Review Articles

Epigenetics in lung cancer: What do DNA-methyltransferases do?
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Keywords: lung cancer, epigenetics, DNA-methyltransferase
Abstract

Despite recent advances in molecular characterization and targeted therapy approaches, lung cancer still remains the number one killer among malignant diseases worldwide. After understanding the impact of genetic mutations on malignant transformation, epigenetic changes have been focused on in recent times. Several studies could elucidate the potential of epigenetic alterations to not only increase invasiveness of cancer cells in cell culture and animal models but also to contribute to autonomous cellular growth and thus malignant transformation itself. Thus, epigenetic changes are nowadays acknowledged as a hallmark in cancer. Several enzymes are involved in the epigenetic equilibrium of DNA methylation and demethylation, one family being DNA methyl transferases (DNMT). Here, we give a review of the impact of DNMTs on the biology of lung cancer and additionally present some of our results within this context. Further, we are also giving a perspective on future treatment options arising from the current literature and our results.
Introduction

Cancer is the second most cause of death in industrial countries following cardiovascular diseases [1]. Of the variety of malignancies, lung cancer is with approximately 1.800.000 diagnoses per year worldwide the neoplasm with the highest incidence rate. In comparison to other cancer-types, lung cancer has a high mortality rate of 0.87, i. e. in almost 90% of lung cancer patients this disease is responsible for their death. This results in approximately 1.600.000 lung cancer related deaths per year, or in other words almost 4.400 lung cancer deaths every day worldwide [2] [3]. One reason for lung cancer’s high mortality is the lack of screening possibilities and thus, about 60 to 70 percent of patients are diagnosed with locally advanced or metastatic disease. Lung cancer, therefore, represents a major socio-economic challenge for most health care and welfare systems. It is thus understandable, that new effective treatment options for lung cancer are urgently needed. In the last two decades, research focused on molecular mechanisms to better understand tumor biology of lung cancer, and use this knowledge to develop targeted therapies (e. g. tyrosine kinase inhibitors targeted against mutated EGFR or translocated ALK) with promising results for overall survival, too. Since these new therapies are eligible for only a small subset of patients, accounting for about 15 %, other, more general mechanisms in carcinogenesis of lung cancer have been investigated, lately. Of these, epigenetic alterations are common in many cancers and may also serve as a therapeutic target in the near future.

Epigenetics and DNMT

Epigenetics are defined as “the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence.” [4] These epigenetic modifications include histone variants, posttranslational modifications of amino acids on the amino-terminal tail of histones and covalent modifications of DNA bases. [5] Histones play a crucial role in DNA-folding and therefore gene expression. There are many histone-modifying enzymes, such as histon-acetyltransferases, histon-lysindemethylases and histon-deacetylases (HDAC). The other major part of epigenetic modifications is the specific methylation of DNA. This methylation process is performed by DNA-methyltransferases (DNMT). [6]

DNMT catalyze the addition of a methyl-group to the fifth C-atom of a pyrimedin ring of cytosine in a CpG-dinucleotide. DNA methyltransferases use S-adenosyl methionine (SAM) as the methyl group donor. (Figure 1)

99% of the human genome are poor of CpG-dinucleotides. The other 1% with a high amount of CpG-dinucleotides are called CpG-islands. These CpG-islands are often connected to promotor regions. Approximately 50% of all promotor regions include CpG-islands. [7] By targeting these promotor connected CpG-islands, DNMT can directly influence protein expression.
There are variants of DNMT that can be found in the human body (DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L). The major difference between these DNMTs is their varying degrees of specificity toward unmethylated and methylated DNA substrates. They all share their catalytic domain with conserved signature motifs. [8]

DNMT1 was the first discovered. It copies methylation patterns during DNA-replication from the paternal DNA strand to the daughter strand. Its affinity to hemimethylated DNA is 5- to 30-times higher than to non-methylated DNA. Therefore, DNMT1 works mainly as maintenance DNMT [9] (Figure 2).

DNMT2 does not methylate DNA but rather tRNA [10].

DNMT3A and DNMT3B are an important part of the early embryonal development and the repression complex. Both have the same affinity to non-methylated as to hemi-methylated DNA. Because of this they are considered as the real “de novo” DNMTs. They are capable of establishing new methylation patterns without a pre-existing model [11]. In addition to this, there are also studies that describe a de novo function of DNMT1 in cancer cells. This finding suggests a major role of DNMT1 in epigenetic alterations that lead to carcinogenesis [12] (Figure 3).

DNMT3L is enzymatically inactive. The enzyme influences other methyltransferases in meiotic processes. DNMT3L is the first described stimulating factor for methylation processes without own enzymatic activity. [13]

Feinberg et al proposed a functional differentiation between different epigenetic regulators: Upon this, epigenetic regulators are divided into modulators, modificators and mediators. In this classification, DNMTs act as modificators. An epigenetic modificator is defined as a mutated or non-mutated gene product, which modifies DNA methylation or chromatine structures [14].

There are different theories how DNMTs and their methylation patterns contribute to carcinogenesis. It is assumed that both, hyper- and hypomethylation, can attain a carcinogenic effect [15].

Hypermethylation of promoter regions leads to transcriptional silencing. The gene connected to the methylated promoter will be no longer expressed. If this occurs at tumor suppressor gene sites their expression level is no longer capable of influencing the cell cycle in their protective function [16]. It is assumed that in a tumor about 400 genes are silenced through hypermethylation [17]. In lung cancer, various tumor suppressor genes are suppressed through hypermethylation. These include p16/INK4a, APC, RASSF1A, FHIT, 3-OST-2 and DAPK. The silencing leads also to specific features of the tumor cell. For example, it was described that hypermethylation of the FHIT promotor correlates with tumor staging, capability of tissue invasion and a poor prognosis [18].

There is also an interaction between DNMT and DNA repair mechanisms. Reduced expression of several repair enzymes increases the risk of a DNA mutation and following malignant transformation. Epigenetic hypermethylation influences multiple repair pathways. In non-small
cell lung cancer (NSCLC), epigenetic pathway inactivation of DNA mismatch repair (MLH1 and MSH2), homologous recombination (BRCA1, BRCA2 and FANCF), and nonhomologous end-joining (XRCC5) has been shown. In addition, epigenetic silencing of the DNA repair enzyme O6-Methyguanine-DNA-Methyltransferase (MGMT) can frequently be found in NSCLC [19].

Additionally to loci specific hypermethylation, global hypomethylation can often be found in tumor cells. Almost all of major human cancers, including colon, gastric, lung, liver, breast, bladder, ovarian and endometrial carcinomas, are characterized by a profound cancer-linked hypomethylation of the genome. Many studies support the early loss of normal methylation patterns in pre-neoplastic cells [20, 21]. According to this theory, global hypomethylation increases during the transformation to a malignant cell and in the progress of tumor development [22].

The underlying mechanism between global hypomethylation and carcinogenesis includes induction of chromosomal instability, reactivation and transposition of transposons, loss of imprinting and activation of previously silenced genes (e.g. S100A4, Cyclin D2, CD133). The healthy human genome consists of relatively short unmethylated sequences that are embedded in a matrix of methylated domains. Especially repetitive sequences are methylated therein. However, how does hypomethylation of these repetitive sequences lead to tumor development? This question is answered by two accepted consequences of hypomethylation. Firstly, transcriptional activity is directly influenced by demethylation near the centromeric region, which causes permissive transcriptional activity at the centromere [23, 24]. Secondly, hypomethylation leads to activation and possible transposition of transposable elements (SINEs, LINEs, IAP retroviral elements), and thus results in chromosomal instability [21, 25].

**DNMT overexpression and lung carcinogenesis**

There are several hypotheses how overexpression of DNMTs affects the organism and carcinogenesis. An accepted theory in lung cancer development is hypermethylation of promotor regions resulting in lower expression of tumor suppressor genes. A widely studied example is the expression of p16, an inhibitor of CDK4. This gene is often transcriptionally silenced in NSCLC samples and shows a high frequency of hypermethylation [15, 26]. However, is hypermethylation of promotor regions connected to DNMT expression?

Pastuszak-Lewandoska et al showed a decreased mRNA-expression of RASSF1A in lung cancer compared to healthy lung tissue. Their analysis of histological subtypes showed a significantly reduced expression of RASSF1A in squamous cell carcinoma (SCC) than in lung adenocarcinoma (LAC). In the majority of tumor samples hypermethylation of the gene’s promotor region was shown. Additionally, tumor tissue showed elevated mRNA expression level of DNMT1 and -3b. Nevertheless, they could not show a correlation between mRNA-expression of DNMT and the tumor suppressor genes analyzed (RASSF1A, NPRL2/G21 and FUS1) [27].
Another study showed elevated mRNA expression of DNMT1, -3B and also -3A in NSCLC tissue. At the same time, hypermethylation of important tumor suppressor genes (APC, DAPK and MGMT) was observed. Again, in this study there was no significant correlation between DNMT expression and hypermethylation status of the studied tumor suppressor genes [28].

Kim et al. showed elevated expression levels of DNMT1 and -3B in lung tumor tissue. There was increased hypermethylation of p16, RARb2, H-cadherin and FHIT in NSCLC cells. In further analysis, DNMT1 showed the strongest correlation to hypermethylation of the p16 promotor. But there was also no statistical significance [29].

All these studies examined the expression level on mRNA basis followed by correlation of the promotor methylation patterns. mRNA expression is influenced by posttranscriptional processes and modifications so that mRNA expression often does not truly reflect protein based expression. This could be a possible reason for missing correlations between DNMT mRNA expression and hypermethylation of tumor suppressor gene promoters.

In a lung cancer mouse model, a gradual overexpression of DNMT1 and -3B on protein level could be shown after induced lung cancer development. At the same time, hypermethylation induced lower expression levels of adhesion proteins (CADM1, TIMP3). There was a significant correlation between DNMT expression and hypermethylation of CADM1 (DNMT1 and -3A) and TIMP3 (DNMT3A). Correlation between the expression of adhesion molecules and DNMT was confirmed by treatment of the cells with a DNMT inhibitor (5-Aza-CdR). After treatment, the expression level of previously silenced CADM1 increased [30]. Another animal study showed an overexpression of DNMT and a simultaneous suppression of tumor suppressor genes (p16 and MLH1) in urethane-induced lung tumors of mice. An early increase in DNMT expression after tumor induction was accompanied by decreased expression of p16. Lower expression of MLH1 followed afterwards [31].

In patient tissue a correlation between DNMT expression and methylation patterns of tumor suppressor gene promoters could be shown, too. Lin et al proved elevated protein expression levels of DNMT1, -3A and -3B in NSCLC probes by immunohistochemical analysis. There were significant correlations between overexpression of DNMT1 and promotor hypermethylation of FHIT, p16 and RARbeta. The strongest correlation was shown in squamous cell carcinomas of smokers [32]. A follow up study showed significant DNMT1 overexpression and reduced expression level of p53 [33]. In a smaller cohort of 57 NSCLC patients, a positive correlation between immunohistochemical expression of DNMT3B and hypermethylation of p16 could be revealed. In the context of this study there also was a positive correlation between DNMT overexpression and HPV16/18 infection as possible inductor of lung cancer [34].

Variants of DNMT3B, which should be the predominant DNMT enzyme in NSCLC cell lines, have been detected, too. These variants are described as deltaDNMT3B and originate through alternative promotor regions or alternative splicing. High expression levels of these variants could be proven in NSCLC tumor tissue. Those variants do not exist in normal lung tissue. In
NSCLC the mRNA based overexpression of deltaDNMT3B correlates significantly with promoter hypermethylation of RASSF1A and p16 [35, 36].

In lung cancer cell lines, a lower expression of GST-M2, a tumor suppressor gene, could be shown. The low expression is linked to a specific promoter hypermethylation, which inhibits the binding of the transcription factor sp1. Treatment of the cell line with the DNMT inhibitor 5-Aza-CdR increased the previously decreased expression of GST-M2. This finding proves an involvement of DNMT in this carcinogenic methylation process. Subsequently DNMT3B expression levels were correlated with GST-M2 expression in 73 NSCLC tumors (UICC stage I and II). The study showed an inverse correlation of these enzymes. The strongest correlation was found in female NSCLC patients in UICC stage I [37].

Dammann et al have shown that promotor regions of important tumor suppressor genes as RASSF1A are hypermethylated in lung cancer cell lines and subsequently showed decreased expression levels. The connection to DNMT was also established by adding a DNMT inhibitor (5-Aza-CdR). Treatment of the cells with the inhibitor induced a re-expression of previously suppressed RASSF1A. They could also show that adding 5-Aza-CdR prevents promotor methylation of another tumor suppressor gene (TIMP4) [26].

Downregulation of DNMT1 (through RNA interference) in NSCLC cell lines lead to a reduced promoter methylation of tumor suppressor genes (RASSF1A, p16, CDH1 and HPP1) [38].

Another important aspect how DNMT overexpression affects carcinogenesis is an increased development of ribosomes. Through this pathway, DNMTs can directly affect the cell cycle. DNMTs play an important role in processing pre-rRNA to functional rRNA. If pre-rRNA will not be methylated, the ribosome cannot be synthesized. The following depletion of rRNA leads to a cell cycle arrest. Therefore, DNMTs can directly affect proliferation. This leads to the hypothesis that increased expression of DNMTs leads to a higher proliferation rate [39]. In our own study, we have shown a positive correlation of immunhistochemical expression of DNMT3A and DNMT3B to histological grading in NSCLC patients. The higher the DNMT expression level, the higher the grading and therefore the aggressiveness and possible proliferation rate of the tumor.

Some studies showed a connection between DNMT expression and survival. They published shorter survival rates of NSCLC patients in stage I and II-III with an overexpression of DNMT1. DNMT1 was shown to be an independent prognostic factor. However, at the same time there was no significant correlation between mRNA expression of DNMT and promoter hypermethylation. It was postulated that a simple analysis of DNMT is insufficient in regard of hypermethylation processes. It was assumed that overexpression of DNMT is only one factor in a complex interaction of multiple factors [29]. Lin et al showed that overexpression of DNMT1 leads to poorer survival in lung cancer [32]. This finding was confirmed in another analysis of 102 NSCLC patients in 2010. This study showed shorter survival with concurrently overexpression of DNMT1 and the transcription factor Sp1 [33]. Fabbri et al. showed a poor prognosis of lung cancer patients with DNMT3A overexpression [40]. Our own studies also revealed a significantly reduced survival of lung cancer patients with immunohistochemical
overexpression of DNMTs (Figure 4). Single overexpression of DNMT3A as well as multiple overexpression of DNMT led to a significant shortened overall survival of NSCLC patients. This proved to be an independent prognostic factor, too.

Transcriptional silencing of tumor suppressor genes follows methylation of promotor sites catalyzed by DNMTs. The lack of tumor suppressor genes leads to accumulation of mutations because of insufficient repair mechanisms. These mutations play a crucial role in carcinogenesis and tumor progression. In this way, high expression of DNMT can lead to an elevated methylation-mutation-frequency (Figure 5). A high mutation count in cancer cells is also associated with a high histological grading and therefore with the tumor’s aggressiveness [41].

**Epigenetic induction and interaction of epigenetic regulators**

Since we know that a connection between DNMT expression, hypermethylation and thus silencing of tumor suppressor genes exists in lung cancer, the question arises, what are the factors inducing these modifications of methylation patterns and overexpression of epigenetic modulators. There are many existing theories to this question. An often-analyzed inductor is tobacco smoke, which is a proven risk factor for lung cancer. It has been shown that specific gene loci in lung cancer and even in healthy lung tissue are hypermethylated in smokers [42]. In mouse models tobacco smoking induced an increased expression of DNMT1 in lung tissue [43]. Overexpression of DNMT1 in tumor tissue of smokers has also been published in an immunohistochemical analysis of Lin et al [32].

It is well known, that in the overall picture of epigenetic changes the interaction between HDAC and DNMT is important. Tobacco-induced lung cancer cells showed an elevated expression of HDAC and DNMT1 as well as simultaneous DNA hypermethylation. Through treatment with the HDAC-inhibitor valproic acid, not only HDAC expression, but also DNMT1 expression decreased. This finding confirmed the interaction between these DNA-methylation regulators [44]. Both enzymes are required for genetic silencing and influence each other in terms of an epigenetic “cross-talk”. But there is no consent which of these enzymes starts the epigenetic cascade [45].

There is an existing cycle model, which starts with recruiting of different binding proteins (e.g. lymphoid-specific helicase (LSH)) and DNMT3B at methylated histone segments. During the next step DNMT1 and HDAC bind together to the DNA and build a protein complex. HDAC initiates the stop of transcription through deacetylation of the associated histones. Followed by increasing concentration of DNMTs and establishing the specific methylation pattern to maintain transcriptional silencing [8]. The model is supported by other findings that DNMT1 and DNMT3B bind directly to promotor regions [32].

It is a fact that certain tumor entities and histological subtypes show specific methylation patterns [46]. Therefore, tumors can be classified by their specific methylation phenotype, which reflects specific biological tumor features (e.g. therapy response) [47, 48].
Another important aspect in epigenetic changes and tumor development is the interaction between DNMT and DNA repair mechanisms. It was shown that important DNA repair enzymes are epigenetically downregulated in different tumor entities. In NSCLC, hypermethylation of promotor regions and consecutive downregulation of DNA repair enzymes has been shown. Epigenetic downregulation occur for mismatch repair (MLH1), base-/nucleotide-excision-repair (XRCC1, ERCC1) non-homologous end joining (XRCC5) as well as for the repair methyltransferase MGMT [19]. Missing DNA repair mechanisms lead to an accumulation of genetic mutations with subsequent possible malignant transformation [41].

There is also a discussed correlation between lower expression of certain microRNA and lung cancer development. An important factor in this hypothesis is the interaction between DNMTs and microRNA. NSCLC cell lines showed inverse correlation of miR-29s and mRNA-level of DNMT3A and -3B. Induced expression of miR-29s in vitro and in animal models recovered normal methylation patterns and re-expression of previously methylation based silenced tumor suppressor genes [40]. The inverse correlation between DNMT expression and microRNA-29b was confirmed in a mouse model [31].

It is clearly visible that through carcinogenesis and in different tumor entities many epigenetic regulation processes exist. Epigenetic modifications allow a more complete understanding of tumor development. DNMT expression and resulting methylation patterns as wells as interaction with several other regulators (e.g. pathways of DNA repair, expression of HDAC and micro-RNA) have to be brought together for an overall picture of the complex system of gene expression regulation.

**Time of epigenetic changes**

Another interesting question in relation to the epigenetic changes in tumor tissue is the moment of their occurrence. Generally speaking, epigenetic alterations can occur at every point of time during cancer development. One of the first steps to proof the connection between hypermethylation and cancer was to show that certain regions in cancer cells have denser methylation patterns than normal, non-neoplastic lung tissue. Certainly, hypermethylation occurred even in histological healthy lung tissue of cancer patients. Whereas lung tissue samples of non-cancer patients did not show this kind of hypermethylation changes. Dammann called this phenomenon “epigenetic field defects” and could prove that epigenetic changes occur in surrounding lung tissue even before the classic cancer cells develop [26]. This is one hint that epigenetic changes can be recognized as an early step in carcinogenesis and that they potentially occur in precancerous cells.

The early occurrence of epigenetic changes was shown in another mouse model. DNMT expression was analyzed during tumor development for the period of 36 weeks. After one week an early overexpression of DNMT1, -3A and -3B could be observed in urethane induced lung
cancer. At this point of time, there were no histological changes detectable in this lung tissue [31].

The epigenetic progenitor model of Feinberg et al. of the year 2006 describes the early occurrence of epigenetic changes during carcinogenesis. A healthy precursor-/stem cell is modified by epigenetic processes and a new pool of modified progenitor cells emerges. Based on this new progenitor cells the classic tumor cell originates stepwise by several genetic and epigenetic influences. According to this accepted model, epigenetic regulators play a crucial role in (early) tumor development and later in its further progression [49].

Another example for the early occurrence of epigenetic changes is the promoter hypermethylation of O6-alkylguanine DNA alkyltransferase (MGMT). Silencing of MGMT leads to a failure of repair mechanisms of DNA adducts. Thus, mutations arise after silencing of MGMT and cancer development starts. Promotor hypermethylation of MGMT plays an important role in development of glioblastoma multiforme and different lymphomas. But also in lung cancer as well as breast and prostate cancer, hypermethylation of MGMT is often detectable [50]. In the case of promoter hypermethylation of MGMT genetic changes arise from preceding epigenetic alterations. These results also lead to the hypothesis that epigenetic alterations mark an early step in malignant cellular transformation.

One major problem in fighting lung cancer is the lack of early detection methods and consideration of epigenetic markers could establish possibilities of screening. There is already a test based on epigenetic factors commercially available (Epi proLung © Epigenomics AG 2015). It offers detection of hypermethylation of SHOX2 in bronchial fluid aspirated during bronchoscopy. DNA methylation of SHOX2 allowed to differentiate between benign lung diseases (i.e. abscesses, infections, obstructive lung diseases, sarcoidosis and scleroderma) and malignant lung cancer with 68% sensitivity and 95% specificity [51].

**Therapeutic possibilities**

The interaction of epigenetic regulators also offers a target for individualized tumor therapies. There are many available DNMT inhibitors. Their classical representative is 5-azacytidine (5-Aza). 5-Aza is a chemical analog of the nucleoside cytidine. These analogs will be built into the DNA and bind covalent to DNMTs. This leads to inhibition and decreasing activity of the enzymes [52]. Based on the basic substance 5-Aza there are many chemical modifications which can be used as DNMT inhibitors. One example is the corresponding deoxynucleotide 5-Aza-2'-deoxycytidine (5-Aza-CdR; syn. Decitabine). Another promising development is the dinucleotide SGI-110, which contains decitabine. This agent is resistant to the fast hepatic elimination through cytidine deaminase unlike decitabine [53]. Decitabine is in clinical use since 2006, 5-Aza since 2004. Both of them are approved in the treatment of myelodysplastic syndrome in the US. It is also effective in the treatment of acute myeloic leukaemia. The actual use of these drugs is therefore currently limited to malignancies of the hematopoietic system. However, their effectiveness was also
studied in early trials of solid tumors. Preclinical studies have shown a significant demethylation of promotor in lung cancer cells via DNMT inhibitors. An increased effect was shown in combination with HDAC inhibitors as for example trichostatin A and entinostat [53, 54]. Monotherapy of DNMT inhibitors are clinical effective in hematopoeitic diseases but not curative. Therefore, the actual approach in lung cancer (and other solid tumors) is also a combination of the epigenetically effective drugs with already established chemotherapeutics. DNMT inhibitors act as sensitizer so that cytotoxic drugs can be more efficient. This was already studied in different pre- and early clinical trials.

Mateen et al have shown the effectiveness of DNMT inhibitors in decreasing the potential of metastasis and invasiveness of NSCLC cells. Loss of e-cadherin is considered as an essential step in tumor development in relation to metastasis and invasiveness. NSCLC cells were treated with a combination of silibinin and a DNMT inhibitor. Silibinin is an extract of mary thistle which has a HDAC inhibitory effect and preclinical anti-cancerous potential in NSCLC [55]. It has been shown that the combination of silibinin and 5-Aza-CdR leads to re-expression of e-cadherin in NSCLC cells. Thus, migration and invasive potential of these cells were reduced [56]. In a lung cancer model of the rat a reduction of tumor burden could be achieved by combined treatment with 5-Aza and entinostat. At the same time, demethylation of several genes of important signal transduction pathways of cell cycle, DNA repair and apoptosis was observed [57]. Another study analyzed the effect of TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) in SCLC cells. A reduced efficiency of TRAIL was observed because of a lowered expression of caspase 8, which plays a central role in the execution-phase of cellular apoptosis. Low expression of caspase 8 could be traced back to a specific promotor hypermethylation. After combined therapy with decitabine and an HDAC inhibitor (valproic acid), caspase 8 expression was restored. Therefore, the cells were sensitized for their TRAIL treatment by the epigenetic drugs [58]. Fueller et al have discovered a synergistic effect of 5-Aza and cisplatin/gemcitabine in NSCLC cells. 5-Aza induced global DNA hypomethylation as well as a specific hypomethylation of CpG islands of tumor suppressor genes, MGMT and THRD, could be shown. Simultaneously there was a synergistic effect between 5-Aza and the cytotoxic drugs in reducing tumor growth [59]. Based on these in vitro tested results, a synergistic effect of demethylating agents and established chemotherapeutics can be considered. A small patient group of 15 stage IV NSCLC patients was treated with 5-Aza-CdR (two to five cycles). Median survival was at 6.7 months. Three of the patients survived longer than 15 months [60]. Among them was even a long term survivor with 81 months of overall survival [61]. This study showed that certain NSCLC patients could profit by a treatment with DNMT inhibitors.

Nevertheless, the treatment with decitabine also resulted in several problems and side effects. There is a certain hematopoietic toxicity [62], risk of neutropenia [63], fast hepatic elimination through cytidine deaminase [53] and chromosomal instability caused by global hypomethylation [8].

Thus, new approaches will treat the epigenetic alterations caused by DNMT with less toxic agents or lower doses, respectively.
Fast hepatic elimination of decitabine and 5-aza makes it difficult to establish pharmacologically active doses without systemic toxicity. Combinations with HDAC inhibitors lead to low dose applications of classical DNMT inhibitors (5-Aza) with pharmacological activity without systemic toxicity [64]. Synergistic effects of HDAC and DNMT inhibitors as chemo sensitizer were already shown in a phase II clinical trial. Patients who showed progressive tumor disease under cytotoxic chemotherapy were subsequently treated with hydralazine (DNMT inhibitor) and valproate (HDAC inhibitor). This cohort included patients with several solid tumor manifestations (including one lung cancer patient). The previously failed chemotherapy regime was continued after the treatment with the epigenetically effective drugs. In 12 of 15 cases (80%) there was a clinical benefit. After the pretreatment, four patients showed a partial tumor regression and eight patients a constant tumor burden (including the lung cancer patient) [65]. A phase II study limited on NSCLC patients analyzed the effectiveness of treatment with low dose 5-Aza in combination with entinostat. 45 patients with already multiple unsuccessfully pretreated advanced NSCLC were included. Median survival was 6.5 months. This is comparable to the standard therapy of refractory NSCLC with erlotinib. However, because of the epigenetic therapy there was a patient in complete remission (for 14 months), one with partial remission (for 8 months) and ten cases with stable tumor burden. A biomarker analysis of methylation of tumor suppressor genes (APC, RASSF1a, CDH13 and CDKN2a) was performed for all included patients at multiple times during therapy. Especially the patients in remission showed a demethylation of the analyzed tumor suppressor genes during their treatment. It is also to mention that 19 patients received systemic chemotherapy again after their “epigenetic” treatment with 5-Aza and entinostat. Four of them (21%) showed a response to the previously failed cytotoxic agents. The results showed that certain patient subgroups can profit by the treatment with DNMT and HDAC inhibitors and that these drugs can also function as sensitizer for classical chemotherapeutics [66].

An approach to avoid fast hepatic elimination of decitabine is the invention of new DNMT inhibitors. Effective half-life of decitabine is reduced by cytidine deaminase from 10h in vitro to less than 10 minutes in vivo. SGI-110 is a second-generation DNMT inhibitor, which is not deactivated by cytidine deaminase. It is a so-called pro drug of decitabine, thus, SGI-110 will be transformed to decitabine in vivo. In a lung cancer model of the rat, combination therapy with SGI-110 and entinostat showed a significant reduction in tumor burden. SGI-110 was quickly transformed into decitabine in vivo and showed a pharmacological half-time of 4 hours. At the same time, there was a re-expression of previously suppressed tumor suppressor genes [53].

Low doses of cucurbitacine B inhibit simultaneously epigenetic regulators HDAC and DNMT in NSCLC cell lines. Cucurbitacines belong to the group of bitter-tasting compounds which can be naturally found in cucurbits [67]. Their inhibition in NSCLC cells leads to a reactivation of suppressed tumor suppressor genes (CDKN1A and CDKN2a). At the same time oncogenes (c-MYC and KRAS) were downregulated. In a lung cancer mouse model, treatment with cucurbitacine b inhibited lung cancer development [68].
Growth stop of NSCLC cell lines was observed by targeted inhibition of DNMT transcription by antisense oligonucleotides (ASO). Genotoxicity led to apoptosis. In this study no re-expression of tumor suppressor genes through ASO was shown which was observed by treatment with decitabine. ASO are transcription inhibitors so that protein expression of DNMT is completely reduced. Decitabine however binds covalent to the replication site and inhibits demethylation. These new results led to the hypothesis that DNMT not only contribute to carcinogenesis by methylation but also by their contribution to large multi protein complexes [69]. The anti-tumorous activity of ASO could not be confirmed in a phase II study in patients with metastatic renal cell cancer [70].

Interaction of DNMTs with the anti-cancer-antibiotics mithramycine A (MMA) is also discussed. The possible interaction is based on the same binding site of both agents on GC- and CG-rich DNA sequences. In lung cancer cell lines with a high metastatic potential, treatment with MMA showed a reduction in invasiveness. Promotor hypermethylation of anti-metastatic tumor suppressor genes (SLIT2 and TIMP3) was also reduced. At the same time, there was a reduction of DNMT1 protein levels during the treatment. Protein analysis showed a possible direct inhibition of DNMT1 by binding of MMA to their catalytic domain. By using low doses of MMA, there were no cytotoxic effects observed in healthy lung cells. A possible reason is the reversible binding of MMA at the DNA binding site as opposed to the covalent and irreversible binding of classic DNMT inhibitors [71].

In conclusion, consideration of epigenetic alterations in carcinogenesis provides a more profound view of tumor biology and its development. The complex epigenetic crosstalk and the connection between epigenetic regulators and classic genetic mutations extend the understanding of tumor development. The knowledge about early appearance of epigenetic alterations and their possible impact on carcinogenesis enables new methods of early lung cancer detection. Early detection would profoundly improve therapeutic options and prognosis in lung cancer patients and could possibly reduce its high mortality rate. The new insights in the field of epigenetic also offer new therapeutic options. Actual postoperative therapies in lung cancer are limited to radiation and cytotoxic chemotherapy. Epigenetic drugs could establish new possibilities. An interesting aspect of epigenetic methylation processes is their possible reversibility. Drug induced demethylation could function as sensitizer for classic, cytotoxic chemotherapeutics. It seems conceivable that certain patients with a DNMT overexpression could profit by a treatment with DNMT inhibitors. These patients should be carefully selected and our results of the effect of DNMT-overexpression on NSCLC patient’s overall survival may offer a predictive test for these new agents.
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Figure legends

Figure 1: DNMT catalyze the transfer of a methyl group to cytosine

![Diagram showing the transfer of a methyl group to cytosine](image)

Cytosine → 5-Methylcytosine

* DNMT enzymes catalyze the transfer of a methyl group from SAM to cytosine.

Figure 2: Maintenance methylation activity of DNMT1 at hemimethylated CpG sites

![Diagram showing maintenance methylation activity of DNMT1](image)

* DNMT1 maintains methylation at hemimethylated CpG sites during DNA replication.
Figure 3: "de novo" methylation activity of DNMT3A/-3B at non-methylated CpG sites
Figure 4: Examples of different levels of nuclear immunohistochemical expression of DNMT in various NSCLCs
A: no expression in LAC; B: weak expression in SCC; C: moderate expression in LAC; D: strong expression in large cell neuroendocrine carcinoma (LCNEC)
Figure 5: DNMT and its role in carcinogenesis