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Methylation status of selected genes in non-small cell lung carcinoma – current knowledge and future perspectives

Review

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DNA methylation is recognized as an early event in cancer initiation and progression. This review aimed to compare the methylation status of promoter regions in selected genes across different histological subtypes of non-small cell lung cancer (NSCLC), including adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and the rare but highly aggressive large-cell neuroendocrine carcinoma (LCNEC). A comprehensive literature search was conducted in the PubMed database until August 17, 2024, using standardized keywords to identify reports on promoter methylation in NSCLC. Seventy-five studies were reviewed, focusing on the promoter methylation of key genes, such as *APC*, *BRCA1*, *CDH1*, *CDH13*, *DAPK1*, *DLEC1*, *FHIT*, *GSTP1*, *hMLH1*, *MGMT*, *CDKN2A*, *RARβ*, *RASSF1*, *RUNX3*, and *TIMP3*. These studies explored diagnostic, prognostic, epidemiological, and therapeutic aspects across NSCLC subtypes. Additionally, mutational profiles of *TP53*, *RB1*, *KEAP1*, and *STK11* and expression patterns of *ASCL1*, *DLL3*, and *NOTCH* were analyzed. The findings suggest that LCNEC may serve as a biological bridge between non-small cell and small-cell lung carcinoma. Our analysis highlights that the methylation status of selected genes could enhance diagnosis, prognosis, and personalized treatment strategies in patients with NSCLC, particularly those with LCNEC.

Key words: gene mutation; expression profile; NSCLC subtypes; LCNEC; biomarkers

Lung cancer is a very aggressive and highly prevalent disease worldwide. It is the leading cause of cancer mortality in men and is the second highest cause of cancer death in women, behind only breast cancer [1]. Despite advances in early diagnosis and standard treatment, most patients are diagnosed at an advanced stage and have a poor prognosis, with an overall 5-year survival rate of 10–15% [2]. Lung cancer is divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), with histological subtypes, namely adenocarcinoma (AD), squamous cell carcinoma (SCC), and large cell carcinoma (LCC) as the most frequent subtypes. The recognition of histological subtypes has become important as a determinant of therapy [3, 4]. The identification of molecular abnormalities in a substantial number of lung cancer patients has given rise to personalized targeted therapies, opening novel therapeutic perspectives [5, 6]. Large cell neuroendocrine carcinoma (LCNEC), another subtype of NSCLC, is a rare and highly aggressive type of lung cancer with a complex biology that shares similarities with both SCLC and NSCLC. The LCNEC clinical management is still controversial, and there is currently a lack of standardized treatment strategy [7].

DNA methylation is a heritable modification based on the addition of a methyl group through a covalent bond to the 5'carbon of the cytosine ring. This bond is mediated by DNA methyltransferases (DNTMs) [8]. In 98% of cases, DNA methylation occurs in the CpG pattern in somatic cells, whereas approximately 25% methylation occurs in the

non-CpG pattern in embryonic stem cells [9]. Most DNA methylation is necessary for normal development, including X-chromosome inactivation, genomic imprinting, and suppression of repetitive element transcription or transposition. Changes in methylation patterns can contribute to malignancies during impaired gene regulation [10]. DNA methyltransferases share a common catalytic domain consisting of six conserved amino acid motifs in the C-terminus, whereas significant differences occur at the N-terminus. DNMTs can be divided into two major groups in mammals based on their structure and function [11]. The first type is DNMT1 with a maintenance methylation function, which binds to hemimethylated DNA and maintains a DNA methylation pattern after replication [12]. DNTM1 is also a crucial element of the transcription suppression complex that interacts with DNMT1-associated proteins, such as transcription factor E2F1 and histone deacetylases (HDACs) [13]. DNMT3A and DNMT3B are responsible for *de novo* methylation as they do not require hemi-methylated DNA for binding activities. They display an equal affinity for hemi- or non-methylated DNA and can cooperate with DNMT1 to extend DNA methylation [14]. Hypermethylation at promoter gene regions mostly suppresses gene transcription, whereas demethylation leading to hypomethylation increases gene transcriptional activity and its expression. Gene expression can be suppressed by inhibition of binding transcription factors sensitive to methylated CpG [15], such as E2F or MYC [16]. Hypomethylation, on the other hand, underlines gene activation with followed gene overexpression. Genome hypomethylation often accompanies terminal stages of tumor progression and is accompanied by gene instability or transposon expression [8].

DNA methyltransferases are methylation enzymes and play a crucial role in DNA methylation. Consequently, suppressing their methylation function can result in a demethylation characterized by a gradual reduction of DNA methylation in newly divided cells [17]. Azacytidine and decitabine are nucleoside analogs applied as demethylation drugs that are used to treat myelodysplastic syndrome [18] and acute leukemia [19]. These DNA methyltransferase inhibitors (DNMTi) affect the demethylation process, where they are able to reverse gene silencing induced by hypermethylation [20]. Zebularine is another drug that falls in the category of nucleoside analogs with inhibition of DNA methylation function [21]. The mechanism of action is similar to azacytidine, but zebularine has lower toxicity and higher stability in aqueous solutions in comparison to both azacytidine and decitabine, however, it is associated with radioactivity [22]. Hydralazine and procainamide are the first drugs reported under the category of non-nucleoside agents with a demethylation effect. Hydralazine decreases the expression of both DNMT1 and DNMT3A, and the procainamide effect results in DNA hypomethylation [23]. Furthermore, pyrazolone and pyrazine have been identified as DNMT3A inhibitors. The main reason for the development of other non-nucleoside drugs is their effectiveness at lower concentrations compared to the cytotoxicity of high concentrations of nucleoside analogs [24].

Here, we have presented a review of data to determine the methylation profile of genes associated with NSCLC progression. We have searched in PubMed from inception to August 17, 2024, for publications on topics focusing on i) methylation of selected genes in histological subtypes of NSCLC and ii) expression and mutation profiles of selected genes in NSCLC. The result of methylation analysis can thus improve our understanding of the molecular characteristics of NSCLC in terms of diagnosis, prognosis, and targeted therapy.

Materials and methods

Search strategy. This study followed the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) 2020 statements checklist (Supplementary Table S1) [25]. A literature search was conducted in PubMed, which is the most recommended database for reviews because of its extensive range of articles, advanced search filters, and systematic search systems. Studies published from inception to August 17, 2024, were considered. To perform a comprehensive search, we used the following keywords in different patterns: ("NSCLC") AND ("methylation") AND ("pattern" OR "CDKN2A" OR "RASSF1" OR "CDH13" OR "DAPK1" OR "TIMP3" OR "hMLH1" OR "APC" OR "BRCA1" OR "DLEC1" OR "CDH1" OR "RUNX3" OR "FHIT" OR "MGMT" OR "RAR beta" OR "WIF1") OR ("expression) OR ("mutation") AND ("RB1" OR "TP53" OR "STK11" OR "LKB1" OR "KEAP1" OR "ASCL1" OR "DLL3" OR "NOTCH") (Supplementary Table S2).

Eligibility criteria. To be included in the review, studies had to meet the following criteria: i) patients with NSCLC or in NSCLC cell lines; ii) to be aimed at diagnostic, prognostic, therapeutic, predictive biomarkers, and or epidemiology, iii) methylation analyses of NSCLC histological subtypes. The search terms used in this review are presented in Supplementary Table S3.

Studies were excluded if one of the following existed: i) duplicate publications; ii) non-human studies or animal studies; iii) reviews, case reports, letters, editorials, or expert opinions; iv) studies without data available or with incomplete or retracted text.

Study selection and data extraction. The selection process of publications that complied with the inclusion criteria was performed manually by author A.P. without the application of automation tools. Four thousand nine hundred fifty items were selected. After removing 2,122 duplicates, we excluded 2,343 citations by title and screened 484 abstracts for retrieval, from which 484 eligible studies were included. The queries and the sections of the paper pertaining to methylation of selected genes in histological subtypes and expression and mutational profiles of selected genes yielded 75 disjunctive studies that were used (Figure 1). Data were

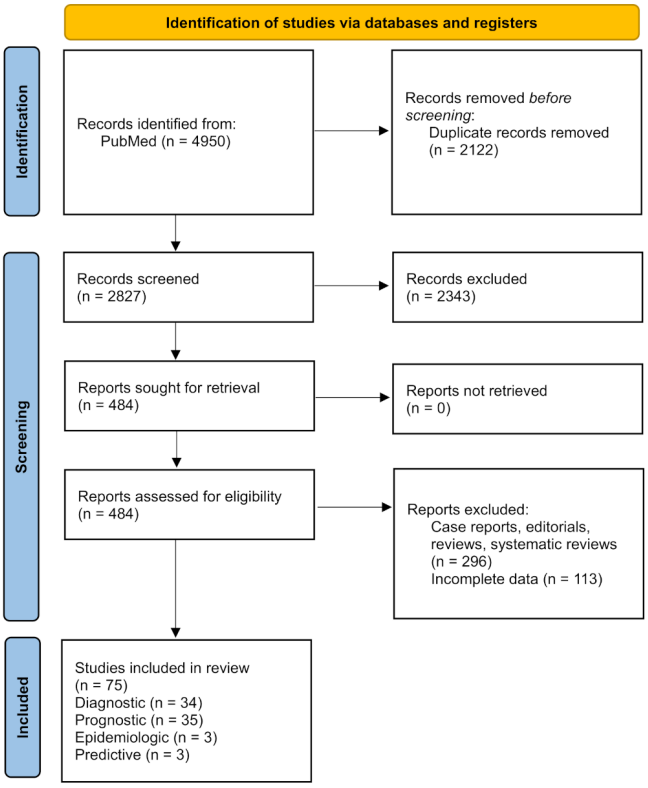

Figure 1. PRISMA 2020 flow diagram for systematic reviews, including searches of databases and registers alone.

collected by author A.P. without the utilization of automation. The extracted data included a summary of methylation of selected gene promoters in histological subtypes of NSCLC and expression and mutational profiles in NSCLC.

For the main topic of investigation, the method for evaluating the results was to summarize data from multiple studies on methylation of selected gene in histological subtypes of NSCLC, namely AD, SCC, and LCC. All authors judged the inclusion of the studies in the review. Comparisons of methylation in NSCLC subtypes of the studies included are presented in Supplementary Table S4.

Results

The search terms used in the review are summarized in Supplementary Table S3. For the research topic, the approach to evaluating the results was to collect methylation data in NSCLC and its subtypes, mainly AD, SCC, and when included, LCNEC. All authors assessed the inclusion of studies in the review based on these parameters.

The independent review authors included only studies in which the measures described above were identifiable and explicit results were reported. Comparisons of the region gene methylation status analyzed in NSCLC and NSCLC subtype patients have been complicated because of the lack of data on LCNEC.

There were 34 items in the diagnostic research area, including *APC, BRCA1, CDH1, CDH13, DAPK1, DLEC1, FHIT, hMLH1, MGMT, CDKN2A, RAR β, RASSF1, RUNX3, TIMP3, WIF1, STK11, RB1* genes, and ASCL1 and DLL3 proteins; in the prognostic area, there were 35 items with genes *APC, BRCA1, CDH1, CDH13, DAPK1, DLEC1, FHIT, hMLH1, MGMT, CDKN2A, RAR β, RASSF1, RUNX3, TIMP3, TP53, RB1, STK11, KEAP, WIF1;* and NOTCH protein; in the field of epidemiology, there were 3 studies: i) in the first study, authors compared promoter methylation level of *GSTP1, CDKN2A, FHIT, APC, RASSF1A, hMLH1, hMSH2, AGT* gene between Korean and Western populations; ii) *CDKN2A* aberrant methylation was analyzed between female smokers and female never-smokers and *CDKN2A, RASSF1A, APC, RAR β, CDH13, MGMT* and *GSTP1* between smokers and never-smokers; iii) in the third, *CDH13* and *RASSF1* genes were studied to monitor efficacy and sensitivity of chemotherapy (Figures 1 and 2).

The following section provides a summary of the data and its interpretation. At the end of each subsection, a brief discussion of the methylation state of the studied genes is given.

Detailed results and discussion

Gene methylation and gene inactivation

Genes with potential diagnostic and prognostic implications

DAPK. DAPK (death-associated protein kinase) is a calcium/calmodulin-dependent serine/threonine kinase that mediates interferon-γ-induced cell death [26]. DAPK downregulation is commonly observed in tumors and in some cases, this downregulation correlates with metastatic progression. The spontaneously occurring rare lung metastases derived from low metastatic clones *in vivo* display loss of *DAPK* expression [26]. These findings demonstrate

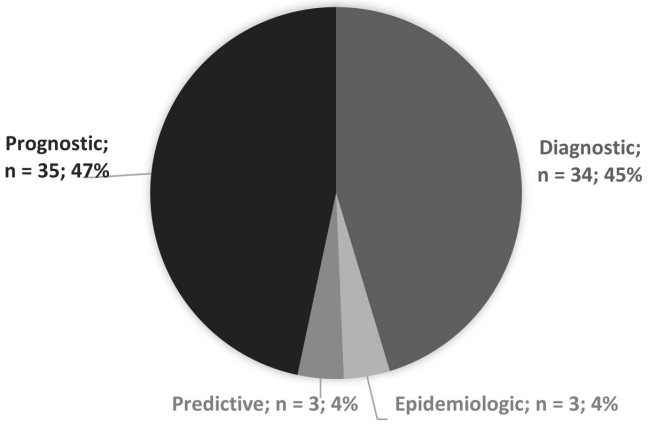

Figure 2. For the 75 studies in the review, the research focus was in the following categories: 45% of the studies were diagnostic (n=34), 47% were prognostic (n=35), 4% were epidemiologic (n=3), and 4% were predictive (n=3).

a causal effect of DAPK on suppressing tumor metastasis. Downregulation of *DAPK* expression in lung cancers correlates with advanced disease stage and lymph node involvement [27]. *DAPK* promoter methylation was found in 18.69–40.98% (20/107 [28], 26/101 [29], 24/70 [30], 50/122 [31]), 50% (14/28) [32], 58.12% (68/117) [33], and 76.47% (13/17) [34] of NSCLC cases, specifically 15.56% (7/45) [28], 22.58–47.89% (14/62 [28], 5/20 [30], 7/15 [32], 34/71 [31]), 57.37% (35/61) [33], and 78.57% (11/14) [34] in AD, 20.93–41.46% (9/43 [28], 12/39 [28], 16/51 [31], 17/41 [30]), 58.93% (33/56) [33], 66.67% (2/3) [34] and 71.43% (5/7) [32] in SCC, 0% (0/4;0/3) [28, 32] and 22.22% (2/9) [30] in LCC (Supplementary Table S4). Finally, *DAPK* promoter methylation could be an important indicator of tumor progression, which is associated with poor prognosis and aggressive metastatic phenotype in patients with NSCLC [27, 33, 35].

RAR-β. RAR-β (retinoic acid receptor β) is required for normal lung development because of the spatiotemporal regulation of retinoic acid levels that assures the formation of a fully functional organ. The RAR-β, as a member of the thyroid-steroid hormone receptor superfamily, localizes to the cytoplasm and subnuclear compartments. It binds to retinoic acid (RA), the biologically active form of vitamin A, which mediates cellular signaling in embryonic morphogenesis, cell growth, and differentiation. Signal transduction involves the binding of RA to nuclear RA receptor (RAR), which forms a heterodimer complex with retinoid X receptor (RXR). The RAR-RXR modulates transcription by binding to DNA at RA response elements (RAREs) located in enhancer regions of the target gene [36].

In the classical model of RA-dependent gene activation, unliganded RAR-RXR heterodimers repress transcription of their associated genes. However, additional co-regulators and epigenetic changes contribute to transcriptional regulation. In the repressive unliganded state, the RAR-RXR heterodimer recruits co-repressors such as nuclear receptor co-repressor 1 (NCOR1) and NCOR2 (also known as SMRT), which in turn recruit HDAC protein complexes and Polycomb repressive complex 2 (PRC2). This results in histone H3 lysine 27 trimethylation (H3K27me3), chromatin condensation, and gene silencing [37, 38]. *RAR-β* gene is frequently epigenetically silenced in tumor progression: the *RAR-β* gene hypermethylation was found in 30.56–56.25% (11/36 [39], 38/101 [29], 43/107 [28], 22/53 [40], 45/80 [41]), 80.24% (134/167) [42], and 80.36% (45/56) [43] of NSCLC cases, more specifically in 26.67–60% (8/30 [39], 22/45 [28], 32/62 [29], 13/22 [40], 15/25 [41]), 81.48% (66/81) [42], and 88.24% (15/17) [43] of AD, in 15.38% (6/39) [29], 33.33–66.67% (15/43 [28], 14/36 [39], 8/17 [40], 30/45 [41]), 79.1% (68/86) [42], and 88.46% (23/26) [43] SCC, and in 25% (1/4) [28, 40] of LCC (Supplementary Table S5). *RAR β* hypermethylation correlates well with an increased risk in NSCLC patients. It also contributes to NSCLC tumorigenesis and may serve as a potential risk factor, diagnostic marker, and drug target for NSCLC [44].

BRCA1. BRCA1 (breast cancer 1) is a multifunctional tumor suppressor that plays a key role in cell cycle regulation, replication, mitotic spindle assembly, chromatin hierarchical control, transcription regulation [45], and DNA damage response and apoptosis [46]. The inactivation of *BRCA1* by epigenetic alterations is a critical event in breast tumorigenesis. Aberrant methylation of *BRCA1* has been observed in association with relatively poor clinical outcomes [47]. It may potentially be used as a prognostic marker relating to the overall survival and disease-free survival of patients with breast cancer [48, 49]. Various meta-analyses have provided evidence that *BRCA1* methylation is associated with poor survival of breast cancer patients [50–52]. *BRCA1* is hypermethylated in 18.57% (13/70) [53], 21% (6/28) [32], and 37.08% (33/89) [54] of NSCLC cases. Hypermethylation occurred in 13.33% (2/15) [32], 20.41% (10/49) [53], and 42.31% (22/52) [54] of AD, 13.33% (2/15) [53], 14.29% (1/7) [32], and 29.73% (11/37) [54] of in SCC and 33.33% (1/3) [32] of LCC (Supplementary Table S6). Patients with *BRCA1* methylation demonstrated significantly poorer recurrence-free survival. Considering that *BRCA1* plays a role in chemotherapy-induced apoptosis, it is plausible that the identification of methylated *BRCA1* could provide information that is clinically relevant to tailored adjuvant therapy [53].

TIMP3. TIMP3 (tissue inhibitor of metalloproteinase 3) protein functions in a wide range of tissue cytoplasm and extracellular matrix. It is an inhibitor of matrix metalloproteinases (MMPs), which are involved in the degradation of the extracellular matrix. The balance between MMPs and TIMPs is important to ensure the integrity of the extracellular matrix. Any alteration of the latter may affect several biological processes, including carcinogenesis [55]. Aberrant promoter methylation of TIMP3 gene promoter frequently occurred in BRCA1ness (tumors that share molecular features of BRCA1-mutant tumors) breast cancer [56], gastric cancer [57], and NSCLC [58]. Hypermethylation in *TIMP3* gene promoter occurred in 18.52% (10/54) [59], 26.17% (28/107) [28], 39.29% (11/28) [32], and 47.29% (61/129) [58] of NSCLC cases, more particularly in 9.38% (3/32) [59], 24.44% (11/45) [28], 38.46% (20/52) [58], 40% (6/15) [32] of AD, 23.26% (10/43) [28], 33.33% (7/21) [59], 42.86% (3/7) [32], 53.25% (41/77) [58] of SCC, and 0% (0/3) [32] and 25% (1/4) [28] of LCC (Supplementary Table S7). TIMP3 has an important role in metastasis and cell invasion in cancer. Hypermethylation of the *TIMP3* gene promoter is associated with improved survival in NSCLC [58, 59].

DLEC1. DLEC1 (deleted in lung and esophageal cancer) is a tumor-suppressor gene, which plays a role in cell-cycle control by inducing G1 arrest [60]. Promoter methylation was associated with downregulation or loss of DLEC1 expression in lung cancer [61]. *DLEC1* has been seen to be methylated in prostate cancer [62], renal cell carcinoma [63], gastrointestinal tumors [64], and lung cancer [65]. Promoter hypermethylation occurred in 37.14% (26/70) [66], 38.66% (92/238) [61], 41.03% (32/78) [39], and 70.59% (12/17) [34]

of NSCLC cases, in particularly 28.57% (10/35) [66], 31.52% (29/92) [61], 40% (12/30) [39], and 71.43% (10/14) [34] promoter hypermethylation occurred in AD, 44.44% (16/36) [39], 45.71% (16/35) [66], 47.83% (44/92) [61], and 66.67% (2/3) [34] in SCC, and 35.19% (19/54) [61] in LCC (Supplementary Table S8). *DLEC1* promoter hypermethylation was associated with lung cancer risk. *DLEC1* methylation detection in patients with lung cancer exhibited a potential diagnostic utility [65].

CDH1. *CDH1* encodes E-cadherin, which is an adhesion molecule of the epithelial cells that plays a key role in stabilizing and maintaining intercellular connections [67]. E-cadherin is bound to the cytoskeleton through catenin, and the reduction of *CDH1* expression may be involved in the invasion and metastasis of several cancers [68–70]. *CDH1* methylation occurs in many types of cancer [71–79], including NSCLC [80]. *CDH1* promoter is hypermethylated in 32.56–58.33% (42/129 [58], 30/91 [81], 8/17 [34], 24/42 [82], 119/204 [83]) of NSCLC cases. Hypermethylation occurred in 30.77–59.52% (16/52 [58], 11/35 [81], 6/14 [34], 13/25 [82], 75/126 [83]) of AD cases, 33.77–66.67% (26/77 [58], 19/56 [81], 6/12 [82], 44/78 [83], 2/3 [34]) of SCC cases, and 100% (5/5) [82] of LCC cases (Supplementary Table S9). *CDH1* promoter hypermethylation may play an important role in carcinogenesis and NSCLC progression and may serve as a potential drug target in lung cancer. However, CDH1 methylation does not correlate with other factors, such as smoking history, clinical stage, pathological type, sex status, lymph node metastasis, or degree of differentiation [80, 84].

RUNX3. RUNX3 (Runt-related transcription factor 3) is a regulator in the transforming growth factor (TGF)-β signaling pathway, which is a known tumor suppressor [85]. Loss of *RUNX3* through deletion or expression inhibition results in the limited function of SMAD proteins and the promotion of TGF-β signaling, which leads to tumor development [86]. Promoter methylation of the *RUNX3* gene was shown in gastric cancer [87], esophageal cancer [88], breast cancer [89, 90], or NSCLC [91]. *RUNX3* promoter was hypermethylated in 20–52.94% (15/75 [92], 30/119 [93], 23/54 [59], 26/58 [94], 9/17 [34]) of NSCLC cases. Among the NSCLC cases, *RUNX3* methylation was observed in 27.91–57.14% (12/43 [92], 10/32 [59], 26/72 [93], 13/32 [94], 8/14 [34]) of AD, 6.67% (3/45) [93], 6.9% (2/29) [92], 33.33% (1/3) [34], 38.1% (8/21) [59], and 50% (13/26) [94] of SCC, and 50% (1/2) [93] of LCC (Supplementary Table S10). *RUNX3* is strongly associated with NSCLC and the histological type of the NSCLC since it was less frequent in SCC compared with AD [95]. Further, *RUNX3* hypermethylation is associated with an increased risk and worse survival in NSCLC and plays an important role in lung carcinogenesis and clinical outcomes [91].

Genes with potential diagnostic, prognostic, and therapeutic implications

WIF1. The Wnt/β-catenin signaling pathway is an evolutionarily conserved pathway that regulates crucial aspects of cell fate determination, cell migration, cell polarity, neural patterning, and organogenesis during embryonic development [96]. Dysregulation of Wnt/β-catenin signaling is often caused by mutations of various components in the pathway, particularly mutations or silencing of the Wnt tumor suppressor. Wnt inhibitors may have a broader role in cancers such as melanoma, lung, and renal cancer, where immunotherapy has come to the forefront [97]. New studies have firmly established the importance of this pathway in regulating the expression of various checkpoints in immune cells and tumor cells to promote immune escape. WIF1 (Wnt inhibitory factor1) acts as an inhibitor of the Wnt/β-catenin signaling pathway. *WIF1* functions as a tumor suppressor gene and has been found to be epigenetically silenced in various cancers [98]. Hypermethylation of the *WIF1* gene promoter was shown in endometrial cancer [99], hepatocellular carcinoma [100], colorectal cancer [101], cervical cancer [102], or NSCLC [103]. *WIF1* promoter hypermethylation occurred in 15.91% (7/44) [104], 27.66% (65/235) [105], 35.29% (6/17) [34], 47.48% (66/139) [106], 69.44% (50/72) [107] NSCLC, specifically in 13.33% (4/30) [104], 21.43% (3/14) [34], 22.22% (30/135) [105], 45.57% (36/79) [106], 63.33% (19/30) [107] of AD, and 27.27% (3/11) [104], 32.18% (28/87) [105], 50% (30/60) [106], and 100% (3/3; 2/2) [34, 107] of SCC. LCC was evaluated in only two studies, with 0% (0/3) [104] and 53.8% (7/13) [105] (Supplementary Table S11). *WIF1* hypermethylation contributes to the development of NSCLC and is a potential marker for the diagnosis of NSCLC and the prediction of prognosis in patients with NSCLC [103].

Genes with potential diagnostic, prognostic, and epidemiological implications

CDKN2A. Several tumor suppressor genes are silenced by promoter gene hypermethylation. Tumor suppressors play key roles in normal cellular functions, such as $p16^{INK4}$ through cell cycle regulation. The $p16^{INK4}$ is a tumor suppressor and cyclin-dependent kinase inhibitor that is essential for regulating the cell cycle by inactivating cyclin-dependent kinases that phosphorylate pRB [108]. Hypermethylation of the *CDKN2A* (Cyclin-dependent kinase inhibitor 2A), which encodes protein $p16^{INK4}$, promoter region may represent an early event, followed by heterozygous deletion of the $p16^{INK4}$ locus [109]. The *CDKN2A* promoter hypermethylation occurred in 25.23–42.42% (27/107 [28], 22/99 [110], 27/101 [29], 16/53 [40], 10/28 [32], 8/22 [111], 14/33 [112]) and 58.82% (10/17) [34], 78.69% (48/61) [113], and 79.49% (31/39) [114] of NSCLC samples, specifically 13.33–33.33% (6/45 [28], 9/62 [29], 3/13 [111], 9/38 [110], 10/32 [40], 5/15 [32]), 61.53% (8/13) [112], 64.29% (9/14) [34], 70.59% (12/17) [114], and 83.33% (30/36) [113] in AD, 11.76–37.21% (2/17 [40], 1/7 [32], 16/61 [110], 4/15 [112], 1/3 [34], 16/43 [28]) and 46.15% (18/39) [29], 55.56% (5/9) [111], 86.36% (19/22) [114], and 87.5% (14/16) [113] in SCC, and 25% (1/4; 1/4) [28, 113], 33.33% (1/3) [32], and 100% (4/4) [40] in LCC (Supplementary Table S12). The finding of used studies

indicates that the *CDKN2A* promoter gene hypermethylation is associated with poor prognosis of NSCLC patients [115].

APC. Wnt/β-catenin signaling is essential for intestinal homeostasis and is aberrantly activated through mutation of the tumor suppressor APC. The APC (Adenomatous Polyposis Coli) gene encodes tumor suppressor protein that acts as an inhibitor of the Wnt signaling pathway. The APC tumor suppressor controls the switch between transcriptional coactivator and corepressor complexes at Wnt target genes in which β-catenin and APC have opposing actions. The β-catenin C-terminal activation domain associates with chromatin-modifying complexes *in vitro* and promotes H3K4 trimethylation at the *c-Myc* gene *in vivo* [116]. In the absence of Wnt signal, casein kinase 1 (CK1) and glycogen synthase kinase-3β (GSK-3β) in a multiprotein complex with β-catenin, and the APC, phosphorylate APC, which increases its affinity for β-catenin. The β-catenin is then ubiquitinated and destroyed by proteasome-mediated proteolysis [116].

Because the Wnt signaling pathway is often mutated or epigenetically altered in tumors, inhibitors of Wnt signaling are being intensively studied. Non-mutational changes include all levels of epigenetic modifications – DNA methylation, histone modification, and mRNA interference. Both types of changes promote tumor formation and invasiveness, which usually happens because of the loss of function of corepressors or the overexpression of coactivators of the Wnt signaling pathway. It has been shown that knocking down specific components of the Wnt signaling pathway has inhibitory effects on tumor growth *in vivo* and *in vitro*. For example, it has been observed that the silencing of β-catenin by siRNA has an inhibitory effect on the growth of colorectal cancers [117]. Loss of Wnt inhibitors may play a major role in NSCLC, although unlike in colon cancer, loss-of-function mutations in the Wnt pathway inhibitor APC are uncommon in NSCLC [118, 119].

Methylation-sensitive high-resolution melting analysis (MS-HRM) is commonly used as a method for promoter methylation analysis, which can be further validated with bisulfite pyrosequencing or next-generation sequencing (NGS). The *APC* gene promoter is hypermethylated in various types of cancers [120–122], including NSCLC, where it is frequently found to be hypermethylated in 49% of cases [123]. Moreover, the APC promoter hypermethylation occurred in 25–48.48% (9/36 [38], 15/53 [124], 7/17 [34], 216/514 [125], 48/99 [110]), 67.86% (19/28) [32], and 89.01% (81/91) [126] cases of NSCLC cases, specifically in 14.29% (3/21) [38], 40.51–73.33% (32/79 [124], 6/14 [34], 20/38 [110], 161/299 [125], 11/15 [32]), and 100% (43/43) [126] cases of AD, 23.2–57.14% (45/194 [125], 1/3 [34], 6/15 [38], 28/61 [110], 4/7 [32]), and 100% (33/33) [126] cases of SCC and in 33.33% (1/5, 5/15) [32, 126] cases of LCC (Supplementary Table S13).

As shown by Virmani et al. (2001), *APC* is underexpressed in lung and breast cancer cells, when 5-aza-3´deoxycytidine enhances the APC level and decreases the *APC* methylation level in lung and breast cancer cells [127]. The methylation status of the *APC* promoter is strongly associated with NSCLC carcinogenesis [128]. Further, the *APC* methylation could not facilitate the distinction between early NSCLC and advanced NSCLC, nor between AD and SCC [129].

MGMT. MGMT (O-6-methylguanine-DNA methyltransferase) is a specific DNA damage reversal repair protein that has been demonstrated to protect tissues against the toxic and carcinogenic effects of alkylating agent chemotherapy by removing adducts from the O^6 position of guanine [130]. Methylation of the gene's promoter has been associated with several cancer types, including colorectal cancer [131], gastric cancer [132], breast cancer [133], ovarian cancer [134], or NSCLC [135–137]. The *MGMT* promoter is hypermethylated in 8.95–19.63% (46/514 [125], 8/65 [138], 14/101 [29], 11/75 [139], 21/107 [28]), 29.79% (28/94) [140], 37.78% (34/90) [141], and 49.54% (109/220) [142] of NSCLC cases. Hypermethylation occurred in 8.03–30.55% (24/299 [125], 2/18 [138], 8/62 [29], 7/44 [139], 12/45 [28], 22/72 [140]), 40% (46/115) [142], and 46.88% (15/32) [141] of AD, 7.73–27.27% (15/194 [125], 4/31 [139], 6/39 [29], 8/43 [28], 6/29 [138], 6/22 [140]), 37.21% (16/43) [141], and 60% (63/105) [142] of SCC, and 20% (3/15) [141] and 25% (1/4) [28] of LCC (Supplementary Table S14). Advanced-stage NSCLC patients showed higher methylation than early-stage patients. MGMT methylation is indeed associated with an increased NSCLC risk and thus has the potential to be a good biomarker for NSCLC diagnosis [143].

hMLH1. The hMLH1 (human mutL homolog 1) plays a role in DNA damage repair, it is a component of the DNA mismatch repair pathway [144]. MLH1 is epigenetically inactivated via methylation of the gene promoter, leading to the deficiency of mismatch repair, resulting in microsatellite instability. In colorectal cancer, microsatellite instability resulting from methylation of the *hMLH1* gene promoter can cause its transcriptional silencing, leading to the carcinogenesis of colorectal cancer [145]. *hMLH1* promoter methylation occurs in other types of cancers as well [146–148], including NSCLC [149]. The promoter of the *hMLH1* gene is hypermethylated in 6.67–35.71% (5/75 [92], 17/99 [110], 14/78 [39], 8/28 [32], 85/238 [61]), 53.33–72.41% (56/105 [150], 38/70 [151], 84/116 [152]) of NSCLC cases. Hypermethylation occurred in 4.65–22.83% (2/43 [92], 4/30 [39], 7/38 [110], 21/92 [61]), 40–71.88% (6/15 [32], 17/28 [151], 43/65 [150], 23/32 [152]) of AD, 10.34–22.22% (3/29 [92], 1/7 [32], 11/61 [110], 8/36 [39]), and 35.29–72.62% (6/17 [150], 42/92 [61], 21/42 [151], 61/84 [152]) of SCC and 33.33% (1/3) [32] and 40.74% (22/54) [61] of LCC (Supplementary Table S15). The promoter of the *hMLH1* hypermethylation should be an early diagnostic marker for NSCLC and also a prognostic index for NSCLC [153]. Additionally, cisplatin-based adjuvant chemotherapy is more beneficial for NSCLC patients without hMLH1 methylation. hMLH1 methylation may have the potential to become a biomarker of individualized therapy for NSCLC patients [149].

FHIT. The fragile histidine triad gene (FHIT), whose product is known as bis (5'-adenosyl)-triphosphatase, is one of the histidine triad gene family members [154], which encodes hydrolase of Ap3A, and the FHIT-Ap3 enzyme-substrate complex appears to be the tumor suppressor signal. Lack of expression of FHIT protein by promoter methylation has been found to play an important role in epithelial tumorigenesis [157, 158]. Apart from NSCLC [159–161], *FHIT* promoter methylation was shown in cervical cancer [162], breast cancer [163], or liver cancer [164]. *FHIT* is hypermethylated in 27.2–36.13% (68/250 [165], 28/91 [166], 19/56 [167], 34/99 [110], 38/109 [168], 43/119 [169]), 53.92% (110/204) [83], and 59.62% (31/52) [170] of NSCLC cases. Hypermethylation occurred in 25–40.91% (10/40 [166], 24/93 [165], 11/38 [110], 13/41 [168], 24/72 [169], 6/17 [167], 9/22 [170]), and 52.38% (66/126) [83] of AD cases, 30.77–56.41% (12/39 [83], 40/125 [165], 9/26 [167], 25/68 [168], 23/61 [110], 18/45 [169], 18/44 [166], 44/78 [83]), and 73.33% (22/30) [170] of SCC cases and 0% (0/4) [166] and 50% (1/2) [169] of LCC cases (Supplementary Table S16). *FHIT* hypermethylation is associated with an increased risk and worsened survival in NSCLC patients. *FHIT* hypermethylation, which induces the inactivation of the *FHIT* gene, plays an important role in carcinogenesis and clinical outcomes and may serve as a potential drug target of NSCLC [160].

Genes with potential diagnostic, prognostic, therapeutic, and epidemiological implications

RASSF1A. RASSF1A (Ras association domain-containing protein 1) is one of the prototypical tumor-suppressor genes universally inactivated in human malignancies. RASSF1 is a protein encoded by the *RASSF1* gene. The *RASSF1* gene has eight isoforms, of which *RASSF1A* and *RASSF1C* are the most abundantly expressed [171]. As a regulator of key cancer pathways, namely Ras/Rho GTPases and Hippo signaling without ignoring strong interaction with microtubules, RASSF1A is one of the guardians of cell homeostasis [172]. As shown by Xie et al. (2022), RASSF1A is underexpressed in lung cancer tissue and cells. Treatment with 5-aza-3'-deoxycytidine increases RASSF1A levels and reduces its methylation in lung cancer cells [173]. Although promoter hypermethylation and loss of heterozygosity of the remaining allele are the most common molecular mechanisms of silencing the *RASSF1* gene, RASSF1A can also be inactivated by protein degradation or point mutation [174]. Promoter gene methylation was found in 21.43–41.59% (6/28 [32], 18/70 [30], 39/112 [175], 21/53 [40], 31/78 [39], 26/65 [176], 40/99 [110], 41/100 [177], 42/101 [29]), 52.38% (22/42) [178], and 85.71% (48/56) [43], cases of NSCLC cases. Further, 6.67% (1/15) [32], 17.07% (7/41) [30], 32.95% (28/85) [175], 43.54–68.18% (27/62 [29], 14/30 [39], 18/33 [176], 34/72 [177], 18/38 [110], 17/32 [178], 21/33 [176], 15/22 [40]), and 82.35% (14/17) [167] cases of hypermethylation were presented in AD, 25–50% (7/28 [177], 5/20[176], 2/7 [32], 12/36 [39], 6/17 [40], 22/61 [110], 15/39 [29], 11/27 [175], 9/20 [30], 5/10 [178]), and 89.66% (26/29) [43, 167] in SCC and 0% (0/4) [40], 22.22% (2/9) [30], 25% (3/12) [176], and 66.67% (2/3) [32] in LCC (Supplementary Table S17). *RASSF1* promoter hypermethylation is associated with an increased risk of NSCLC and with the differentiation state of NSCLC [179, 180] and can reflect the drug sensitivity of tumors to individualized treatment [178]. There is a significant relationship between *RASSF1A* promoter methylation and lung cancer risk (OR, 16.12, 95% CI, 11.40–22.81; $p>0.001$) with no evidence of between-study heterogeneity. In subgroup analyses, the increased risk of RASSF1A methylation in cases compared to controls for the NSCLC group (OR, 13.66, 95% CI, 9.529–19.57) and for the SCLC group (OR, 314.85, 95% CI, 48.93–2026.2) [179].

CDH13. Cadherin 13 (CDH13) is a unique member of the cadherin superfamily as it lacks transmembrane and cytoplasmic domains. CDH13 is anchored to the cell membrane through the glycosylphosphatidylinositol anchor [181]. CDH13 is involved in low-density lipoproteins, hormone-like effects on Ca^{2+} mobilization and increased cell migration, insulin-dependent signaling, eNOS activation, phenotype changes, and angiogenesis [182, 183]. The presence of *CDH13* methylation was shown in various cancers [184–187], including NSCLC [188]. Toyooka et al. (2001) showed that all methylated breast and lung carcinoma cell lines lacked expression irrespective of whether the unmethylated form was present, confirming biallelic inactivation in methylated lines. Gene expression was restored in all five methylated cell lines tested after treatment with the demethylating agent 5'-aza-2-deoxycytidine [189]. *CDH13* is hypermethylated in 16.92% (11/65) [138], 25.74–65.57% (26/101 [29], 40/150 [124], 172/514 [125], 13/28 [32], 121/251 [188], 23/42 [178], 35/54 [190], 40/61 [113]) of NSCLC cases. Hypermethylation occurred in 29.11–53.13% (23/79 [124], 20/62 [29], 6/15 [32], 123/299 [125], 62/122 [188], 17/32 [178]), and 63.41–69.44% (26/41 [190], 8/12 [138], 25/36 [113]) of AD cases, 2.94–22.68% (1/34 [138], 6/39 [29], 44/194 [125]), and 42.86–69.23% (3/7 [32], 9/16 [113], 6/10 [178], 93/142 [188], 9/13 [190]) of SCC cases and in 25% (1/4) [113] and 33.33% (1/3) [32] of LCC cases (Supplementary Table S18). *CDH13* hypermethylation is associated with an increased risk and worse survival in NSCLC and can reflect drug sensitivity of tumors to individualized treatment [178, 191].

Methylation sensitive genes

Dammann et al. found that methylation in NSCLC occurred in genes *TIMP4* (94%), *SOX15* (100%), *EGFL7* (100%), *CD105* (69%), *SEMA2* (93%), *DLC1* (61%), and *SLIT2* (100%) [192]. Castro et al. analyzed multiple gene promoter hypermethylation, which resulted in *PRDM2* (41%), *SCGB3A1* (50%), *BNIP3* (44%), *HLTF* (15%),

H2AFX (19%), *ID4* (46%), *CCND2* (48%), *TWIST1* (39%), *SFRP4* (39%), *SFRP5* (32%), *CACNA1G* (39%), *TGIF* (19%), *CACNA1A* (33%) NSCLC methylation [59]. Hypermethylation also occurred in *CALCA* (68%), *ER* (68%), *ECAD* (16%), *hTERT* (36%), *IGF* (21,4%), *p15* (7%), *CD44* (18%) [32], *BRCA2* (36%), *XRCC5* (34%) [54], *hMSH2* (8%), *AGT* (21%) [110], *TBX5* (84%), *PITX2* (77%) [66], *RASGFR2* (38%) [175], *TFPI-2* (27%) [94], *WIF1* (35%) [34], *p14* (9%) [28, 29], *ESR1* (9%) [138], *XPC* (35%) [193] in NSCLC.

LCNEC subtypes based on gene mutations

Recent studies have evaluated several genes, including *TP53*, *RB1*, *KEAP1*, *STK11*, *MEN1*, *ASCL1*, *DLL3,* and *NOTCH,* which play a key role in classifying LCNEC into two molecular subtypes. Those genes are the determining factor in subtyping LCNEC into two subtypes. Type I, or NSCLC-like LCNEC, is characterized by biallelic alterations in *TP53, STK11,* or *KEAP1* (genes commonly associated with AD and SCC) and *KRAS* mutations (typical of AD), along with an $ASCL1^{high}/DLL3^{high}/NOTCH^{low}$ expression profile, which is more similar to SCLC. Type II, or SCLC-like LCNEC, presents with biallelic *TP53* and *RB1* alterations and an $ASCL1^{low}/DLL3^{low}/NOTCH^{high}$ expression profile, aligning more closely with the molecular characteristics of SCLC [194–197]. In addition, a third subset of carcinoid-like LCNEC (4%) was identified, which lacked *RB1* and *TP53* alteration and was characterized by *MEN1* mutations, hallmarks of carcinoids. *STK11* mutations appeared more in NSCLC-like LCNEC compared to AD. *STK11* is associated with rapid tumor growth and metastasis in lung AD, which can explain the aggressive clinical behavior of LCNEC [198]. SCLC-like subset harbored *MYCL* amplification, *SOX2* amplification, *PTEN* mutation/loss, and *FGFR1* amplification, with a complete absence of *STK11* and *KRAS* mutations. NSCLC-like subset occasionally showed SCLC alterations (e.g., *MYCN* amplification). Both NSCLC-like and SCLC-like LCNEC had higher mutational burden than conventional NSCLC and SCLC, suggesting LCNEC could be sensitive to immune checkpoint inhibitors [199]. Miyoshi et al. also showed alterations in the PI3K/AKT/mTOR pathway, *FGFR1* (5%), *ERBB2* (4%), and *EGFR* (1%) [200].

Signaling pathways of DLL3, NOTCH, ASCL1

DLL3 (Delta-like 3) is a member of the NOTCH receptor ligand family that inhibits NOTCH receptor activation, promoting neuroendocrine differentiation [201]. In healthy cells, DLL3 is localized to the Golgi apparatus and cytoplasmic vesicles, whereas in SCLC, it is typically found on the plasma membrane. DLL3 does not initiate signaling between cells but functions only when co-expressed with the NOTCH receptor on the same cell. Upon binding to DLL3, the NOTCH receptor is sequestered in the Golgi apparatus, rendering it inactive [202].

ASCL1 is a basic helix-loop-helix transcription factor that drives the expression of several oncogenes, including *BCL-2, SOX2,* and *MYCL* [203]. DLL3 is a direct downstream target of ASCL1, which interacts with the promoter of the *DLL3* gene [204]. ASCL1 plays a critical role in the development and differentiation of neuroendocrine cells, particularly in the lung. It is essential for the formation of normal pulmonary neuroendocrine cells, which are precursors to SCLC tumor-initiating cells [205]. ASCL1 may cooperate with the biallelic loss of pRB and p53 in neuroendocrine precursors during primary SCLC carcinogenesis and may also contribute to secondary SCLC that arises from NSCLC following cancer therapy [206].

NOTCH signaling activation promotes tumor cell proliferation or survival and tumorigenesis through multiple processes, e.g., upregulation of *CCD1* and *MYC* [207], repression and subsequent cellular proliferation [208], *DUSP1* (dual specificity phosphatase one) repression and *ERK* activation [209] or JAK-STAT signaling activation [210]. Conversely, NOTCH signaling activation blocks tumor cell proliferation or survival and tumorigenesis through multiple processes, e.g., direct upregulation of *CDKN1A* [211], GLI (glioma-associated oncogene) family zinc finger one repression [212] or downregulation and subsequent depletion of cancer stem cells [213]. NOTCH signaling is also involved in the development and homeostasis of immune cells. For example, JAG1-NOTCH signaling promotes the self-renewal of long-term hematopoietic stem cells, DLL4-NOTCH1 signaling supports early T-lymphocyte progenitor differentiation, and DLL1-NOTCH2 signaling is essential for the differentiation of marginal zone B lymphocytes [214].

Gene alterations in NSCLC

TP53. The p53 protein functions as a transcription factor, localized in both the nucleus and cytoplasm, where it specifically binds to DNA [215]. It is negatively regulated by MDM2 and MDMC, which promote its degradation via ubiquitination [216]. In response to oncogenic stress, such as *BCL2*, *BRCA1*, or *CDKN1A* activation [217, 218], as well as ribosomal stresses [219], nutrient deprivation [220], and hypoxia [221], p53 drives the expression of various genes. Under these stress conditions, p53 ubiquitination is inhibited, resulting in increased p53 protein levels. Additionally, p53 stability is enhanced through post-translational modifications, including phosphorylation, acetylation, and methylation [222]. Once stabilized, p53 forms a tetramer in the nucleus and binds to specific DNA sequences to regulate gene transcription [216].

In the presence of DNA damage, p53 acts as a guardian of the genome by coordinating multiple DNA damage response mechanisms [223]. The p53 protein activates the expression of DNA repair proteins DDB2 and XPC [224]. The gene encoding p21, which inhibits cell cycle proteins and phosphorylation of pRB, is the first transcriptional target

identified for p53 [225]. Furthermore, p53 represses cyclin-dependent kinases and cyclin B, both of which are essential for the G2/M phase progression and mitotic entry [226]. p53 also induces apoptosis by transcriptionally activating pro-apoptotic genes such as Puma, Bax, and Noxa [227, 228]. Additionally, p53 binds to the promoter region of the retrotransposon element LINE1, inhibiting the expression of transposon sequences [229].

Mutations in *TP53* are predominantly missense mutations, involving a single amino acid substitution, with the DNA-binding domain being the most frequently mutated region [229]. Mutant p53 proteins tend to be more stable than the wild-type protein, leading to their accumulation in cells [230]. These mutations not only result in the loss of normal p53 function but also promote cancer metastasis and contribute to treatment resistance in cancer [231].

A meta-analysis revealed that *TP53* mutations occur in 42.5% (569/1338) of NSCLC cases, with 35.35% (245/693) in AD, 49.44% (267/540) in SCC, and 54.29% (57/105) in LCC. Moreover, p53 protein expression was found to be negative in 52.45% (1347/2568) of NSCLC cases, with 58.86% (618/1050) negative in AD, 48.03% (645/1343) in SCC, and 48% (84/175) in LCC. Notably, *TP53* mutations are present in 92% of LCNEC cases. NSCLC with *TP53* alterations is associated with a poorer prognosis and may exhibit increased resistance to chemotherapy and radiation therapy [232].

RB1. The Retinoblastoma gene (*RB1*) encodes the retinoblastoma protein (pRB), which plays a crucial role in regulating the cell cycle by interacting with E2F transcription factors. In its unphosphorylated form, pRB binds to E2F, suppressing its transcriptional activity. The phosphorylation status of pRB, governed by the tumor suppressor p21 (regulated by p53), controls the formation of pRB-E2F complexes [233]. pRB is phosphorylated at multiple sites by cyclin-dependent kinases, including cyclin D-CDK4/6, cyclin E-CDK2, cyclin A-CDK2, and cyclin B-CDK1 [225]. pRB-E2F complexes repress the transcription of numerous cell cycle genes, many of which are required for the G1/S transition [234]. pRB is attributed to the regulation of epithelial to mesenchymal transition [235] and plays a possible role in immune response [236].

RB1 mutations are observed in 9.2% (16/174) of NSCLC cases, with 7.14% (12/168) in AD, 23.08% (3/13) in SCC, and 25% (1/4) in LCC. Although *RB1* mutations occur in a minority of NSCLC cases and are associated with poor prognosis, they are found in the majority of SCLC patients and are linked to a more favorable prognosis [237]. Furthermore, adenocarcinoma patients with concurrent mutations in *EGFR*, *RB1*, and *TP53* are prone to transformation into SCLC [238].

The protein expression of pRB is absent in 28.05% (207/738) of NSCLC cases, with 31.16% (110/353) negative in AD, 24.65% (88/357) in SCC, and 33.33% (2/6) in LCC [239–245]. *RB1* mutations occur in 42% of LCNEC cases. Deficiency in RB1 may also enhance sensitivity to chemotherapy [246].

STK11/LKB1. The *STK11/LKB1* gene encodes a serine-threonine kinase that regulates cellular metabolism, energy homeostasis, growth, and cell polarity through the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) and 12 AMPK-related kinases [247]. AMPK activation can also occur through elevated intracellular Ca^{2+} levels and DNA damage. In conditions of glucose deprivation, leading to reduced fructose-1,6-bisphosphate (FBP) levels, aldolases promote the formation of a lysosomal complex composed of v-ATPase, Ragulator, AXIN/STK11, and AMPK. This complex activates AMPK through STK11 before energy levels drop [248]. Once activated, AMPK shifts metabolism toward reduced anabolism and increased catabolism by phosphorylating key proteins involved in lipid metabolism, glycolysis, protein synthesis, and mitochondrial homeostasis [249].

AMPK serves as a metabolic checkpoint, inhibiting cell growth under low-nutrient conditions by modulating the mammalian target of the rapamycin (mTOR) pathway, the central regulator of cellular growth [250, 251]. AMPK also enhances T-cell survival by maintaining intracellular ATP levels in the absence of glucose, promoting genes involved in glutamine uptake and metabolism [252]. Additionally, AMPK regulates mitochondrial function, which may support sustained glycolysis and anti-tumor T-cell activity [253].

STK11 is altered in 12.37% (46/372) of NSCLC cases, with mutations observed in 12.11% (31/256) of AD, 8.93% (5/56) of SCC, and 42.86% (3/7) of LCC. In LCNEC, STK11 mutations were detected in 30% of cases [194]. Inactivation of STK11 is associated with a reduced infiltration of cytotoxic CD8+ T-lymphocytes [254]. To date, the expression status of STK11 in NSCLC has not been evaluated.

KEAP1. The *KEAP1* (Kelch-like ECH-associated protein 1) gene is a key negative regulator of the cellular adaptive response to reactive oxygen species (ROS) and xenobiotics, a process mediated by the transcription factor NRF2. Under normal physiological conditions, KEAP1 is part of an E3 ubiquitin ligase complex that controls the activity of NRF2 by promoting its ubiquitination and subsequent proteasomal degradation [255]. In response to cellular stress, KEAP1 allows NRF2 to evade ubiquitination, leading to its accumulation and translocation into the nucleus, where it activates the transcription of antioxidant genes. KEAP1 contains stress sensors and inactivation mechanisms that regulate NRF2 activity in response to oxidative stress, cellular metabolites, and dysregulated autophagy [256].

KEAP1 mutations are present in 18.29% (207/1132) of NSCLC cases, with a higher frequency in AD (19.23%, 160/832) and 15.61% (32/205) in SCC. LCC cases were not assessed in these studies [257, 258]. In LCNEC, *KEAP1* mutations occur in 20% of cases. Additionally, low or absent KEAP1 expression is observed in 56% of NSCLC cases, with a higher prevalence in AD (62%) compared to SCC (46%). Loss of KEAP1 expression is associated with poor overall survival and resistance to chemotherapy [259].

ASCL1. *ASCL1* (achaete-scute homolog 1) encodes transcription factors critical for neuronal differentiation and the development of olfactory and autonomic neurons. It is selectively expressed in normal fetal pulmonary neuroendocrine cells [205]. The ASCL1 protein is overexpressed in SCLC, whereas its expression is reduced or absent in AD and SCC [260]. In LCNEC, ASCL1 expression helps define two subtypes: one with high ASCL1 expression and the other with low ASCL1 expression [261].

NOTCH. The NOTCH signaling pathway consists of four NOTCH receptor isoforms (NOTCH 1–4) that regulate cell fate determination, proliferation, differentiation, and apoptosis [262]. Approximately 50% of NSCLC cases exhibit NOTCH signaling activity, although the specific expression patterns vary by subtype [263, 264]. For instance, studies by Li et al. (2010) found higher NOTCH1 expression in SCC compared to AD [265], while Donnem et al. reported lower NOTCH1 expression in SCC than in other subtypes [266]. Overexpression of NOTCH1 and NOTCH3 is associated with metastasis and reduced overall survival in NSCLC patients [267]. In contrast, NOTCH expression is significantly reduced in SCLC cases with high neuroendocrine features. Dysregulation of the NOTCH pathway is a critical factor in SCLC tumorigenesis, disease progression, and chemoresistance [268].

DLL3. DLL3 (Delta-like 3) is an inhibitory ligand of the NOTCH receptor. The binding of DLL3 to NOTCH prevents the receptor from translocating to the cell surface, leading to its accumulation on the cell membrane, particularly when overexpressed. This mechanism promotes the growth of neuroendocrine tumor cells. Tanaka et al. reported high DLL3 expression in 83% of SCLC cases, while only one out of eight NSCLC cases showed DLL3 expression, indicating that DLL3 is preferentially expressed in SCLC [269].

Methylation status of mentioned genes

To date, methylation studies in NSCLC have primarily focused on the *KEAP1* gene, with methylation occurring in 47% of cases, suggesting that deregulation of the NRF2/KEAP1 pathway plays a significant role in NSCLC carcinogenesis [270, 271]. Methylation of other key genes, including *TP53, RB1, STK11, MEN1, ASCL1, DLL3*, and *NOTCH*, has not yet been evaluated in NSCLC.

Limitations of the studies and future research directions

The limitations of this review primarily stem from the heterogeneity and sample size constraints present in the included studies. Many studies had a relatively low number of patients, which may limit the statistical power and generalizability of the findings. Additionally, most studies did not consistently distinguish between the LCC subtype and other NSCLC subtypes, and when LCC was included, the sample sizes were often small. The variation in clinicopathological data and the diverse populations across different geographic regions also contributed to inconsistencies in the results, complicating direct comparisons. To establish the diagnostic, prognostic, and therapeutic implications of gene methylation in NSCLC, future research should focus on multicentric studies involving larger and more diverse patient cohorts. This would help validate findings across different populations and strengthen the clinical relevance of methylation markers in NSCLC.

Furthermore, future research should also focus on expanding our understanding of DNA methylation patterns across different histological subtypes, particularly the rare and aggressive LCNEC. This involves comprehensive epigenetic profiling to uncover subtype-specific methylation markers that could improve diagnosis and prognosis. Importantly, DNA methylation holds significant potential for the early detection of NSCLC through the identification of specific biomarkers, a crucial factor in reducing lung cancer mortality [272, 273]. Longitudinal studies are also essential to validate methylation as a biomarker, potentially facilitating its integration into liquid biopsy platforms for non-invasive monitoring of disease progression and treatment response, as methylation sequencing in plasma cell-free DNA from patients with advanced cancers may detect the presence of cancer and its subtype [274]. Furthermore, postoperative cell tumor DNA methylation levels could serve as an indicator of molecular residual disease in patients with resected NSCLC [275].

The clinical translation of methylation research could significantly impact NSCLC management. Multi-gene methylation panels could enhance diagnostic accuracy and guide personalized treatment strategies, especially for subtypes like LCNEC, which currently lack standardized protocols. Furthermore, research into how methylation status affects drug resistance could lead to novel therapeutic targets, improving the efficacy of existing treatments. Overall, a focus on standardizing methylation testing and integrating it into routine clinical practice could pave the way for more precise and effective management of NSCLC.

In conclusion, this paper provides a comprehensive overview of the aberrant methylation of key genes (*APC, BRCA1, CDH1, CDH13, CDKN2A, DAPK1, DLEC1, FHIT, hMLH1, MGMT, RARβ, RASSF1, RUNX3, WIF1,* and *TIMP3*) and the expression and mutation profiles of key genes (*TP53, RB1, STK11, KEAP1, ASCL1, DLL3,* and *NOTCH*) in NSCLC, based on 75 studies covering histological subtypes such as AD, SCC, and, where available, LCC (Table 1). Of these studies, 34 addressed diagnostic gene methylation, 35 focused on prognostic factors, and six covered epidemiology and predictive biomarkers.

Furthermore, the paper reviews the current understanding of mutational and expression profiles in LCNEC. Notably, only two studies have investigated *KEAP1* methylation in NSCLC, and no studies to date have examined the methylation of *TP53, RB1, STK11, ASCL1, DLL3,* and *NOTCH* in

Table 1. Methylation summary in selected genes in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC), and large cell carcinoma (LCC).

References	Genes	Methylation summary (%)			
		NSCLC	AD	SCC	LCC
Huang 2018 [34], Wang 2008 [32], Yanagawa 2007 [29], Zöchbauer-Müller 2001 [28], Lee 2012 [40], Seike 2000 [111], Ulivi 2006 [113], Ng 2002 [112], Liu 2003 [114], Kim 2007 [110]	*CDKN2A*	25.23–79.49	13.33–70.59	11.76–87.5	25–100
Huang 2018 [34], Wang 2008 [32], Yanagawa 2007 [29], Zöchbauer-Müller 2001 [28], Yang 2018 [33], Niklinska 2009 [30], Tang 2000 [31]	*DAPK1*	18.69–76.47	15.56–78.57	20.93–71.43	0–22.22
Wang 2008 [32], Yanagawa 2007 [29], Lee 2012 [40], Kim 2007 [110], Niklinska 2009 [30], Shah 2020 [177], Chen 2006 [175], Zhai 2014 [178], Li 2003 [176], Song 2011 [39], Li 2012 [43]	*RASSF1*	21.43–85.71	6.67–82.35	25–89.66	0–66.67
Yanagawa 2007 [29], Zöchbauer-Müller 2001 [28], Lee 2012 [40], Song 2011 [39], Li 2012 [43], Li 2014 [42], Feng 2016 [38], Li 2014 [167], Zhao 2012 [41]	*RAR β*	30.56–80.36	26.67–88.24	15.38–88.46	25
Huang 2018 [34], Wang 2008 [32], Kim 2007 [110], Feng 2016 [38], Brabender 2001 [126], Toyooka 2003 [125], Suzuki 2006 [124]	*APC*	25–89.01	14.29–100	23.2–100	33,33
Wang 2008 [32], Yanagawa 2007 [29], Ulivi 2006 [113], Zhai 2014 [178], Toyooka 2003 [125], Suzuki 2006 [124], Hsu 2007 [190], Seuk Kim 2005 [188], Kontic 2012 [138]	*CDH13*	16.92–65.57	29.11–69.44	2.94–69.23	25–33.33
Wang 2008 [32], Harada 2013 [53], Lee 2007 [54]	*BRCA1*	18.57–37.08	13.33–42.31	13.33–29.73	33.33
Yanagawa 2007 [29], Zöchbauer-Müller 2001 [28], Toyooka 2003 [125], Wu 2008 [142], Liu 2006 [140], Brabender 2003 [141], Chan 2002 [139], Wu 2007 [193]	*MGMT*	8.95–49.54	8.03–46.88	7.73–60	20–25
Wang 2008 [32], Kim 2007 [110], Song 2011 [39], Seng 2008 [61], Wang 2003 [151], Geng 2009 [152], Hsu 2005 [150], Yanagawa 2003 [92]	*hMLH1*	6.67–72.41	4.65–72.41	10.34–72.62	33.33–40.74
Yanagawa 2007 [29], Kim 2007 [110], Li 2014 [167], Li 2010 [168], Li 2009 [170], Kim 2004 [165], Tzao 2004 [166], Tomizawa 2004 [169], Nakata 2006 [83]	*FHIT*	27.2–59.62	25–52.38	30.77–73.33	0–50
Wang 2008 [32], Zöchbauer-Müller 2001 [28], Damman 2005 [192], Castro 2010 [59], Gu 2006 [58]	*TIMP3*	18.52–47.29	9.38–40	23.26–53.25	0–25
Huang 2018 [34], Song 2011 [39], Seng 2008 [61], Nawaz 2014 [66]	*DLEC1*	37.14–70.59	28.57–71.43	44.44–66.67	35.19
Huang 2018 [34], Nakata 2006 [83], Gu 2006 [58], Shimamoto 2004 [82], Kim 2007 [81]	*CHD1*	32.56–58.33	30.77–59.52	33.77–66.67	100
Huang 2018 [34], Yanagawa 2003 [92], Castro 2010 [59], Yu 2012 [94], Sato 2006 [93]	*RUNX3*	20–52.94	27.91–57.14	6.67–50	50
Huang 2018 [34], Lee 2013 [106], Yang 2009 [107], Suzuki 2007 [105], Yoshino 2009 [104]	*WIF1*	15.91–69.44	13.33–63.33	27.27–100	0–53.8

NSCLC. LCNEC, positioned as a "bridge" between small SCLC and NSCLC, underscores the necessity for further research to determine the methylation status of these genes in both NSCLC and LCNEC. Such investigations could enhance diagnostic, prognostic, and therapeutic strategies, leveraging gene methylation modifications.

Supplementary information is available in the online version of the paper.

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Methylation status of selected genes in non-small cell lung carcinoma – current knowledge and future perspectives

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Supplementary Information

Supplementary Table S1. PRISMA 2020 main checklist.

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Page 1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Page 2 and 3
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Page 3
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Page 5
Information sources	6	Specify all databases, registers, websites, organizations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Page 3
Search strategy	7	Present the full search strategies for all databases, registers, and websites, including any filters and limits used.	Page 2 and 4
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Page 5 and 6
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Page 5 and 6
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g., for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Page 5 and 6
	10b	List and define all other variables for which data were sought (e.g., participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	N/A
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Page 6
Effect measures	12	Specify for each outcome the effect measure(s) (e.g., risk ratio, mean difference) used in the synthesis or presentation of results.	N/A
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g., tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	N/A
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	N/A
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	N/A
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	N/A
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g., subgroup analysis, meta-regression).	N/A
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	N/A

Supplementary Table S1. ***Continued ...***

Section and Topic	Item #	Checklist item	Location where item is reported
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	N/A
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	N/A
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Page 6 and 7
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	N/A
Study characteristics	17	Cite each included study and present its characteristics.	Page 8–18
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Page 7
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimates and its precision (e.g., confidence/credible interval), ideally using structured tables or plots.	Page 8–18
Results of syntheses	20a	For each synthesis, briefly summarize the characteristics and risk of bias among contributing studies.	N/A
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g., confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	N/A
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	N/A
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	N/A
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	N/A
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	N/A
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Page 8–18
	23b	Discuss any limitations of the evidence included in the review.	Page 8–18
	23c	Discuss any limitations of the review processes used.	N/A
	23d	Discuss implications of the results for practice, policy, and future research.	Page 19
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	N/A
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	N/A
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	N/A
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	N/A
Competing interests	26	Declare any competing interests of review authors.	N/A
Availability of data, code, and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	N/A

Supplementary Table S2. Search strategy used for the PubMed database.

Database	PubMed
Date	From inception to 15 October 2023
#1	"NSCLC" AND ((methylation) OR (hypermethylation))
#2	((epigenetics) AND (ASCL1) OR (DLL3) OR (NOTCH) OR (TP53) OR (RB1) OR (STK11) OR (LKB1) OR (KEAP1))
#3	(((methylation) OR (hypermethylation)) AND ((pattern) OR (gene promoter) OR (CDKN2A) OR (RASSF1) OR (CDH13) OR (DAPK1) OR (TIMP3) OR (hMLH1) OR (APC) OR (BRCA1) OR (DLEC1) OR (CDH1) OR (RUNX3) OR (FHIT) OR (MGMT) OR (RAR beta) OR (WIF1)))
#4	(((expression) OR (mutation)) AND ((ASCL1) OR (DLL3) OR (NOTCH) OR (TP53) OR (RB1) OR (STK11) OR (LKB1) OR (KEAP1)))

Supplementary Table S3. The search items used for the systematic review.

Search Terms Used in the Systematic Review				
NSCLC methylation profiling	NSCLC methylation brca1	NSCLC epigenetic tp53	NSCLC expression p35	NSCLC mutation tp53
NSCLC methylation cdkn2a	NSCLC methylation dlec1	NSCLC epigenetic rb1	NSCLC expression rb1	NSCLC mutation rb1
NSCLC methylation rassf1	NSCLC methylation cdh1	NSCLC epigenetic stk11	NSCLC expression stk11	NSCLC mutation stk11
NSCLC methylation cdh13	NSCLC methylation runx3	NSCLC epigenetic lkb1	NSCLC expression lkb1	NSCLC mutation lkb1
NSCLC methylation dapk1	NSCLC methylation rar beta	NSCLC epigenetic keap1	NSCLC expression keap1	NSCLC mutation keap1
NSCLC methylation timp3	NSCLC methylation fhit	NSCLC epigenetic ascl1	NSCLC expression ascl1	
NSCLC methylation hmlh1	NSCLC methylation mgmt	NSCLC epigenetic dll3	NSCLC expression dll3	
NSCLC methylation apc	NSCLC methylation wif1	NSCLC epigenetic notch	NSCLC expression notch	

Supplementary Table S4. Methylation of *DAPK* in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).

Histological subtype	Percentages (%)	Proportions
NSCLC	18.69–76.47	20/107 [28], 26/101 [29], 24/70 [30], 50/122 [31], 14/28 [32], 68/117 [33], 13/17 [34]
AD	15.56–78.57	7/45 [28], 14/62 [28], 5/20 [30], 7/15 [32], 34/71 [31], 35/61 [33], 11/14 [34]
SCC	20.93–71.43	9/43 [28], 12/39 [28], 16/51 [31], 17/41[30], 33/56 [33], 2/3 [34], 5/7 [32]
LCC	0–22.22	0/4; 0/3 [28, 32], 2/9 [30]

Supplementary Table S5. Methylation of *RAR-β* in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).

Histological subtype	Percentages (%)	Proportions
NSCLC	30.56–80.35	11/36 [39], 38/101 [29], 43/107 [28], 22/53 [40], 45/80 [41], 134/167 [42], 45/56 [43]
AD	26.67–88.24	8/30 [39], 22/45 [28], 32/62 [29], 13/22 [40], 15/25 [41], 66/81 [42], 15/17 [43]
SCC	15.38–88.46	6/39 [29], 15/43 [28], 14/36 [39], 8/17 [40], 30/45 [41], 68/86 [42], 23/26 [43]
LCC	25%	1/4 [28, 40]

Supplementary Table S6. Methylation of *BRCA1* in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).

Histological subtype	Percentages (%)	Proportions
NSCLC	18.57–37.08	13/70 [53], 6/28 [32], 33/89 [54]
AD	13.33–42.31	2/15 [32], 10/49 [53], 22/52 [54]
SCC	13.33–29.73	2/15 [53], 1/7 [32], 11/37 [54]
LCC	33.33	1/3 [32]

Supplementary Table S7. Methylation of *TIMP3* in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).

Histological subtype	Percentages (%)	Proportions
NSCLC	18.52–47.29	10/54 [59], 28/107 [28], 11/28 [32], 61/129 [58]
AD	9.38–40	3/32 [59], 11/45 [28], 20/52 [58], 6/15 [32]
SCC	23.26–53.25	10/43 [28], 7/21 [59], 3/7 [32], 41/77 [58]
LCC	0–25	0/3 [32], 1/4 [28]

Supplementary Table S8. Methylation of *DLEC1* in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).

Histological subtype	Percentages (%)	Proportions
NSCLC	37.14–70.59	26/70 [66], 92/238 [61], 32/78 [39], 12/17 [34]
AD	28.57–71.43	10/35 [66], 29/92 [61], 12/30 [39], 10/14 [34]
SCC	44.44–66.67	16/36 [39], 16/35 [66], 44/92 [61], 2/3 [34]
LCC	35.19	19/54 [61]

Supplementary Table S9. Methylation of *CDH1* in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).

Histological subtype	Percentages (%)	Proportions
NSCLC	32.56–58.33	42/129 [58], 30/91 [81], 8/17 [34], 24/42 [82], 119/204 [83]
AD	30.77–59.52	16/52 [58], 11/35 [81], 6/14 [34], 13/25 [82], 75/126 [83]
SCC	33.77–66.67	26/77 [58], 19/56 [81], 6/12 [82], 44/78 [83], 2/3 [34]
LCC	100	5/5 [82]

Supplementary Table S10. Methylation of *RUNX3* in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).

Histological subtype	Percentages (%)	Proportions
NSCLC	20–52.94	15/75 [92], 30/119 [93], 23/54 [59], 26/58 [94], 9/17 [34]
AD	27.91–57.14	12/43 [92], 10/32 [59], 26/72 [93], 13/32 [94], 8/14 [34]
SCC	6.67–50	3/45 [93], 2/29 [92], 1/3 [34], 8/21 [59], 13/26 [94]
LCC	50	1/2 [93]

Supplementary Table S11. Methylation of *WIF1* in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).

Histological subtype	Percentages (%)	Proportions
NSCLC	15.91–69.44	7/44 [104], 65/235 [105], 6/17 [34], 66/139 [106], 50/72 [107]
AD	13.33–63.33	4/30 [104], 3/14 [34], 30/135 [105], 36/79 [106], 19/30 [107]
SCC	27.27–100	3/11 [104], 28/87 [105], 30/60 [106], 3/3; 2/2 [34, 107]
LCC	0–53.8	0/3 [104], 7/13 [105]

Supplementary Table S12. Methylation of *CDKN2A* in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).

Histological subtype	Percentages (%)	Proportions
NSCLC	25.23–79.49	27/107 [28], 22/99 [110], 27/101 [29], 16/53 [40], 10/28 [32], 8/22 [111], 14/33 [112], 10/17 [34], 48/61 [113], 31/39 [114]
AD	13.33–83.33	6/45 [28], 9/62 [29], 3/13 [111], 9/38 [110], 10/32 [40], 5/15 [32], 8/13 [112], 9/14 [34], 12/17 [114], 30/36 [113]
SCC	11.76–87.5	2/17 [40], 1/7 [32], 16/61 [110], 4/15 [112], 1/3 [34], 16/43 [28], 18/39 [29], 5/9 [111], 19/22 [114], 14/16 [113]
LCC	25–100	1/4; ¼ [28, 113], 1/3 [32], 4/4 [40]

Supplementary Table S13. Methylation of *APC* in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).

Histological subtype	Percentages (%)	Proportions
NSCLC	25–89.01	9/36 [38], 15/53 [124], 7/17 [34], 216/514 [125], 48/99 [110], 19/28 [32], 81/91 [126]
AD	14.29–100	3/21 [38], 32/79 [124], 6/14 [34], 20/38 [110], 161/299 [125], 11/15 [32], 43/43 [126]
SCC	23.2–100	45/194 [125], 1/3 [34], 6/15 [38], 28/61 [110], 4/7 [32], 33/33 [126]
LCC	33.33	1/5, 5/15 [32, 126]

Supplementary Table S14. Methylation of *MGMT* in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).

Histological subtype	Percentages (%)	Proportions
NSCLC	8.95–49.54	46/514 [125], 8/65 [138], 14/101 [29], 11/75 [139], 21/107 [28], 28/94 [140], 34/90 [141], 109/220 [142]
AD	8.03–46.88	24/299 [125], 2/18 [138], 8/62 [29], 7/44 [139], 12/45 [28], 22/72 [140], 46/115 [142], 15/32 [141]
SCC	7.73–60	15/194 [125], 4/31 [139], 6/39 [29], 8/43 [28], 6/29 [138], 6/22 [140], 16/43 [141], 63/105 [142]
LCC	20–25	3/15 [141], 1/4 [28]

Supplementary Table S15. Methylation of *hMLH1* in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).

Histological subtype	Percentages (%)	Proportions
NSCLC	6.67–72.41	5/75 [92], 17/99 [110], 14/78 [39], 8/28 [32], 85/238 [61], 56/105 [150], 38/70 [151], 84/116 [152]
AD	4.65–71.88	2/43 [92], 4/30 [39], 7/38 [110], 21/92 [61], 6/15 [32], 17/28 [151], 43/65 [150], 23/32 [152]
SCC	10.34–72.62	3/29 [92], 1/7 [32], 11/61 [110], 8/36 [39], 6/17 [150], 42/92 [61], 21/42 [151], 61/84 [152]
LCC	33.33–40.74	1/3 [32], 22/54 [61]

Supplementary Table S16. Methylation of *FHIT* in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).

Histological subtype	Percentages (%)	Proportions
NSCLC	27.2–59.62	68/250 [165], 28/91 [166], 19/56 [167], 34/99 [110], 38/109 [168], 43/119 [169], 110/204 [83], 31/52 [170]
AD	25–52.38	10/40 [166], 24/93 [165], 11/38 [110], 13/41 [168], 24/72 [169], 6/17 [167], 9/22 [170], 66/126 [83]
SCC	30.77–73.33	12/39 [83], 40/125 [165], 9/26 [167], 25/68 [168], 23/61 [110], 18/45 [169], 18/44 [166], 44/78 [83], 22/30 [170]
LCC	0–50	0/4 [166], 1/2 [169]

Supplementary Table S17. Methylation of *RASSF1A* in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).

Histological subtype	Percentages (%)	Proportions
NSCLC	21.43–85.71	6/28 [32], 18/70 [30], 39/112 [175], 21/53 [40], 31/78 [39], 26/65 [176], 40/99 [110], 41/100 [177], 42/101 [29], 22/42 [178], 48/56 [43]
AD	6.67–82.35	1/15 [32], 7/41 [30], 28/85 [175], 27/62 [29], 14/30 [39], 18/33 [176], 34/72 [177], 18/38 [110], 17/32 [178], 21/33 [176], 15/22 [40], 14/17
SCC	25–89.66	7/28 [177], 5/20 [176], 2/7 [32], 12/36 [39], 6/17 [40], 22/61 [110], 15/39 [29], 11/27 [175], 9/20 [30], 5/10 [178], 26/29 [43, 167]
LCC	0–66.67	0/4 [40], 2/9 [30], 3/12 [176], 2/3 [32]

Supplementary Table S18. Methylation of CDH13 in NSCLC and its subtypes adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma (LCC).

Histological subtype	Percentages (%)	Proportions
NSCLC	16.92–65.57	11/65 [138], 26/101 [29], 40/150 [124], 172/514 [125], 13/28 [32], 121/251 [188], 23/42 [178], 35/54 [190], 40/61 [113]
AD	29.11–69.44	23/79 [124], 20/62 [29], 6/15 [32], 123/299 [125], 62/122 [188], 17/32 [178], 26/41 [190], 8/12 [138], 25/36 [113]
SCC	2.94–69.23	1/34 [138], 6/39 [29], 44/194 [125], 3/7 [32], 9/16 [113], 6/10 [178], 93/142 [188], 9/13 [190]
LCC	25–33.33	1/4 [113], 1/3 [32]