Cancer Metastasis Rev (2010) 29:95–107
DOI 10.1007/s10555-010-9203-x

Lung cancer: From single-gene methylation to methylome profiling

Gerwin Heller · Christoph C. Zielinski · Sabine Zöchbauer-Müller

Published online: 23 January 2010

Abstract DNA methylation as part of the epigenetic gene-silencing complex is a universal occurring change in lung cancer. Numerous studies investigated methylation of specific genes in primary tumors, in serum or plasma samples, and in specimens from the aerodigestive tract epithelium of lung cancer patients. In most studies, single genes or small numbers of genes were analyzed. Moreover, it has been observed that methylation of certain genes can already be detected in samples from the upper aerodigestive tract epithelium of cancer-free heavy smokers. These findings indicated that methylation of certain genes may be a useful biomarker for prognosis, disease recurrence, early detection, and lung cancer risk assessment. So far, several genes were identified which seem to be of worse prognostic relevance when they were found to be methylated. In addition, it has been shown that a panel of markers may be relevant to predict disease recurrence after surgery. In comparison to analysis of single or small numbers of genes, methods for genome-wide detection of methylation were developed recently. These approaches are focused on either pharmacological re-activation of methylated genes followed by expression microarray analysis or on microarray analysis of sodium bisulfite-treated or affinity-enriched methylated DNA sequences. With currently available methods for the simultaneous detection of methylation, up to 28,000 CpG islands 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.

G. Heller · C. C. Zielinski · S. Zöchbauer-Müller (✉)
Department of Medicine I, Clinical Division of Oncology,
Medical University of Vienna,
Währinger Gürtel 18-20,
1090 Vienna, Austria
e-mail: sabine.zoechbauer-mueller@meduniwien.ac.at

Keywords Lung cancer · Epigenetics · DNA methylation · High throughput analysis

1 Introduction

Despite enormous effort in early detection programs and development of new drugs and innovative treatment strategies in recent years, lung cancer is still very often a deadly disease with 5-year survival rates of about 14% [1]. While numerous genetic abnormalities have been described in lung carcinomas during the past decades, the detection of epigenetic changes in lung cancer started significantly later. At the beginning of this era, methylation analysis of only single genes was performed in a small number of primary tumors of lung cancer patients. However, rapidly, an increasing number of genes were investigated for their methylation status and researchers reported that methylation can also be detected in blood samples and in exfoliative material of the aerodigestive tract epithelium from lung cancer patients. Moreover, it became evident that methylation of certain genes can already be detected in cancer-free smokers. In the meantime, data about worse prognostic impact of methylation of certain genes have been reported. Moreover, it has been demonstrated that the methylation status of certain genes may predict disease recurrence in non-small cell lung cancer (NSCLC) patients. Recently, high throughput approaches to detect methylation have been developed. These methods provide researchers with a huge amount of information which will help to better understand the role of epigenetic changes in the pathogenesis of lung cancer. We now need to translate our findings about DNA methylation in lung cancer into clinical practice which hopefully will result in a better outcome for these patients.

In this review, we summarize results from single-gene methylation analysis as well as from high throughput methylation approaches in lung cancer cell lines and in specimens from lung cancer patients and cancer-free smokers.

2 DNA methylation and regulation of gene expression

Epigenetic mechanisms largely contribute to the regulation of the transcriptional activity of a gene. Among these mechanisms, DNA methylation and various chemical modifications of histone proteins (acetylation, methylation, ubiquitination, phosphorylation, and ADP-ribosylation) are key regulators which affect the binding of transcription factors to DNA and change the structure of chromatin resulting either in gene activation or gene silencing [2, 3]. In recent years, the interaction between DNA methylation and histone modifications resulting in gene silencing became more and more evident [4]. Methyl-cytosines bind methyl-CpG binding domain proteins. Through interactions of them with histone deacetylases, histone methyltransferases, and chromatin-remodeling enzymes, methylated DNA is translated into compacted chromatin which is repressive for transcription. Several studies revealed that DNA methylation directly influences both the acetylation and the methylation state of histone proteins [5–7].

In mammalian cells, DNA is methylated by DNA methyltransferases (DNMTs). DNMT1 is associated with the replication complex and responsible for both methylating fully unmethylated DNA (*de novo* methylation) and maintaining pre-existing DNA methylation patterns in a cell [8]. DNMT3A and DNMT3B are *de novo* DNMTs [9, 10]. The target position for the addition of a methyl-group is the 5′-carbon of cytosines within CpG dinucleotides. The CpG dinucleotide is relatively rare in the mammalian genome [11]. However, certain regions of the genome, 0.5-4 kb in length, harbor CpG dinucleotides at high density and are called CpG islands (CGIs). CGIs are found in approximately 60% of the human gene-promoter regions and may extend into the 5′ coding end of the gene [12]. Computational analysis predicts 29,000 CGIs in the human genome and most chromosomes harbor 5-15 islands per Mb [13, 14]. While 70-80% of all non-CGI CpG dinucleotides in the human genome are methylated, CGI CpG dinucleotides usually remain unmethylated. Exceptions are CGIs associated with imprinted, X-linked, and tissue-specific expressed genes [15–17]. Alterations in CGI methylation and histone modifications leading to silencing of certain tumor suppressor genes (TSGs) frequently occur in cancer cells [18, 19].

DNA methylation is a reversible process and expression of methylated genes can be restored by the use of DNMT inhibitors such as 5-aza-2′-deoxycytidine (Aza-dC). In addition, histone deacetylase (HDAC) inhibitors such as trichostatin A (TSA) synergistically enhance the re-activating effect of DNMT inhibitors [20].

3 Interaction between microRNAs and DNA methylation in lung cancer

Recently, it has become apparent that microRNAs (miRNAs) are involved in tumorigenesis [21]. miRNAs are noncoding, 18-25 nt long RNAs which regulate a variety of biological processes including development, cellular differentiation, proliferation, and apoptosis by silencing specific target genes [22]. miRNAs induce translational repression by binding to complementary sequences in the 3′ untranslated regions (3′ UTR) of target mRNAs or by directing mRNA degradation [23]. Deregulation of miRNA expression resulting in abnormal activation or repression of oncogenes and TSGs is a common event in various human cancers [21]. It has been shown that DNA methylation is one mechanism for miRNA gene silencing [24–27]. Examples for methylated miRNA genes in lung cancer are *miR-34a* and *miR124a* [27, 28]. Interestingly, both of these miRNAs are part of molecular pathways whose depletion may contribute to the development of a malignant phenotype. While miR34a is part of the p53 network, miR124a is supposed to regulate levels of the cell cycle progression factor CDK6 [27, 29].

Interestingly, Fabbri et al. [30] demonstrated recently that miRNAs are not only targets but also regulators of DNA methylation. They reported that DNMT3A and DNMT3B are regulated by miR29 and that increased expression of miR-29 in lung cancer cells results in restoration of normal DNA methylation and expression patterns of the silenced TSGs *FHIT* and *WWOX*.

4 DNA methylation of single or small numbers of genes in lung cancer patients

Different methods were developed to determine the methylation status of genes. The most frequently used methods include bisulfite genomic sequencing and methylation-specific polymerase chain reaction (MSP) [31–33]. In addition, quantitative real-time polymerase chain reaction (RT-PCR) approaches are being used [31, 34, 35]. These methods are based on the treatment of genomic DNA with sodium bisulfite which converts unmethylated cytosine bases into uracil while methylated cytosines remain unchanged. Subsequently, methylation can be detected using specific primer sets for either methylated or unmethylated target sites.

To date, a large number of genes have been investigated for their methylation status in lung cancer patients. Most of the studies investigated surgically obtained tumor samples

from NSCLC patients. Genes which were intensively studied in primary NSCLC samples include *p16*, *RASSF1A*, *APC*, *RARβ-2*, *CDH1*, *CDH13*, *DAPK*, and *MGMT* [36–43]. While *p16* and *RASSF1A* are involved in cell cycle regulation, *CDH1* and *CDH13* play a role in regulation of cell adhesion, *APC* inhibits *β-catenin*, *RARβ-2* is involved in growth regulation, *DAPK* acts in regulation of apoptosis and *MGMT* functions in DNA repair. Other genes investigated in primary NSCLCs include for instance *ASC/TMS1*, *FHIT*, *hSRBC*, *TSLC1*, and *DAL-1* [44–49]. *ASC/TMS1* and *FHIT* have pro-apoptotic function, *hSRBC* is a cell cycle regulator and *TSLC1* and *DAL-1* are putative TSGs involved in a cell adhesion cascade. The frequencies of methylation of these genes in primary NSCLCs were detected in up to 96%. Details about some of these genes are shown in Table 1.

A majority of the studies also investigated if methylation of certain genes occurs in corresponding non-malignant lung tissue samples. It has been shown that most genes were found to be methylated only in tumors but not at all or only in a very low percentage in the corresponding non-malignant lung tissues [38, 41, 43, 47]. Overall, these findings demonstrate that methylation of cancer-related genes is a frequent event in the pathogenesis of lung cancer.

However, DNA methylation cannot only be detected in primary tumors but also in blood, sputum, bronchial brushings, and bronchioloalveolar lavage (BAL) samples from lung cancer patients. A major advantage is that these specimens can be obtained in a non-invasive or minimally invasive way. Methylation in blood can usually only be detected if the primary tumor is methylated. Sputum can be obtained from smokers. BAL samples represent lung-specific material, however, a bronchoscopy needs to be performed. Overall, detecting methylation in these samples is potentially useful for predicting disease recurrence after surgery, for monitoring response to therapy, and for early detection of lung cancer.

Numerous manuscripts have been published which describe the frequency of methylation of genes (for instance *MGMT*, *p16*, *DAPK*, *APC*, *CDH13*, *FHIT*, *RARβ-2*, *RASSF1A*) in serum or plasma samples and in the corresponding primary tumors [50–56]. In most studies, the frequencies of methylation in serum or plasma samples were lower compared to primary tumors. Usually, methylation in serum or plasma samples was detected only if the corresponding tumors were methylated [50, 52]. In the majority of the studies, methylation was not detected in serum or plasma samples from healthy controls [51, 54].

Several genes were found to be methylated in sputum samples from lung cancer patients. These genes include *hMLH1*, *p16*, *CDH13*, *DAPK*, *LAMC2*, *MGMT*, *PAX5ά PAX5β*, *RASSF1A* [55–59]. Another gene which is frequently methylated in NSCLC patients is the *ASC/TMS1* gene. Interestingly, *ASC/TMS1* methylation was observed in 14% of primary tumors and in 17% of sputum samples of stage I NSCLC patients, but in 60% of primary tumors and in 41% of sputum samples of later stages NSCLC patients suggesting that methylation of *ASC/TMS1* is a marker for late-stage lung cancer [60]. The majority of studies describe similar or slightly lower frequencies of methylation of certain genes in sputum samples compared to primary tumors.

Table 1 Genes reported to be of unfavorable prognostic relevance if methylated in primary NSCLCs

Gene	Gene name	Function	Percent of methylation[a]
APC	Adenomatosis polyposis coli	Inhibitor of β-catenin	46-96
ASC/TMS1	Target of methylation-induced silencing 1	Pro-apoptotic	40-47
CDH1	Cadherin 1, E-cadherin (epithelial)	Cell adhesion	18-58
DAL-1	Erythrocyte membrane protein band 4.1-like 3	Cytoskeleton organization	55-57
DAPK	Death-associated protein kinase 1	Pro-apoptotic	16-44
DLEC1	Deleted in lung cancer protein 1	Cell cycle regulation	39
FHIT	Fragile histidine triad protein	Pro-apoptotic	37-38
hMLH1	DNA mismatch repair protein Mlh1	DNA repair	36
MGMT	O6-methylguanine-DNA methyltransferase	DNA repair	16-38
p16	Cyclin-dependent kinase 4 inhibitor A	Cdk inhibitor	25-41
RASSF1A	Ras association domain family 1	Cell cycle regulation	30-40
RRAD	Ras-related associated with diabetes	Signal transduction	42
RUNX3	Runt-related transcription factor 3	Pro-apoptotic	25
TSLC1	Tumor suppressor in lung cancer 1	Cell adhesion	37-44

Data extracted from the following references [37–39, 42–45, 48–50, 56, 61, 68, 72, 74, 75, 77, 79, 82, 114–123]

[a] Includes not only the frequency of methylation from the manuscript which described a prognostic relevance but also frequencies from other manuscripts which did not find a prognostic relevance for a certain gene

Methylation of eight genes was evaluated in different kinds of material including primary tumor biopsies, serum, and sputum samples from stage III NSCLC patients [59]. The prevalence for methylation of the genes in sputum was similar to that seen in tumors, but was higher than that in serum. The authors conclude that sputum can be used as a surrogate for tumor tissue to predict the methylation status of NSCLCs. Shivapurkar et al. [61] analyzed the methylation status of 11 genes in primary NSCLCs, in adjacent non-malignant lung tissues, in peripheral blood mononuclear cells from cancer-free patients, in sputum samples of cancer patients and in controls. The gene *3-OST-2* showed the highest levels of methylation in tumors combined with lowest levels of methylation in control tissues. Moreover, the authors reported that quantitative analysis of *3-OST-2*, *RASSF1A*, *p16*, and *APC* methylation in sputum samples may have excellent potential as a biomarker assay for lung cancer patients.

Methylation of genes, for instance, *p16*, *APC*, *RASSF1A*, *CDH13*, *DAPK*, *MGMT*, *RASSF1A*, and *RARβ-2*, was also detected in bronchial lavage samples [56, 62–66]. However, the frequency of methylation in this kind of samples was usually lower compared to the frequencies in primary tumors and sputum samples. In addition, the frequencies of methylation were lower in peripherally located tumors compared to centrally located tumors [62].

Recently, Han et al. [67] investigated methylation of *DAPK*, *RASSF1A*, and *PAX5β* in exhaled breath condensate from lung cancer patients and control individuals. Both groups contained current, former, and never smokers. The *RASSF1A* methylation density was different regarding the smoking status. Former smokers had higher methylation density versus never smokers and current smokers. Methylation of specific CpG sites of *DAPK* and *PAX5β* promoter were associated with lung cancer status. The authors concluded that DNA methylation can be detected in exhaled breath condensate, however, additional studies are necessary to define the potential impact of this observation.

5 Differences in the methylation patterns due to histology and genetic abnormalities

Toyooka et al. [68] investigated the frequencies of methylation of eight genes in a large number of lung tumors consisting of small cell lung cancers (SCLC), NSCLCs and bronchial carcinoids. The frequencies of methylation of *APC*, *CDH13* and *p16* were significantly higher in NSCLCs compared to neuroendocrine tumors. On the other hand, the frequencies of methylation of the genes *RASSF1A*, *CDH1* and *RARβ-2* were higher in SCLCs compared to carcinoids. In addition, the methylation index, a parameter which reflects the methylation status of all genes tested, was significantly higher in SCLCs compared to carcinoids. However, differences in the frequencies of methylation were seen not only between NSCLCs, SCLCs and carcinoids, but also between NSCLC subtypes. The frequencies of *APC* and *CDH13* methylation were significantly higher in adenocarcinomas compared to squamous cell carcinomas. On the contrary, methylation of *p16* was more frequently detected in squamous cell carcinomas compared to adenocarcinomas. Methylation of nine genes including *p16*, *CDH1*, *TIMP3*, *RASSF1A*, *FHIT*, *APC*, *DAPK*, *MGMT*, and *GSTP1* was analyzed in primary NSCLCs by Gu et al. [69]. Overall, the methylation index was significantly higher in adenocarcinomas than in squamous cell carcinomas. A comparison of the methylation pattern of 47 gene-promoter regions between primary NSCLCs and corresponding adjacent normal lung tissues was performed by Ehrich et al. [70]. Six genes with statistically significant differences in methylation between tumor and normal tissue were identified. Moreover, using a hierarchical clustering algorithm normal lung could be distinguished from lung cancer tissue with >95% sensitivity and specificity. These studies indicate that DNA methylation is tissue type-specific. Distinct methylation signatures distinguish the major histologic lung cancer types and also tumor tissues from non-malignant lung tissues.

Differences in the methylation patterns were also found between lung tumors with certain genetic abnormalities. Toyooka et al. [71] reported that in lung adenocarcinomas the probability of having *EGFR* mutations was significantly lower among tumors with *p16* and *CDH13* methylation than in tumors without. In addition, the methylation index was significantly lower in *EGFR* mutant cases compared to wild-type cases. In contrast, the frequency of *K-RAS* mutations was significantly higher in *p16* methylated cases than in unmethylated cases. The methylation index was marginally higher in *K-RAS* mutant cases compared to wild-type cases. Overall, the authors concluded that there are differences in the involvement of methylation between *EGFR*- and *K-RAS*-mediated tumorigenesis. These findings suggest that genetic and epigenetic changes interact systematically to promote tumorigenesis of lung adenocarcinomas.

6 DNA methylation as biomarker

In general, detecting methylation of certain genes seems to be a powerful biomarker for prognosis of patients, disease recurrence, early detection and risk assessment. In addition, it also might be a therapeutic target. However, there are some open questions and limitations which still need to be discussed, e.g., is there sufficient tumor DNA in blood, especially in early stages of disease? Tumor markers in blood are not organ-specific and DNA methylation detected

in blood may indicate a tumor other than lung cancer. Satisfactory sputum specimens can usually only be obtained from smokers but not from non-smokers or long-term former smokers. Therefore, this sample type is useful only for a subgroup of patients. Sputum which is obtained from the central lung, however, may not detect peripheral tumors. Another point is that there are several methods by which methylation can be detected and the question is which of them is optimal. Moreover, it has been shown that the sensitivity and the specificity are higher if panel of markers are investigated rather than single genes. However, which panel of markers has the highest sensitivity and specificity? In the following sections, we describe findings about how DNA methylation can function as a biomarker.

6.1 DNA methylation markers with unfavorable prognostic relevance

A worse prognostic impact of methylation in primary tumors of NSCLC patients has been reported for several genes. Several years ago, Tang et al. [72] reported that methylation of *DAPK* is strongly associated with survival in stage I NSCLC patients. This finding was confirmed later by Lu et al. [73]. Methylation of *RASSF1A* as unfavorable prognostic parameter in NSCLC patients was reported by Burbee et al. [43]. Similar findings were observed by Kim et al. [74] and by Tomizawa et al. [75] in patients with stage I lung adenocarcinomas. Toyooka et al. [76] reported that *p16* methylation was significantly related to unfavorable prognosis in patients with lung adenocarcinomas. Two genes (*RASSF1A*, *RUNX3*) out of ten tested were identified to be of poor prognostic relevance when they were found to be methylated in stage I NSCLC patients [77]. The prognostic impact of methylation of five genes located at the chromosomal region 3p was studied by Seng et al. [78] in primary NSCLCs. Interestingly, methylation of *DLEC1* was found to be an independent marker of poor survival in both the whole study population as well as in patients with squamous cell carcinomas. *hMLH1* methylation was also of prognostic impact particularly in patients with large cell carcinomas. Concordant methylation of *DLEC1* and *hMLH1* was a strong indicator for poor prognosis in the whole study population. Genes with a poor prognostic relevance are listed in Table 1.

6.2 DNA methylation markers relevant for disease recurrence

A potential association between DNA methylation in the blood and disease recurrence in stage I NSCLC patients after surgery was investigated by Brock et al. [79]. Methylation of the genes *p16*, *MGMT*, *DAPK*, *RASSF1A*, *CDH13*, *APC*, and *ASC* was determined in tumor and corresponding lymph nodes of the patients. Interestingly, methylation of *p16*, *CDH13*, *RASSF1A*, and *APC* in tumors and in histologically tumor-negative lymph nodes was associated with disease recurrence. If *p16* and *CDH13* were methylated in tumor and mediastinal lymph nodes, the odds ratio for recurrence was 15.5. The authors discussed that methylation of these genes in histologically normal lymph nodes probably indicates the presence of microscopically undetectable micrometastasis. Because the current method of risk assessment for recurrence in stage I NSCLC patients is imprecise, the authors suggested that detection of methylation of certain genes may identify cells with a potential for metastatic spread and could be helpful to predict disease recurrence.

6.3 Smoking, early detection of lung cancer, and risk assessment

Smoking is the major risk factor for lung cancer and about 85-90% of all lung cancers occur in smokers [80]. In addition to the major genetic differences between lung cancers arising in smokers and never smokers [81], it has been shown that differences in the frequencies of methylation of certain genes occur in these two types of lung cancer. Toyooka et al. [82] investigated the methylation status of seven genes in a large number of lung adenocarcinomas. The authors observed higher rates of methylation of *p16* and *APC* as well as a significantly higher methylation index in ever smokers compared to never smokers. In addition, the mean methylation index of tumors arising in former smokers was significantly lower than the mean of current smokers. A comparison of methylation of certain genes in bronchial epithelium between current and former smokers was performed by Belinsky et al. [83]. Interestingly, *p16* and *DAPK* methylation was found frequently in bronchial epithelium from current smokers as from former smokers, suggesting that methylation changes can persist long after smoking cessation.

Recently, researchers tried to elucidate the interaction of tobacco carcinogens and DNA methylation during lung carcinogenesis. Damiani et al. [84] developed an *in vitro* cell transformation model to identify critical mediators of premalignancy. Using this model, they observed increased levels of DNMT1 protein during exposure of immortalized bronchial epithelial cells to the carcinogens methylnitrosurea, benzo(a)pyrene-diolepoxide 1 or both, and an association with methylation of certain genes. The authors conclude that carcinogen-induced methylation is mediated by DNMT1 and is causal for transformation of immortalized bronchial epithelial cells. In addition, it has been shown that carcinogens induce single- and double-strand breaks (DSB) in DNA and reduced capacity for repair of DNA damage has been associated with lung cancer [85].

Thus, Leng et al. [86] investigated the hypothesis that DSB repair capacity and sequence variations in genes involved in this pathway are associated with a high methylation index in current and former cancer-free smokers. A 50% reduction in the mean level of DSB repair capacity was observed in lymphocytes from smokers with a high methylation index compared to smokers with no genes methylated. In addition, single nucleotide polymorphisms within several genes were highly associated with the methylation index. Overall, this study identified an association between DSB DNA repair capacity and alterations of certain genes within this pathway as an important factor for gene methylation in sputum, which finally results in an increased risk for lung cancer.

It has been observed that methylation of certain genes can already be detected in aerodigestive tract samples from cancer-free smokers. Methylation of several genes, for instance *p16*, *DAPK*, *GSTP1*, and *RASSF1A*, were detected in different sample types from this population [83, 87–90]. In most studies, the frequency of methylation of these genes was significantly lower in samples from cancer-free smokers compared to lung tumors. Methylation of four genes was analyzed in oropharyngeal brushes, sputum samples, bronchial brushes, and BAL samples of the upper aerodigestive tract epithelium from heavy smokers without evidence of cancer but with morphometric evidence of sputum atypia [91]. Interestingly, methylation occurred more frequently in samples from the central airways (sputum, bronchial brushes) compared to the peripheral airways (BAL), and only occasionally in the oropharynx. *RARβ-2* was the most frequently methylated gene. Results from sputum samples and bronchial brushes were comparable.

Bhutani et al. [92] investigated if the oral epithelium may be used as a surrogate tissue for assessing tobacco-induced molecular alterations in the lungs. They determined the methylation status of *p16* and *FHIT* in oral and bronchial brush specimens from 127 smokers enrolled in a chemoprevention trial. Specimens were obtained at baseline and three months after intervention. At baseline, methylation in bronchial tissue was present in 23% of samples for *p16* and in 17% of samples for *FHIT*. Comparable results were found for oral tissue. Of these samples, 19% were *p16* methylated and 15% were *FHIT* methylated. The mean bronchial methylation index was far higher in patients with oral tissue methylation compared to patients without oral tissue methylation. Similar findings were observed after three months. The authors conclude that oral epithelium has some potential as surrogate tissue for assessing tobacco-induced molecular damage in the lungs.

Methylation of seven genes was studied in atypical adenomatous hyperplasia (AAH) of the lung (a putative preneoplastic lesion), adjacent normal lung tissue, and in synchronous lung adenocarcinomas [93]. The authors observed a significant increase in the frequency of methylation during the histologic progression from normal to AAH and finally to adenocarcinoma. Multifocal AAHs had distinct patterns of methylation suggesting divergent epigenetic field defects.

Using a highly sensitive PCR approach, Palmisano et al. [94] found methylation of *p16* and/or *MGMT* in DNA from sputum in 100% of patients with squamous cell lung carcinomas up to three years before clinical diagnosis. The prevalence for methylation of several genes in sputum and plasma samples from women at different risk for lung cancer (lung cancer survivors, clinically cancer-free smokers, and never smokers) was investigated by Belinsky et al. [55]. The authors reported that concomitant methylation of multiple genes in sputum is strongly associated with lung cancer risk. In another project from this research group, six genes were identified which are associated with a 50% increased lung cancer risk in a high-risk cohort [58]. The concomitant methylation of three or more of these six genes was associated with a 6.5-fold increased risk for lung cancer.

Overall, these results demonstrate that methylation of certain genes can be an early event in the pathogenesis of lung cancer and that methylation can increase during malignant progression. A panel of certain methylated genes may be useful as biomarker for early detection of lung cancer and risk assessment. In addition, oral epithelium has some potential to serve as surrogate tissue for tobacco-induced methylation changes in the lungs.

7 DNA methylation as potential therapeutic target

While methylation of single genes cannot be reversed at the present time, global approaches for demethylation are currently being tested as therapeutic modalities. The effect of therapies that reverse epigenetic changes in certain malignant diseases has been reported in the past few years. DNMT inhibitors such as Aza-dC or 5-azacytidine (AzaC) have been approved by the Food and Drug Administration of the USA in myelodysplatic syndrome [95, 96], whereas HDAC inhibitors have shown to be active in cutaneous and peripheral T-cell lymphoma [97, 98]. However, in general, the efficacies of these drugs in solid tumors are disappointing. Several reasons for these findings have been discussed: it is not precisely understood how these drugs work, it is not known how to optimally select patients for this therapy, and it is not clear how to find the optimal dosing. Thus, we know very little about the actual or potential efficacy of these drugs in lung cancer. Recently, Juergens et al. [99] reported preliminary results from a phase II study investigating the efficacy of AzaC in combination with the HDAC

inhibitor entinostat in pretreated NSCLC patients. Interestingly, the authors observed a benefit from this treatment in several patients. Overall, this treatment was well tolerated. However, additional clinical studies are necessary for complete evaluation.

8 High throughput DNA methylation profiling in lung cancer

Recently, a new era in detecting DNA methylation has begun by performing genome-wide methylation analysis. Since it has been reported by Costello et al. [100] that on average 600 CpG islands are targets for methylation in tumor cells, researchers have expressed increasing interest in developing techniques to investigate not only methylation of single genes but also large-scale and genome-wide analyses of methylation. The first approach for large-scale DNA methylation analysis of CGIs was Restriction Landmark Genomic Scanning (RLGS). By RLGS, Dai et al. [101] analyzed 1,184 CGIs and discovered 11 genes which are differentially methylated in lung cancer compared to matching non-malignant lung tissue samples. Two of these genes (*GNAL* and *BMP3B*) were methylated in more than 50% of tumors analyzed. Brena et al. [102] used RLGS to identify methylated genes in lung adeno- and squamous cell carcinomas. They found a 47-gene methylation signature which could distinguish these two histologic subgroups. In addition, they identified methylation of *OLIG1* as new prognostic parameter for lung cancer patients.

Other frequently used assays for high throughput methylation analysis include expression microarray analysis of cell lines before and after treatment with DNMT inhibitors +/− HDAC inhibitors ("pharmacological re-activation"), BeadArray-based methylation analysis of a panel of cancer-related genes (Illumina GoldenGate™ methylation assay), and microarray analysis in combination with immunoprecipitation of methylated DNA (5-methylcytidine antibody; MeDIP-chip) [103]. These methods are discussed in detail below.

8.1 Gene-expression profiling of pharmacologically re-activated genes

As already mentioned above, DNA methylation is reversible by the use of DNMT inhibitors such as Aza-dC. *In vitro*, Aza-dC has been shown to efficiently induce re-expression of genes silenced by methylation [41, 43, 45, 47, 104]. Moreover, a synergistic effect in gene re-expression between Aza-dC and the HDAC inhibitor TSA has been reported [20]. Based on this observation, an indirect approach for the identification of a broad range of so far unknown methylated genes has been developed [105]. In this assay, gene expression patterns of cells treated with Aza-dC and/or TSA and of untreated counterparts are compared by expression microarray analysis. Using a similar approach, Shames et al. [106] compared gene expression in seven NSCLC and three human bronchial epithelial cell lines before and after Aza-dC treatment. They identified 132 tumor-specifically methylated genes and some of them were involved in specific molecular pathways whose loss may contribute to the development of a malignant phenotype. Thirty-one out of 45 genes which were further investigated by MSP in primary lung carcinomas and matching non-malignant lung tissue samples were found to be methylated only in tumors. Out of these seven genes with potential clinical relevance, *ALDH1A3*, *BNC1*, *CCNA1*, *CTSZ*, *LOX*, *MSX1*, and *NRCAM* were identified. Similarly, Zhong et al. [107] performed gene-expression microarray analysis of nine NSCLC cell lines before and after treatment with Aza-dC and TSA and identified 214 genes whose expression was up-regulated after drug treatment. Interestingly, some of these genes (*NNAT*, *RASGRP2*, *MT3*, *ATF3*, and *CYLD*) have strong growth suppressing properties suggesting an important role of these genes in lung cancer pathogenesis. A summary of results from these two studies are shown in Table 2.

Overall, these data suggest that gene expression microarray analysis in cells treated with demethylating agents is a powerful approach to identify unknown methylated genes. A major disadvantage of this assay is that it can be performed only in cell lines but not in tissue samples. Because it has been suggested that cancer cell lines can acquire methylation in culture, cell lines may not accurately reflect the methylation patterns of tumors *in vivo* [108, 109]. Therefore, it is necessary to validate data obtained from cancer cell lines in tumor samples of patients.

8.2 High throughput DNA methylation analysis using BeadArrays

As described above, treatment of genomic DNA with sodium bisulfite converts unmethylated cytosine bases to uracil while 5-methylcytosine remains unchanged. Specific primers can then distinguish whether the sequence of interest is methylated or not. Using a sodium bisulfite conversion approach combined with the BeadArray technology (Illumina GoldenGate™ methylation assays), Bibikova et al. [110] analyzed methylation profiles of 1,536 CpG sites from 371 cancer-related genes in cancer cell lines of different histologic types and in primary lung adenocarcinomas and non-malignant lung tissue samples. Several genes of potential clinical interest including *ASCL2*, *CDH13*, *HOXA5*, *HOXA11*, *NPY*, *RUNX3*, *TERT*, and *TP73* were identified and further examined by bisulfite

Table 2 Summary of results from gene expression microarray analysis of lung cancer cell lines before and after drug treatment

Treatment	Number transcripts analyzed	Histology of cell lines, number	Number of methylated CGIs[a]	Number of CGIs further investigated	Genes of potential clinical interest	Reference
Aza-dC	~47,000	ADC, 4 SCC, 1 LCC, 2 SCLC, 1 HBEC, 3	132[b]	45	*BNC1*, *CCNA1, CTSZ*, *LOX*, *MSX1, ALDH1A3*, *NRCAM*	106
Aza-dC/TSA	~18,400	ADC, 2 MUCO, 1 NSCLC, ns, 1	214[c]	9	*NNAT*, *MT3*, *CYLD*, *AFT3*,*RASGRP2*	107

CGI CpG island, *Aza-dC* 5-aza-2′-deoxycytidine, *TSA* trichostatin A, *ADC* adenocarcinoma, *SCC* squamous cell carcinoma, *LCC* large cell carcinoma, *HBEC* human bronchial epithelial cells, *MUCO* mucoepidermoid carcinoma, *ns* not specified

[a] Describes differences between [b] Treated and untreated cancer cells and HBEC or between [c] Treated and untreated cancer cells

genomic sequencing. Moreover, the authors demonstrated that methylation signatures were different between the cancer cell lines of various histologic types. In addition, 55 CpG sites were found which methylation pattern distinguished lung carcinomas from non-malignant lung tissue samples [110]. Similar results were reported by Christensen et al. [111]. This research group analyzed lung adenocarcinomas, mesotheliomas, and non-malignant lung tissue samples for methylation of 1,413 CpG loci associated with 773 cancer-related genes using Illumina's GoldenGate™ methylation assay. Differences in methylation patterns were found between lung tumors and non-malignant lung tissue samples and between lung adenocarcinomas and mesotheliomas.

Recently, Illumina released the new generation of BeadArrays for assessment of DNA methylation where 27,578 CpG loci covering more than 14,000 genes can be analyzed at single-nucleotide resolution. However, data derived from this new generation of BeadArrays have not been reported yet.

Table 3 Summary of results from high throughput methylation analysis in primary tumors

Method	Number CpG sites analyzed	Histology of tumors, number	Number of methylated CGIs[a]	Number of CGIs further investigated	Genes of potential clinical interest	Reference
RLGS	1,184 SCC, 7 LCC, 4 LC, 1	ADC, 4	148[b]	11	*GNAL*, *BMP3B*	101
RLGS	2,590–4,108 SCC, 14	ADC, 11	395[b,c]	47	*OLIG1*	102
GoldenGate™ methylation assay	1,536	ADC, 23	207[b]	55	*ASCL2*, *TP73* *CDH13*, *HOXA11* *HOXA5*, *NPY,* *RUNX3*, *TERT*	110
GoldenGate™ methylation assay	1,413	ADC, 57 MESO, 158	>500[b,c]	-	-	111
MIRA-chip	~27,800	SCC, 5	977[b]	12	*OTX1*, PAX6, *EVX2, BARHL2*, *MEIS1, IRX2*, *TFAP2A, OC2*	113

CGI CpG island, *RLGS* Restriction Landmark Genomic Scanning, *ADC* adenocarcinoma, *SCC* squamous cell carcinoma, *LCC* large cell carcinoma, *LC* lung cancer, *MESO* mesothelioma, *MIRA* methylated CpG island recovery assay

[a] Describes differences between [b] Tumors and corresponding non-malignant tissues and between [c] Tumors of various histologies

8.3 Methylome profiling using tiling microarrays

In the last few years, tiling arrays were developed which are a powerful tool to perform whole-genome analysis including DNA methylation [112]. Recently, Weber et al. [103] established a strategy to isolate methylated DNA fragments by immunoprecipitation (MeDIP) using an anti-5-methylcytidine antibody. Moreover, they combined the MeDIP assay with the microarray technology (MeDIP-chip). Using this approach in SW48 colon carcinoma cells and normal colon mucosa, they obtained genome-wide DNA methylation patterns and identified about 200 genes which are differently methylated between these sample types [103]. However, MeDIP-chip analysis in lung cancer has not been reported yet. A very similar approach is the methylated CpG island recovery assay, which isolates methylated DNA fragments using recombinant MBD2b/MBD3L proteins. These fragments are subsequently hybridized to microarrays. Using this approach, Rauch et al. [113] investigated the methylation status of 27,800 CGIs in primary tumors of five patients with stage I lung squamous cell carcinomas. With a range from 216 to 848 methylated CGIs, the number of methylated genes in this study is in concordance with previously reported data [100]. Further methylation analysis in 20 primary squamous cell carcinomas and non-malignant lung tissue samples identified eight genes (*OTX1*, *BARHL2*, *MEIS1*, *OC2*, *PAX6*, *IRX2*, *TFAP2A*, and *EVX2*) which might be of clinical interest for these patients. Results from high throughput DNA methylation analysis are summarized in Table 3.

In conclusion, using these high throughput approaches, hundreds of genes were found to be methylated in tumors. Methylation patterns between tumors and corresponding non-malignant lung tissues and also between tumors of various histologies are different confirming previously obtained data that DNA methylation is tissue type-specific. In addition, a large number of genes unknown to be methylated in lung cancer so far were identified.

However, it seems to be of interest that genes which are known to be frequently methylated in lung cancer by single-gene methylation analysis are infrequently found to be methylated by these high-throughput approaches. Well-known methylated genes originally identified from single-gene analysis were also found to be methylated by genome-wide approaches (*CDH1* and *TIMP3* [106], *FHIT* [111], *p16* [113], *SLIT2* [111, 113] and *CDH13* [110]). Although Bibikova et al. [110] observed methylation of *RASSF1A* and *ASC/TMS1* in a panel of lung cancer cell lines, methylation of these genes was not detected in primary lung adenocarcinomas. A possible explanation for differences in results obtained by single-gene and high-throughput DNA methylation approaches could be that, so far, mainly small numbers of samples were tested using high-throughput analyses. Other problems with genomic approaches include a continuous scale, as opposed to plus or minus values, multiple probes for single genes which often result in different answers and multiple isoforms, not all of which are analyzed. Further studies addressing these issues are necessary.

9 Summary

Numerous genes have been identified which are involved in different pathways relevant for lung cancer pathogenesis and which are frequently methylated leading together with other epigenetic mechanisms to gene silencing. In addition, it has been shown that methylation of certain genes can be of clinical relevance. While at the beginning of the DNA methylation era mainly single genes were investigated for their methylation status, recently, methods for high-throughput DNA methylation analysis were developed. These approaches provide researchers with a huge amount of information about methylation patterns in lung cancer cell lines and samples from lung cancer patients. However, because of differences in methodology, it is difficult to compare results obtained from these studies. In addition, most studies have analyzed relatively small numbers of samples. Thus, which high throughput approach is the most suitable one needs to be evaluated. It can be hypothesized that using such methods, hundreds of genes could be identified which are of clinical interest. For instance, disease recurrence in NSCLC patients after surgery could be probably much more precisely predicted than today. It could also be helpful in selecting patients who may benefit from an epigenetic therapy. Overall, findings suggest that high throughput methylation profiling could be of enormous help to improve prognosis of lung cancer patients.

9.1 Key unanswered questions

The key unanswered question remains whether it is possible to translate the findings from global methylation profiling to patient care—diagnosis, prognosis, and therapy.

Acknowledgment This work was supported by the Vienna Science and Technology Fund (project number LS07-019).

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