the control group and lung cancer (Table 2) [15]. Schmidt et al. [37] analyzed the methylation of alveolar lavage fluid samples from patients with lung cancer. They found that SHOX2 methylation distinguished benign and malignant lung disease with 95% specificity and 68% sensitivity. In addition, they also found that SHOX2 gene methylation was more suitable for the diagnosis of lung adenocarcinoma (LUAD) and SCLC, with diagnostic sensitivity of 82% and 97% respectively (Table 2). RASSF1A gene is a tumor suppressor gene in experiments, and its methylation is closely related to NSCLC [38]. Hubers et al. [39] proposed that the high specificity of the RASSF1A gene could be maintained by methylation testing every two years. Liu et al. [40] extracted 94 tumor tissue samples, 143 sputum samples, and some blood samples from 155 lung cancer patients. When they tested it, they found that TMEM196 was methylated in the samples, but not in the control group. This finding provides strong evidence for TMEM196 gene as a marker for early diagnosis of lung cancer. Ponomaryova et al.[41] found that the methylation of the RARβ2 gene was associated with the occurrence of lung cancer ($P < 0.05$). Gainetdinov et al.[42] found that LINE-1 appeared hypomethylated in lung cancer patients, and lung cancer patients could be distinguished from normal individuals by the methylation level of LINE-1. In addition, L1RE1 hypomethylation is also thought to distinguish lung cancer tissue from adjacent tissue [43]. Nunes et al. [44] found that the sensitivity and specificity of HOXA9 gene methylation in the diagnosis of squamous cell carcinoma reached 55.2% and 74.3%, and the sensitivity and specificity of SCLC reached 63.9% and 84.2% (Table 2). In addition, they also found that RASSF1A methylation had a sensitivity of 52% and a

specificity of 96.2% for the diagnosis of SCLC (Table 2) [44].

3.2. Multi-gene combined with methylation detection and early diagnosis of lung cancer

In addition to using a single-gene for methylation detection, two or more gene methylation joint detection method also begins to emerge. Ma et al. [45] found that only RASSF1A was used for single-gene methylation analysis, with a sensitivity of 64% for carcinoma tissues and 60% for bronchial brushing samples (Table 2). However, if the three genes RASSF1A, PCDHGB6, and HOXA9 were tested for methylation, the sensitivity could be increased to 92% and 80%, respectively (Table 2). Studies have shown that SHOX2 and RASSF1A double gene methylation detection is greatly helpful in the detection of early lung cancer and the differentiation of benign and malignant pulmonary nodules. Its sensitivity is over 70% and specificity can be as high as 90% (Table 2), far higher than the traditional cytological detection method [46,47]. Other studies have shown that dual gene methylation testing of RARB2 and RASSF1A is helpful in the diagnosis of NSCLC, with sensitivity up to 87% and specificity up to 75% (Table 2) [48]. Xu et al. [49] used Epi ProLung to detect the methylation levels of SHOX2 and PTGER4 in samples to diagnose lung cancer. The results showed that the sensitivity and specificity of SHOX2 and PTGER4 methylation detection reached 75.6% and 84.8%, and the effect of double marker detection was better than single marker detection (Table 2). Weiss et al. [50] conducted the combined detection of SHOX2 and PTGER4 methylation in plasma samples from 118 lung cancer patients and 212 normal people. The results showed that the sensitivity was 67% when the specificity was 90% (Table 2). When the sensitivity was 90%, the specificity was 73% (Table 2). Grote et al. [13] detected the methylation of p16(INK4a) and RARB2 genes, and the sensitivity and specificity for the diagnosis of pulmonary malignant tumors were 69% and 87%, respectively (Table 2). Huang et al. [51] analyzed 151 studies related to adenocarcinoma (AC) and squamous cell carcinoma (SCC) and found that three hypermethylated genes (CDH13, RUNX3, and APC) and two hypomethylated genes (CDKN2A and O-6-methylguanine-DNA methyltransferase (MGMT)) may have the ability to distinguish AC from SCC. Further studies have found that CDH13 and APC have higher sensitivity and specificity for combined detection, and their results are of great significance for distinguishing AC and SCC.

The above studies show that the combined detection of dual-gene or multi-gene methylation has higher sensitivity and specificity than that of single-gene methylation detection. In the future, methylation detection may be more inclined to double-gene or multi-gene combined detection, because it can not only reduce the contingency of results, but also improve the accuracy of diagnosis, which is more conducive to early diagnosis.

3.3. Circulating tumor DNA (ctDNA) methylation: the mainstream of liquid biopsy in early diagnosis of lung cancer

3.3.1. Definition and action of ctDNA

As we all know, early diagnosis is very important for cancer patients. However, conventional imaging examinations such as CT, MRI are characterized by high misdiagnosis rate, difficulty in finding early tumors, and inability to reflect the metastasis of tumors in time [52]. Another method, biopsy, requires frequent sampling, which can cause additional pain [53]. This method also has the risk of promoting tumor metastasis [53]. Now, there is a new way to diagnose lung cancer by detecting the methylation status of certain markers in sputum, blood, bronchial fluid, and urine. This method has high sensitivity and specificity and is also known as the liquid biopsy [54–57]. The advantages of liquid biopsy are easy operation, accurate results, safe detection, and small sample size required for detection. At present, ctDNA methylation is still one of the classic detection objects of liquid biopsy technology.

CtDNA is from fragments of DNA produced by apoptosis, necrosis, or secretion of tumor cells, and is a part of cell-free DNA (cfDNA) [58]. Studies have shown that ctDNA is already present in peripheral blood in the early stage of tumor development, and it is also an excellent prognostic marker [59]. The researchers were able to detect more early lesions and improve lung cancer cure rates by detecting the methylation status of ctDNA than by detecting mutations in ctDNA [60]. Moreover, ctDNA methylation can be tested to find the site of the tumor [61].

3.3.2. Good news and bad news for ctDNA methylation tests

At present, ctDNA methylation detection has continued to have good news. Guo et al. [61] developed a high-throughput noninvasive methylation detection technique. This technique is the first to combine liquid ctDNA biopsy with methylation detection, greatly improving the sensitivity of early tumor screening. Also, it can significantly reduce background noise and localize tumors by screening for CpG methylation haplotypes. Hence the advent of this technology is considered to officially enter the 2.0 era of tumor liquid biopsy. Recently, Chen et al. [62] successfully realized early screening and early diagnosis of five types of cancer by using the independently developed PanSeer technology in China. They were able to detect cancer up to four years earlier by detecting microscale tumor methylation signals in human blood samples, compared with routine tests. Liang et al. [63] used targeted ctDNA methylation high-throughput sequencing technology to detect the methylation of ctDNA or specific CGIs in the blood samples of patients. The diagnostic sensitivity and specificity were both above 80%, and some were even as high as 96%. Some researchers have developed early ultrastructural DNA capture technology for tumors and rapid fluorescence in situ hybridization technology. These two technologies can respectively solve the disadvantages of difficult acquisition and enrichment of ctDNA and long sample detection time, providing a strong guarantee for early diagnosis and rapid diagnosis of lung cancer. Recently, Wang et al. [64] developed a new detection technology based on ctDNA methylated liquid biopsy, which can accurately distinguish benign lung nodules from lung cancer (especially LUAD), and has the potential for early screening of lung cancer.

However, ctDNA methylation detection still faces many difficulties and challenges, including low ctDNA content and the treatment of background noise. Additionally, although there are many methods for ctDNA detection, there is still no unified standard for the detection process. In the future, every link of ctDNA detection from pre-analysis to the analysis process should be standardized.

3.4. CfDNA methylation: a potential cancer screening tool

At present, lots of studies have shown that there is inter-tumor heterogeneity between tumor cells [65,66]. Therefore, the detection of single tumor tissue isn't conducive to the accuracy of treatment, and the detection of multiple samples doesn't meet the clinical requirements. Noninvasive diagnosis by detecting cfDNA methylation in body fluids can effectively solve the problem of inter-tumor heterogeneity. CfDNA is mainly derived from fragmented DNA during apoptosis, necrotic cell DNA fragments, and exosomes secreted by cells. Studies have shown that cfDNA has a methylation profile similar to that of its tissue-derived DNA [67]. Moreover, cfDNA methylation can be used in early diagnosis and screening, organ transplantation evaluation, noninvasive prenatal testing, and other aspects [68]. Studies have shown that early lung cancer can be detected using cfDNA methylation [69,70]. Therefore, the detection of cfDNA methylation is helpful for the accurate diagnosis of cancer.

Epi ProLung is the world's first product for the early diagnosis of lung cancer by detecting cfDNA methylation in the blood. Xu et al. [49] reaffirmed its effectiveness in the diagnosis of lung cancer in the experiment. Liu et al. [71] developed a genome-wide methylation detection technique for detecting cfDNA methylation, which can detect 9223 CpG sites at a time with high detection accuracy. It is very beneficial in the early diagnosis of cancer and the classification of cancer. Xu

et al. [72] used Methylated DNA Immunoprecipitation Sequencing (MeDIP-seq), a genome-wide methylation detection technology, to detect cfDNA methylation and successfully found some methylation markers related to lung cancer. Shen et al. [73] developed a methylation detection technology based on immunoprecipitation and named it cell-free methylated DNA immunoprecipitation and high-throughput sequencing (cfMeDIP–seq). This method only needs 1–10 ng of plasma cfDNA as samples, and the analysis of cfDNA methylation can be used to determine whether there are early tumors in the human body. At present, the detection technology of cfDNA methylation has entered the stage of clinical verification internationally, and the biggest obstacle is still how to ensure the efficient utilization of cfDNA.

With further research, more and more markers of methylation have been discovered. Methylation detection has also gradually shifted from single-gene methylation detection to multi-gene combined methylation detection, and the proportion of minimally invasive and non-invasive detection in detection methods is increasing. The liquid biopsy technology based on noninvasive detection has been proved to be used for the early detection of lung cancer with high sensitivity and specificity in experiments. However, we should also recognize that the sensitivity and specificity of most of the current methylation markers do not meet the clinical requirements. We also need to find more markers with higher sensitivity and specificity. Additionally, these markers should undergo clinical verification of a large number of samples before entering into clinical application, so as to ensure their specificity and sensitivity.

4. DNA methylation is associated with lung cancer progression and metastasis

4.1. DNA methylation may lead to the progression and metastasis of NSCLC

Yu et al. [74] extracted and compared the lung tissues of normal people, NSCLC patients with cancer metastasis, and NSCLC patients without cancer metastasis. They discovered that methylation of the EPHB6 gene has occurred in patients, and that the higher the level of methylation of the EPHB6 gene, the higher the risk of cancer metastasis (Table 1). In the follow-up studies, they also found that the gene was a transfer suppressor gene. Hwang et al. [75] tested tissue samples from 324 NSCLC patients with methylation-specific high-resolution melting (MS-HRM) and EpiTYPER (TM), and found that the promoter of the HS3ST2 gene was highly methylated. They also found that the gene can inhibit cell migration, invasion, and proliferation. Consequently, they believed that HS3ST2 hypermethylation was a significant indicator for the prognosis of NSCLC patients (Table 1). Ma et al. [76] found that TMEM88 gene hypermethylation occurred in 12 NSCLC samples compared with the corresponding 12 non-cancer samples, and the higher the degree of methylation, the shorter the survival time of patients (Table 1). When they treated NSCLC cell lines with demethylating reagents, the ability of cancer cells to proliferate, invade, and metastasize was significantly reduced. Experiments showed that the TMEM88 gene was probably a tumor suppressor gene, and its methylation was closely related to the poor prognosis of NSCLC. These studies suggest that methylation of tumor suppressor genes may be the cause of lung cancer progression and metastasis, and demethylation of tumor suppressor genes may prevent lung cancer metastasis.

Zhang et al. [77] found that deficiency of DAL-1 protein makes NSCLC more likely to metastasize (Table 1). Further studies revealed that the DAL-1 deletion was due to methylation of the DAL-1 gene promoter. Soes et al. [78] extracted tumor samples from NSCLC patients with tumor metastasis and compared them with those without tumor metastasis. They found that promoter hypomethylation of the ELMO3 gene occurred in samples with cancer metastasis, and that the gene expression was much higher than in samples without cancer metastasis. Thus, they hypothesized that the hypomethylation of the ELMO3 gene was associated with metastasis of NSCLC (Table 1). Both studies suggest that the degree of methylation of some genes may be associated with NSCLC metastasis, but it has yet to be tested for clinical use.

4.2. DNA methylation and brain metastases in lung cancer

Lung cancer brain metastases are very common in lung cancer patients, with brain metastases occurring in 10%–36% of lung cancer patients [79]. And the prognosis of such patients is very poor. Consequently, finding a reliable target to predict the risk of brain metastases from lung cancer has always been the goal of scientists. Fan et al. [80] collected data of 60 patients with advanced lung cancer and divided them into groups A, B, and C. Group A included 18 patients with lung cancer with brain parenchyma metastasis, group B included 11 patients with lung cancer with meninges metastasis, and group C was the control group. Through methylation analysis, it was found that there were different methylation sites in groups A and B compared with group C, and there were 15 identical sites in both groups. In other words, the methylation of some genes in these regions leads to brain metastasis of lung cancer, and methylation of different genes leads to different degrees of metastasis of cancer cells. This study is the first to demonstrate that DNA methylation can help predict the risk of brain metastases from lung cancer. Hashimoto et al. [81] examined genomic DNA from 55 NSCLC patients who had undergone a brain operation and continued to observe the prognosis of the patients. They found that the average time for tumor metastasis or local recurrence was four months for patients with the methylated promoter region of MGMT, and 11.5 months for patients without the methylated promoter region of MGMT (Table 1). Thus, they believe that MGMT can be an important prognostic marker for NSCLC patients with brain metastasis. Both studies explored the causes of lung cancer brain metastases from the perspective of DNA methylation and also showed that DNA methylation could be used to predict the risk of lung cancer brain metastases. These findings are of great significance for doctors to detect brain metastases of lung cancer as early as possible and adjust treatment or rehabilitation programs in time.

5. DNA methylation and lung cancer treatment

DNA methylation also plays a significant role in lung cancer treatment. Through the methylation status of markers, we can more clearly define the type and stage of lung cancer, so as to develop the corresponding treatment plan [44]. Currently, 10% of all cancers are of unknown origin or are metastatic. Therefore, it is only by finding the source of such cancers that patients can be effectively treated to maximize their chances of survival and recovery [82]. DNA methylation is expected to solve this problem.

5.1. DNMT and lung cancer therapy

One idea that researchers have proposed for cancer treatment is to demethylate certain tumor suppressor genes and DNA repair genes so that they can express normally and inhibit tumor development. Currently, DNA methyltransferase inhibitors (DNMTis) have been studied the most. Studies have shown that DNMT is a major target of tumor epigenetic drugs [83].

Topper et al. [84] found that the therapeutic schedule of DNMTis combined with histone deacetylase inhibitors (HDACis) is of great help in the treatment of NSCLC. This combination therapy has been shown to inhibit the proliferation and metastasis of cancer cells in both human and mouse models of NSCLC. The clinical application shows that DNMTis 5-Azacytidine (5-AzaC) and its analogue 5-Aza-2'-deoxycytidine (5-Aza-CdR) have the function of demethylating tumor suppressor genes to inhibit tumors. However, 5-Aza-CdR has some defects that can't be ignored, including its weak specificity and the risk that improper dosage may lead to tumor metastasis. Therefore, it limits the clinical use of these two drugs. Besides, it remains to be seen whether the

use of DNMTis will change the methylation of other genes.

5.2. Tumor mutation burden (TMB) combined with DNA methylation in the treatment of lung cancer

Anti-programmed cell death protein 1 (PD-1) and anti-programmed cell death ligand 1 (PD-L1) immunotherapy are effective in the treatment of NSCLC. However, how to accurately screen patients suitable for this therapy has become a major problem. TMB has been proposed as a predictive marker of immunotherapy efficacy. TMB refers to the total number of genic coding errors, base substitutions, and genic insertion or deletion errors that occur per million bases [85]. Studies have shown that TMB can effectively predict the therapeutic effect of PD-L1 antibody [86]. In addition, the higher the TMB, the better the effect of the PD-1 inhibitor [85]. However, it has been found in clinical studies that the performance of this marker needs to be improved. Studies have shown that DNA methylation is also a good marker. So, what is the correlation between TMB and DNA methylation, and would it be better to combine them as molecular markers? Cai et al. [87] conducted relevant studies and found that the epigenetic variation of lung cancer was related to the TMB. The results showed that the correlation between TMB and DNA methylation was mainly manifested in three aspects: 1. TMB degree was positively correlated with DNA methylation level; 2. The higher the TMB, the more unstable the DNA genome; 3. The promoter region of the gene is the main site of DNA methylation in the high TMB group. This finding is the first to confirm the association between TMB and DNA methylation, and it is of great significance for the immunotherapy of NSCLC.

5.3. Epigenetic therapy has led to the development of other cancer therapies

Now, the discovery of DNA methylation in tumorigenesis and progression has also led to the development of targeted therapies. Researchers can develop targeted drugs to demethylate tumor suppressor genes and DNA repair genes or to methylate overexpressed proto-oncogenes in order to suppress tumors. Additionally, epigenetic therapy and immunotherapy are more and more closely combined, and they depend on and promote each other. Currently, combination therapies combining epigenetic regulators such as DNMTis with immune checkpoint PD-1/cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) have shown promising application prospects in the treatment of tumors [88]. We assume that in the future, epigenetic therapy will be more closely combined with immunotherapy, radiotherapy, and chemotherapy, and these combined therapies will become the mainstream of cancer treatment.

The good news for the epigenetic field is that new epigenetic drugs are being put into early clinical trials to treat cancer recurrence and drug resistance. However, the low specificity of the drug and the high side effects are challenges that we need to overcome. In the future, new epigenetic drugs may be used to maintain the effectiveness of chemotherapy in patients. With the development of epigenetic studies, methylation modification signals are bound to play a more important role in cancer treatment.

6. DNA methylation can be an important marker for lung cancer prognosis

Prognostic markers are a kind of marker that can judge whether a patient's tumor will metastasize or relapse and judge a patient's five-year survival rate. DNA methylation can regulate gene expression, and it is helpful to study the mechanism of drug resistance and track the development of cancer. Therefore, DNA methylation is a good prognostic marker.

6.1. DNA methylation is a marker for predicting drug efficacy

Wu et al. [89] found that the promoter of the DNA repair gene HMLH1 was hypermethylated in NSCLC cells resistant to cisplatin. When they treated it with demethylated drugs, the resistance fell significantly. Therefore, they believed that the methylation status of HMLH1 was an important indicator to judge the efficacy of drugs (Table 1). Similarly, high levels of IGFBP-3 methylation were also present in cisplatin resistant cells, but not in cisplatin sensitive cells (Table 1) [90]. Fischer et al. [91] found that methylation of the RASSF1A gene was closely associated with better efficacy of gemcitabine in NSCLC patients (Table 1), and they believed that it was a vital prognostic marker for NSCLC. Hiddinga et al. [92] demonstrated that methylation of MGMT in SCLC patients can make the alkylating agent temozolomide more effective (Table 1). These studies suggest that the methylation status of specific genes can also reflect the effectiveness of drugs, and also provide doctors with a new way to solve the problem of drug resistance in tumor cells.

6.2. Other methylation markers for lung cancer prognosis

Liu et al. [22] found that the binding of DNA methylation sites to transcription factors could be a marker of prognosis in cancer patients. Saito et al. [93] detected and analyzed the pathological tissues of 28 SCLC patients after surgery, and found that the CpG island methylation phenotype (CIMP) was an important indicator of poor prognosis of SCLC patients. SHOX2 gene methylation has been shown to act as a predictive marker for the prognosis of NSCLC [15]. Studies have shown that lung cancer patients with methylated TMEM196 gene [40] and HERC5 gene [94] have a poor prognosis, and their survival rate is significantly lower than that of patients with high expression of this gene (Table 1). Yao et al. [95] found that methylation of the GRK6 gene promoter was associated with poor prognosis (Table 1). Further studies showed that DNA methylation promoted the metastasis of LUAD cells by inhibiting the binding of CCAAT/enhancer-binding protein-α (C/EBPα). Yu et al. [96] found that hypomethylation of the FAM83A gene predicted poor prognosis in patients with LUAD (Table 1). Ponomaryova et al. [48] found that the methylation levels of RARB2 and RASSF1A genes could help in evaluating the effect of treatment in lung cancer patients. In summary, we can judge the prognosis of patients by the methylation level of specific genes.

6.3. CtDNA is an important prognostic marker in patients with advanced NSCLC

In recent years, ctDNA methylation has also made great progress in the study of prognosis. Pecuchet et al. [97] used targeted next-generation sequencing (NGS) to detect ctDNA and found that ctDNA zeroing was a marker of a large reduction in tumor size in patients with advanced NSCLC after treatment. It also indicates that the progression-free survival (PFS) and overall survival (OS) of the patients can be prolonged. Another study also confirmed that a significant reduction in ctDNA predicted better objective response rate (ORR), longer PFS, and longer OS in advanced NSCLC patients treated with durvalumab [98]. Therefore, reducing by leaps and bounds or zeroing of ctDNA is a crucial prognostic marker for patients with advanced NSCLC. Moreover, the researchers believe that the first test after treatment is the most crucial monitoring node. Besides, studies have shown that compared with imaging examination, ctDNA detection can identify residual disease after lung cancer treatment about 5 months earlier, which is of great benefit for follow-up treatment of lung cancer [99].

In summary, DNA methylation can be used as a significant marker of drug efficacy, five-year survival, and lung cancer recurrence. In the future, doctors can judge the prognosis of patients from the perspective of DNA methylation, and then make certain adjustments to the patient's treatment.

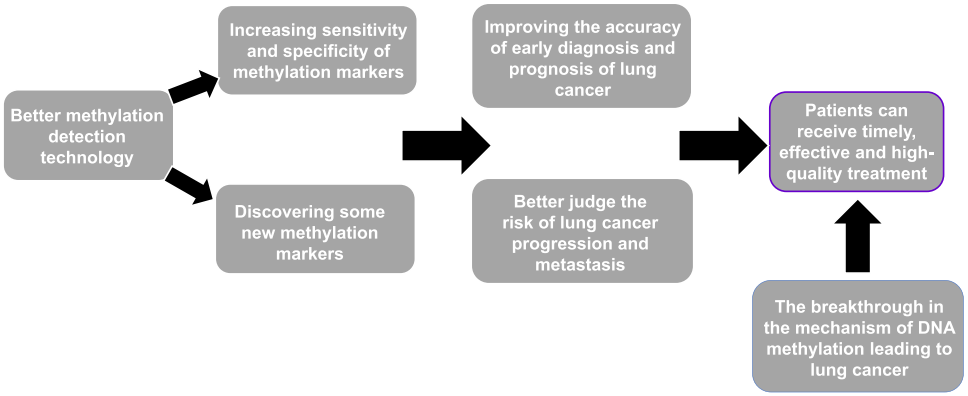

Fig. 4. Association between the pathogenesis, early diagnosis and screening, progression and metastasis, treatment, and prognosis of lung cancer. Better DNA methylation detection techniques can make better use of samples and find highly sensitive and specific methylation markers. Markers can be used for early diagnosis and screening of lung cancer, progression and metastasis, and prognosis. Excellent methylation markers can improve the accuracy of lung cancer diagnosis and prognosis, and doctors can better predict the risk of lung cancer progression and metastasis. With improved accuracy in early diagnosis, prognosis, progression and metastasis, patients can receive timely, effective, and high-quality treatment. In addition, elucidating the mechanism by which DNA methylation leads to lung cancer may also be beneficial for patient treatment. The fundamental goal of all this work is to improve the five-year survival rate for lung cancer patients.

7. Conclusion and perspective

Over the years, DNA methylation has become a hot topic in life science research, with new technologies and discoveries emerging. In this review, the important role of DNA methylation and the latest progress of lung cancer were reviewed in terms of pathogenesis, early diagnosis and screening, progression and metastasis, treatment, and prognosis. Actually, the links between the pathogenesis, early screening, progression and metastasis, treatment, and prognosis of lung cancer are very close (Fig. 4). Additionally, we believe that only when patients receive timely, effective, and high-quality treatment can the five-year survival rate of lung cancer patients be improved. Then, the status of lung cancer as cancer with the highest morbidity and mortality rates in China has been gradually changed.

Currently, there are three main reasons for limiting the clinical application of DNA methylation. First, the specificity and sensitivity of most methylation markers are not high enough to be widely used in the clinic. Second, a small number of markers have high specificity and sensitivity, which may not be true due to the limited test samples. Therefore, large-scale sample trials should be conducted before clinical use for all markers, but the trials in this area are currently still lacking. Third, the lack of highly specific and sensitive detection techniques. Methylation detection methods are usually minimally invasive or non-invasive, and the amount of methylation markers in body fluids is very low, so high specificity and sensitivity detection techniques are required to effectively utilize these samples. However, it is not easy to develop a methylation detection technique with high specificity and sensitivity and suitable for widespread application.

Liquid biopsy, as a part of DNA methylation detection, is a promising method for cancer diagnosis. This paper mainly introduces the progress of ctDNA and cfDNA. At present, sputum methylation detection is widely used. On the one hand, the combination of sputum methylation detection and imaging examination can effectively prevent the missed diagnosis of central lung cancer (Fig. 3); on the other hand, sputum methylation detection can be performed at home, which is more convenient than other invasive operations (Fig. 3). However, the sensitivity and specificity of sputum methylation detection still need to be improved. Liquid biopsy isn't a substitute for tissue biopsy due to its low sensitivity, low DNA content in body fluids, and few methylation markers. The biopsy will remain the gold standard of tumor diagnosis for a long time to come. In the future, the development of liquid biopsy will mainly solve two problems, one is how to efficiently use the limited samples, and the other is how to make sensitivity and specificity of liquid biopsy close to or exceed biopsy. Only by solving these two problems can liquid biopsy become the main force in lung cancer diagnosis.

With the development of research, people will understand more and more about the role of DNA methylation in the pathogenesis, early diagnosis and screening, progression and metastasis, treatment, and prognosis of lung cancer. However, DNA methylation still has a long way to go from scientific research to clinical practice. It is believed that with the efforts of scientists, DNA methylation will be more widely used and more patients will benefit from it.

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CL and XZ conceived the work. RL wrote and drafted the manuscript. XL, WL, XZ and CL discussed and edited the manuscript. All authors read and approved the final version of the manuscript.

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Consent for publication

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