Lung cancer epigenetics: emerging biomarkers

Lung cancer is the leading cause of cancer-related deaths worldwide, and the 5-year survival rate is still very poor due to the scarcity of effective tools for early detection. The discovery of highly sensitive and specific biomarkers highlighting pathological changes early enough to allow clinical intervention is therefore of great importance. In the last decade, epigenetics and particularly research on DNA methylation have provided important information towards a better understanding of lung cancer pathogenesis. Novel and promising molecular biomarkers for diagnosis and prognosis of lung cancer are continuously emerging in this area, requiring further evaluation. This process includes extensive validation in prospective clinical trials before they can be routinely used in a clinical setting. This review summarizes the evidence on epigenetic biomarkers for lung cancer, focusing on DNA methylation.

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Lung cancer is the most common malignancy affecting both genders and remains the main cause of cancer-related deaths worldwide [1]. Only 13% of lung cancer patients survive more than 5 years. Lung cancers are classified according to histological types and this classification has important implications for the clinical management and prognosis of the disease. There are two main histological groups of lung cancer, non-small-cell lung cancer (NSCLC) and small-cell lung cancer; approximately 85% of lung tumors are NSCLC, which comprise three major histological subtypes: adenocarcinoma, squamous cell carcinoma and large-cell carcinoma [2].

Initiation and progression of lung carcinoma is the result of the interaction between genetic, epigenetic and environmental factors. In lung cancer, epigenetic alterations such as promoter DNA methylation that leads to gene silencing are common events. These alterations can take place in defined nuclear positions and chromosome domains after exposure to environmental risk factors such as smoking, drugs and chronic inflammation [3]. Epidemiological studies indicate that cigarette smoking has a strong association [4], since approximately 80–90% of lung cancers are attributable to cigarette smoking [5]. Although the majority of lung cancer patients are smokers, only a minority of lifetime smokers will develop the disease. This strongly indicates that genetic and/or epigenetic susceptibility plays a causal role in lung carcinogenesis [6].

Early detection of lung carcinoma could change the disease outcome; in fact, the survival rate can increase dramatically. In the effort to improve early detection, many imaging and cytology-based strategies have been employed; however, none has yet been highly effective, either because of limited sensitivity or the prohibitive cost they bear to public health systems [7,8]. A report on detection of early-stage cancers using low-dose spiral (helical) computed tomography (CT) described a 10-year survival rate of 88% [7]. The recent low-dose spiral CT protocols can be very efficient in detecting small tumors [9,10]; however, the number of indeterminate nodules is high, posing questions on the appropriate management of these patients [11]. It is now widely accepted that epidemiological risk modeling is required for stratification of individuals for CT screening [12]. In addition, one of the major unmet clinical needs is the inclusion of new molecular biomarkers on non- or minimally invasive samples to assist early diagnosis, prognosis and prediction of response to treatment.

Understanding the molecular pathways within lung cancer, and focusing on their molecular heterogeneity, is the most effective way towards the development of novel diagnostic and therapeutic tools. In the last decade, a plethora of molecular factors all involved in lung carcinogenesis have been evaluated as prognostic biomarkers, such as markers of apoptosis (BCL-2), cellular adhesion

and cellular growth (RAS, EGF receptor) and tumor proliferation [13]. The recent advances in the field of lung cancer epigenetics provide a very promising step towards the direction of novel biomarker development. Epigenetics refer to altered phenotypes (e.g., patterns of gene expression) mediated by changes other than an alteration in the primary nucleotide sequence. Such epigenetic changes are DNA methylation, miRNAs, nucleosome remodeling and covalent histone modifications [14].

This review summarizes the emerging epigenetic biomarkers in lung cancer, focusing on DNA methylation and their potential clinical applications in lung cancer.

Tumor biomarkers

The National Cancer Institute defines a biomarker as a biological molecule found in blood, other body fluids or tissues that is a sign of a normal or abnormal process or of a condition or disease. Tumor biomarkers offer good guidance on many areas of cancer biology. They are not only useful in early diagnosis of cancer but can also provide important prognostic information, forecasting how aggressive the disease process is and distinguish the tumor's outcome [15]. Another important application is to predict the response to therapy; predictive biomarkers evaluate the probable benefit of a particular treatment. The clinical information given by tumor biomarkers is significant in the selection of appropriate treatment, leading to personalized cancer therapy [16]. There are several distinct types of tumor biomarkers based on different areas: genetics, epigenetics, proteomics, metabolomics and imaging technology.

DNA methylation as a tumor biomarker

Epigenetic abnormalities are present in all human cancers and are currently considered to be the hallmark of cancer initiation and progression. There is increasing information on the extent of epigenetic reprogramming of cancer cells, utilizing high-throughput methodology, such as microarrays and next-generation sequencing. Currently, the best-studied epigenetic event in the mammalian genome is DNA methylation, which is a chemical covalent modification of cytosine. In particular, is the addition of a methyl group (CH_3) at the fifth carbon position of cytosine bases that are located 5′ to a guanosine in a CpG dinucleotide. The distribution of CpG dinucleotides in the genome is uneven and CpG dinucleotides are found to be concentrated in short regions called CpG islands (CGIs). Although CGIs comprise less than 1% of the genome, they are most frequently located in gene promoter regions and are generally unmethylated in normal adult cells. Increased methylation (hypermethylation) in the promoter region of a gene leads to reduced expression, whereas methylation in the transcribed region (gene body) has a variable effect on gene expression [17,18].

Cancer cells have an aberrant methylation signature; they present global hypomethylation [19] and hypermethylation of tumor suppressor genes (TSGs) [18–20]. Global hypomethylation is frequent in NSCLC and is associated with genomic instability [21] and aberrant overexpression of oncogenic gene isoforms [22]. A large number of aberrantly methylated genes have also been identified in lung cancer, either by high-throughput methods [23–35] or target-based approaches [36–38]. Furthermore, methylation has been described as an early event in lung tumorigenesis. A well-studied example of early hypermethylation is that of *p16*, contributing significantly to its transcriptional silencing [39–41]. Additional examples include H-cadherin [42], *RASSF1A* [43], *APC* [44,45] and *DAPK1* [46]. This plethora of high-throughput and gene-focused approaches has now provided a very long list of candidate markers for lung cancer diagnosis and prognosis, and predictive information.

The virtually universal presence of DNA hypermethylation in all types of cancer makes it an ideal candidate tumor biomarker. Compared with other molecular marker classes such as mRNA and proteins, DNA methylation has many advantages [47]. First, DNA methylation is a covalent modification of DNA, so it is chemically stable and can survive harsh conditions for long periods of time. Second, through simple procedures it can be readily amplifiable and easily detectable. In addition, contrary to cancer-specific mutations, which are relatively rare and present in different gene positions, the incidence of aberrant methylation of specific CGIs is much higher [48–50], and moreover such methylation can be discovered by genome-wide screening procedures [51,52]. Methylation-specific PCR has been extensively used to detect aberrant methylation even in cases where methylated sequences are rare [53]. Methylation can be a very early indication of neoplastic transformation, since it has been observed even in early stages

of carcinogenesis and in non-neoplastic tissues. For example, methylation of noncore regions of p16 is observed in pulmonary hyperplasia (17%), dysplasia (24%) and lung carcinoma *in situ* (50%) [39,54]. DNA methylation usually affects longer DNA areas within promoters as opposed to point mutations that affect a single nucleotide and could variably occur along the gene sequence. Therefore, methylation profiling is less laborious and less expensive than mutation profiling.

Recent advantages in DNA methylation detection techniques include powerful tools such as sodium bisulfite (SB) conversion, restriction genomic scanning and CGI microarrays [55]. SB conversion by itself is not a DNA methylation detection technique, but just the first step for some of the assays listed, such as methylation-specific PCR. CGI methylation can distinguish the normal and aberrant patterns of methylation, since following SB treatment, 'converted' DNA can be used in many PCR-based techniques. A routine DNA methylation diagnostic tool has to be sensitive and reproducible, with fast and standardized protocols able to facilitate automation [56]. Currently, quantitative methylation-specific PCR (qMSP) seems to hold many of these characteristics and has demonstrated very good performance in clinical samples such as bronchial washings [57] and plasma [58]. However, significant development is required for these assays to reach good clinical laboratory practice standards [59].

Aberrant DNA methylation can be applied to cancer diagnostics in three different levels of clinical management. First, aberrant methylation specifically present in cancer cells can be used in diagnosing cancer cells in biopsies or body fluids, that is, detecting cell-free DNA (cfDNA) circulating in plasma. Second, aberrant methylation at certain CGIs is associated with disease phenotype(s), such as histological type, degree of differentiation, aggressive behavior, response to chemotherapy regimens or association with adverse drug effects; it can be used as a prognostic or predictive marker in managing treatment and follow-up of diagnosed cases. Third, if aberrant methylation of some CGIs in noncancerous tissues is associated with a risk for cancer development, it can be used as a cancer risk marker.

Currently, DNA methylation appears as one of the most promising epigenetic biomarkers, which can improve the early detection of cancer and subsequent management of patients with diagnosed malignancy. However, how simple is the development of a biomarker? How far are we from the clinical validation of methylation biomarkers? Recently the DNA methylation profile distinguishing epithelial-like from mesenchymal-like NSCLC, which display different response to anti-EGFR therapy, was studied [60]. For this purpose, a genome-wide analysis was first performed in epithelial- and mesenchymal-like NSCLC cell lines [60]. The most discriminating differentially methylated regions classified the cell lines as epithelial or mesenchymal and the methylation data were validated by direct sequencing of cloned fragments. When qMSP assays were performed on the 13 most discriminative genes, ten out of the 13 genes were able to classify cell lines, while seven of them were associated with *in vitro* erlotinib resistance [60]. When the methylation status of *ERB2* and *ZEB2* was more precisely investigated by pyrosequencing and qMSP in NSCLC cell lines, primary tumors and biopsies of patients resistant to chemotherapy, it was shown to be predictive of an epithelial-like phenotype and could be further used for diagnostic purposes [60].

To facilitate similar procedures for the discovery and validation of DNA methylation-based biomarkers, computational methods for optimized biomarkers selection, evaluation of performance and assay design must be developed [61]. At this point, the necessity of longitudinal retrospective studies should be underlined. Prior to attempting to develop assays for routine clinical use, these preliminary findings must be confirmed in studies with high statistical power.

DNA methylation has been detected in a number of body fluids of patients with cancer. In some tumor types this may be an alternative to tissue biopsy or paraffin-embedded tissues. These samples may provide a less invasive approach for disease detection and monitoring compared with tissues. In lung cancer, aberrant DNA methylation can be detected in the sputum of patients [62,63], in bronchoalveolar lavage [64–66] and saliva [67,68]. It has been suggested that this may also play a role in early detection and assessment of lung cancer risk. Methylation of the *p16* and *MGMT* genes has been detected in the sputum up to 3 years prior to diagnosis of squamous cell carcinoma [41]. Interestingly, DNA methylation has been reported in exhaled breath from lung cancer patients [69].

Circulating tumor DNA

Over the last decade, there has been a significant boost in blood-based DNA methylation assays in clinical research, as it is a potential source of minimally invasive samples, mainly targeting cfDNA in serum or plasma [70–72], or DNA extracted from peripheral blood leucocytes [73], and since it has become clear that hypermethylation can be detected in tumor-derived DNA circulating in plasma or serum of cancer patients. The precise mechanism by which DNA is released into the bloodstream still remains uncertain. Among the hypotheses explaining the presence of cfDNA in plasma or serum, active release of tumor DNA and passive DNA leakage following necrosis or apoptosis of neoplastic cells are discussed [74]. In a very recent study, a connection between cfDNA and circulating tumor cells from the same patients has been reported for the first time, based on the methylation status of *SOX17* [75]. cfDNA maintains the genetic and epigenetic profile of its primary tumor source [76,77]. It must be noted that cfDNA from plasma is not identical to cfDNA isolated from serum; the most obvious difference is the higher levels of cfDNA in serum than in plasma. Apparently, additional DNA in serum is associated with the clotting process [78,79]. Lower concentrations of cfDNA in plasma compared with serum better reflect cfDNA in circulation and also the disease [80,81]. A plethora of loci have been reported to be methylated in the plasma/serum of NSCLC patients suggesting novel potential diagnostic and prognostic targets. Kneip *et al.* have recently reported that DNA methylation of *SHOX2* could be used as a biomarker to distinguish between malignant lung disease and controls at a sensitivity of 60% and a specificity of 90% [82]. Cancer in patients with stage II (72%), III (55%) and IV (83%) disease were detected at a higher sensitivity when compared with stage I patients. Small-cell lung cancer and squamous cell carcinoma were detected at the highest sensitivity when compared with adenocarcinomas. Based on this study, *SHOX2* DNA methylation could be used as a biomarker for detecting the presence of malignant lung disease in blood plasma from patients with lung cancer [82]. In a recent study, Begum *et al.* tested the methylation status of six genes (*APC, CDH1, MGMT, DCC, RASSF1A* and *AIM1*) in serum of a limited number of lung cancer patients for elucidation of the diagnostic application of this panel of markers [45]. Their results indicate that *DCC*

has 100% specificity, and 35.5% of the 76 lung cancer patients were correctly identified. However, this approach needs to be evaluated in a larger test set to determine the role of this gene set in early detection and surveillance of lung cancer [45]. Several studies have examined DNA methylation in the primary tumor and corresponding plasma/serum and in most cases methylation in blood was only seen in patients with methylated primary tumor DNA [83]. The development of a blood-based test for lung cancer based on gene promoter methylation could augment current early detection approaches such as CT scans and sputum cytology. The methylation markers of lung cancer show great potential in molecular detection approaches. As was very clearly shown, these markers can be detected in the plasma of stage I lung cancer patients and therefore show promise in the early detection of lung cancer [58]. However, it is still very difficult to find these methylation events in serum DNA with a high enough degree of sensitivity and specificity. For this reason, quantitative and reliable estimation of DNA methylation levels for multiple genomic regions pose a major challenge where starting DNA is available in very low quantity, as in plasma or serum samples. Major advances in the development of techniques for the quantitative detection of DNA methylation in a minute amount of DNA, particularly valuable in profiling a large sample series of body fluids from molecular epidemiology studies, were recently reviewed [84]. With currently available methods for the simultaneous detection of methylation, up to 28,000 CGIs can be analyzed. Overall, we are just at the beginning of translating these findings into the clinic and there is hope that future patients will benefit from these results [84]. However, we do not want to overestimate the applicability of methylation tests for diagnosing cancer in serum or plasma-derived DNA; unless follow-up in larger cohorts is available, such evidence remains anecdotal.

DNA methylation as a tumor biomarker in lung cancer

■ Early detection

A diagnostic molecular biomarker would ideally reflect early steps in cancer development. It thus needs to be able to discriminate between neoplastic transformation and preneoplasia. A variety of epigenetic biomarkers have been evaluated so far in lung cancer diagnostics.

The promoter of *p16* was probably the first to be shown hypermethylated early in lung carcinogenesis [54,85] and a number of studies followed showing abnormal hypermethylation of many gene promoters such as *APC*, *RARb*, *NeuroD*, *RASSF1* and *MGMT* [86–91]. Recently, the diagnostic value of *RASSF1A* methylation and *KRAS* mutations in bronchial washings reported a 29% efficiency of detecting malignancy in false-negative or ambiguous cytology outcomes [92]. *SHOX2* quantified methylation showed promising results in bronchial aspirates, with a sensitivity of 78% and a specificity of 96% [93] while *SHOX2* sensitivity and specificity in plasma was 60 and 90%, respectively [82]. A group of genes (*APC*, *RASSF1A*, *KLK10*, *CDH13* and *DLEC1*), when tested in plasma, demonstrated 84% sensitivity and 74% specificity (TABLE 1).

■ Prognosis & prediction

Conventionally, tumor characteristics such as pathological subtype, nodal invasion and metastasis are used to predict disease outcome. In addition to these traditional prognostic factors, aberrant DNA methylation could refine prognostic information [94]. Brock *et al.* found aberrant patterns of promoter methylation of *APC*, *RASSF1A*, *p16* and *CDH13* associated with early recurrence in stage I NSCLC [95]. In support of this notion, Yamamoto *et al.* indicated that synchronous methylation of *CDH13* and *p16* is a more effective prognostic biomarker than *p16* alone [96]. Hypermethylation of TSGs is mostly associated with worst outcome. Zhang *et al.* explored the methylation status of 15 genes by methylation-specific PCR in 64 paired NSCLC and adjacent normal tissues [97]. The authors found that patients with a simultaneous methylation of four or more genes had poorer 2-year progression-free survival [97]. Recently, promoter methylation of *BRMS1* was shown to correlate with smoking history and poor survival in NSCLC [98]. Similarly, DNA methylation on ten genes has been reported to predict survival of stage I NSCLC patients [99] while *SHOX2*, analyzed in 474 paraffin-embedded samples from NSCLC patients, also seems to contribute in predicting disease outcome (TABLE 2) [100].

Methylation of miRNA loci as novel biomarkers in lung cancer

miRNAs are considered novel epigenetic regulators of gene expression, playing important roles in various biological processes

such as development, proliferation, cellular differentiation and apoptosis, through silencing of specific target genes [101–105]. Changes in miRNA expression levels have been detected in many human tumor types and recent studies have demonstrated their critical role in cancer pathogenesis [106–108]. Numerous recent studies indicated that miRNAs may function as TSGs or oncogenes [109]. Investigating the expression patterns and functions of miRNAs in lung cancer is still at an early stage, although some of them, such as *let-7* and *miR-128b*, are known to be aberrantly expressed in lung carcinoma. *let-7*, a well-understood example since it is one of the first identified miRNAs, seems to function as a tumor suppressor in lung cancer [110]. Overexpression of this miRNA may inhibit the expression of Ras [111] and repress proliferation of lung cancer cells [112] while overexpression of *miR-206* can inhibit migration and invasion of lung cancer cells [113]. *Let-7a3* and *miR-17-92* are oncogenic miRNAs, and are upregulated in lung cancer cells [114].

DNA methylation has been shown to affect the expression levels of miRNAs [115], while miRNAs can be regulators as well as targets of DNA methylation. Through aberrant DNA methylation miRNAs can be activated or silenced [116–118]. When *let-7-a3* is hypomethylated it is overexpressed in lung cancer

Table 1. DNA methylation as a diagnostic marker in non-small-cell lung cancer.

Study (year)	Gene	Tissue/body fluid	Ref.
Belinsky *et al.* (1998)	*p16*(*INK4a*)	Biopsy samples, sputum	[39]
Topaloglu *et al.* (2004)	*FHIT*, *H-cadherin RARβ*, *CDH1*, *APC*	Bronchial lavage	[87]
Geng *et al.* (2012)	*NEUROG2*, *NID2*	Lung tumor tissue	[88]
Schiemann *et al.* (2005)	*MGMT*, *RASSF1A*, *GSTP1*, *CDKN2A*, *RARβ2*, *ARF*, *APC*	Bronchial aspirates	[89]
Grote *et al.* (2006)	*CDKN2A*, *RARβ2*, *RASSF1A*	Bronchial aspirates	[90]
Fujii *et al.* (2012)	*RASSF1A*, *p16INK4a*, *RARβ*, *MGMT*, *DAPK*	Pleural fluid	[91]
Van der Drift *et al.* (2012)	*RASSF1A*	Bronchial washings	[92]
Schmidt *et al.* (2010)	*CDKN2A*, *RARβ*, *SHOX2*	Primary NSCLC tumor	[93]
Kneip *et al.* (2011)	*SHOX2*	Plasma	[82]

NSCLC: Non-small-cell lung cancer.

Table 2. DNA methylation as a prognostic marker in non-small-cell lung cancer.

Study (year)	Gene	Tissue/body fluid	Ref.
Brock *et al.* (2008)	*MGMT, ASC, DAPK, APC, RASSF1A, p16, CDH13*	Paraffin-embedded tumor tissue	[95]
Yamamoto *et al.* (2009)	*p16, RASSF1A, CDH13*	Lung tumor tissue	[96]
Zhang *et al.* (2011)	*RASSF1A, DAPK RARβ, APC CDH13, KLK10 DLEC1, RASSF1A SFRP1, RARβ CDKN2A*	Primary NSCLC tumor/plasma	[97]
Yang *et al.* (2011)	*BRMS1*	Lung tumor tissue	[98]
Dietrich *et al.* (2012)	*PITX2, SHOX2*	Lung tumor tissue	[100]
NSCLC: Non-small-cell lung cancer.			

cells, thus enhancing cancer phenotypes and oncogenic alterations [119]. In lung adenocarcinomas hypermethylation of *miR-124a* mediates Rb phosphorylation and CDK6 activation [120], while the *miRNA-29* family may restore aberrant methylation in lung cancer by targeting the 3′-untranslated region of *DNMT3A* and *DNMT3B*, which are frequently upregulated in lung cancer and are associated with poor prognosis [120]. When miR-29 is overexpressed it can inhibit lung carcinogenesis by normalizing aberrant methylation in NSCLC through the induction of re-expression of methylated silenced TSGs [120]. Taken together, elucidating the relationship between DNA methylation and miRNAs will provide an improved understanding of the development of more complete tumor biomarkers.

Future perspective

DNA methylation represents one of the most important areas for the discovery of novel biomarkers for the diagnosis, prognosis and prediction of therapy. The frequency of DNA methylation aberrations in lung cancer and the technical advantages in detecting this modification in body fluids seems to provide an advantage over other types of biomarkers. However, despite the encouraging results obtained so far, significant challenges still lie ahead in this field. While the exact contribution of aberrant methylation to lung carcinogenesis and the mechanism underlying cancer-specific methylation alteration remain to be clarified, methodological issues have also to be solved. Technical procedures for different types of

DNA isolation (e.g., cfDNA from serum, total DNA from sputum) require standardization and analytical validation before any application in the clinical setting. For the vast majority of the suggested biomarkers, the data available is at the proof-of-principle level with inadequate retrospective validation. Finally, following the optimization of assays at good clinical laboratory practice standards, multicenter prospective validation is required to prove the immediate patient benefit and, in longer terms, cost–effectiveness for the public health system. It seems inevitable that in order to utilize the great potential of DNA methylation to provide specific and sensitive diagnostic tools for early detection of lung cancer, large multicenter consortia have to be formed to achieve the statistical power needed for clinical biomarker validation.

Moreover, the ever-growing number of genes that show epigenetic alterations in disease emphasizes the crucial role of these epigenetic alterations – particularly DNA methylation – for future diagnosis, prognosis and prediction of response to therapies [121]. It is crucial to note that epigenetic modifications such as DNA methylation are intrinsically reversible, in contrast with genetic mutations. This is a very promising approach towards the treatment of cancer patients via targeting epigenetic mechanisms. Intensive investigations have been performed to evaluate epigenetic drugs as novel therapeutic interventions. These DNA demethylating agents, used either alone or in combination with other agents such as chemotherapeutic drugs and histone deacetylase inhibitors, have been shown to be effective in treatment of cancer, although only in a small set of patients [122]. However in this case, we have to point out the potential risk of creating hypomethylation and the associated risks of genomic instability and activating epigenetically silenced oncogenes.

Executive summary

DNA methylation as a tumor biomarker

- Epigenetic abnormalities are present in all human cancers and are currently considered to be the hallmark of cancer initiation and progression.
- Currently, the best-studied epigenetic event in the mammalian genome is DNA methylation, which is a chemical covalent modification of cytosine.
- The virtually universal presence of DNA hypermethylation in all types of cancer makes it an ideal candidate tumor biomarker.
- DNA methylation is chemically stable, it can be readily amplifiable and is easy to detect.
- In lung cancer, aberrant DNA methylation can be detected in tissues, in plasma/serum, in sputum, in bronchial lavage and saliva.

Circulating tumor DNA

- Cell-free DNA maintains the genetic and epigenetic profile of its primary tumor source.
- A plethora of loci have been reported to be methylated in the plasma/serum of non-small-cell lung cancer (NSCLC) patients, suggesting novel potential diagnostic and prognostic targets such as *APC*, *CDH1*, *MGMT*, *DCC*, *RASSF1A* and others.

DNA methylation as a tumor biomarker in lung cancer

- A variety of epigenetic biomarkers have been evaluated so far in lung cancer diagnostics.
- The diagnostic value of *RASSF1A* methylation and *KRAS* mutations in bronchial washings reported a 29% efficiency of detecting malignancy in false-negative or ambiguous cytology outcomes.
- *SHOX2* quantified methylation showed promising results in bronchial aspirates, with a sensitivity of 78% and a specificity of 96% while *SHOX2* sensitivity and specificity in plasma was 60 and 90%, respectively.
- Promoter methylation of *APC*, *RASSF1A*, *p16* and *CDH13* associated with early recurrence in stage 1 NSCLC.
- Promoter methylation of *BRMS1* correlates with smoking history and poor survival in NSCLC.

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