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Diese Diplomarbeit ist ihr gewidmet!

Table of content

Hiermit erkläre ich, dass ich die vorliegende Diplomarbeit selbstständig an der Medizinischen Universität Wien, Universitätsklinik für Innere Medizin I, Klinische Abteilung für Onkologie durchgeführt, mit äußerster wissenschaftlicher Sorgfalt verfasst und keine anderen als die angegeben Hilfsmittel verwendet habe. Sämtliche Inhalte wie Texte und Bilder von fremden Quellen sind durch einen Verweis auf die jeweilige Quelle gekennzeichnet. Sollte dennoch der unwahrscheinliche Fall einer Verletzung von Urherberrechten eintreten, so bitte ich zwecks Klärung möglicher Zusammenhänge um Kontaktaufnahme mit der Verfasserin.

1. Abstract

Despite a better understanding of its molecular pathogenesis and advances in developing new treatment strategies, lung cancer is still one of the leading causes of cancer-related deaths worldwide with 5-year overall survival rates of about 17%. With a growing understanding of epigenetic mechanisms, the interaction between DNA methylation and histone tail modifications, which results in gene silencing, became evident. Several studies revealed that DNA methylation is a frequently occurring event in non-small cell lung cancer (NSCLC) and that aberrant DNA methylation of promoter regions may lead to silencing of crucial tumor suppressor genes. Numerous genetic and epigenetic abnormalities which are known to be involved in the pathogenesis of NSCLC were already identified. Recently, a relatively new abnormality was observed in many cancer types including lung cancer: the deregulated expression of microRNA (miRNA) genes. MiRNAs are small, non-coding RNAs, which regulate a large number of biological processes by silencing specific target genes. Certain miRNA genes were found to be associated with CpG islands in their promoter regions suggesting that aberrant DNA methylation may lead to deregulated expression of miRNAs and to the development of a malignant phenotype. Using miRNA expression analyses, several miRNAs were identified to be tumor-specifically up- or downregulated. However, knowledge about miRNA gene methylation in NSCLC is still limited.

Thus, we performed a high-throughput screen for methylated miRNA genes in primary tumors and corresponding non-malignant lung tissue samples of 50 NSCLC patients using methylated DNA immunoprecipitation (MeDIP) combined with microarray technique (MeDIP-chip). Overall, 138 tumor-specifically miRNA genes were identified. Tumor-specific methylation of *miR-10a, miR-10b, miR-129-2, miR-572* and *miR-615* was confirmed by methylation-sensitive high resolution melting (MS-HRM) analyses in additional 47 NSCLC patients. Moreover, we observed that *miR-10a* methylated patients had a statistically significant shorter overall survival compared to *miR-10a* unmethylated patients. In addition, *miR-10a* methylation was found more frequently in adenocarcinomas than in tumors of other histologies. Target prediction revealed that *miR-10a, miR-10b, miR-129-2, miR-572* and *miR-615* are involved in crucial molecular pathways whose depletion may contribute to the development of a malignant phenotype.

Our findings indicate that DNA methylation plays an important role in the pathogenesis of NSCLCs. Furthermore, our results suggest that methylation of certain miRNA genes may be of prognostic significance in NSCLC patients and may serve as a potential biomarker.

2. Zusammenfassung

Mit jährlich über einer Million Todesopfern weltweit ist das Lungenkarzinom eine der häufigsten Krebserkrankungen, die zumeist erst in einem sehr fortgeschrittenen Stadium diagnostiziert wird. Dies geht mit einer geringen Fünfjahresüberlebensrate von ungefähr 17% einher. Bei der Entstehung von Lungenkrebs spielen nicht nur genetische Anomalitäten wie Mutationen sondern auch epigenetische Veränderungen eine wichtige Rolle. Intensiv untersucht wurde das Zusammenspiel von DNA Methylierung und Histonmodifikationen, das eine Kompaktierung der Chromatinstruktur von Genen bewirkt und zu Veränderungen der Genexpression führen kann. Funktionsverlust von Tumorsuppressorgenen durch aberrante DNA Methylierung wurde unter anderem beim nichtkleinzelligen Lungenkarzinom (NSCLC) festgestellt. In den letzten Jahren wurde nicht nur die epigenetische Regulation von protein-kodierenden Genen, sondern auch von microRNA-kodierenden Genen untersucht. MicroRNAs (miRNAs) zählen zu den kurzen, nicht-kodierenden RNAs und spielen eine wichtige Rolle bei der Regulation von Zellproliferation, Differenzierung und Apoptose.

Da es aber noch wenig Kenntnisse über den Zusammenhang der DNA Methylierung und der deregulierten Expression von miRNA Genen in NSCLCs gibt, haben wir das genomweite Methylierungsmuster von miRNA Genen in 50 NSCLC Patienten untersucht. Mittels Kombination von Immunpräzipitation methylierter DNA und der Microarray Technik (MeDIP-chip) konnten wir 138 tumorspezifisch methylierte miRNA Gene entdecken. Das Methylierungsmuster von *miR-10a, miR-10b, miR-129-2, miR-572* und *miR-615* wurde außerdem mittels methylierungssensitiver, hochauflösender Schmelzkurvenanalyse (MS-HRM) untersucht. Mit dieser Methode konnten die Ergebnisse der genomweiten Analyse bestätigt werden. Um die potentielle klinische Relevanz dieser Ergebnisse zu untersuchen, wurden die Methylierungsdaten mit klinisch-pathologischen Charakteristika der NSCLC Patienten verglichen. Diese Analysen ergaben, dass die Methylierung von *miR-10a* im Zusammenhang mit einem kürzerem Gesamtüberleben der NSCLC Patienten steht. Außerdem wurde die Methylierung von *miR-10a* häufiger in Adenokarzinomen als in anderen histologischen Untergruppen gefunden. Eine *in silico* „target prediction“ zeigte, dass alle fünf untersuchten miRNAs in wichtige Signalwege involviert sind, woraus gefolgert werden kann, dass eine Deregulierung dieser Signalwege zur Entstehung von NSCLCs beitragen kann.

Unsere Resultate lassen darauf schließen, dass die DNA Methylierung von miRNA Genen eine wichtige Rolle in der Pathogenese von NSCLC spielt. Außerdem weisen unsere Ergebnisse auf eine potentielle klinische Relevanz von methylierten miRNA Genen hin.

3. Scientific background

3.1. Lung cancer

3.1.1. Lung cancer incidence and mortality rates

In spite of advances in a better understanding of its molecular pathogenesis and developing new treatment strategies in recent years, lung cancer is still the leading cause of cancer-related deaths worldwide, causing over one million deaths each year (Parkin et al. 2005, Hecht 2006). Although efforts have been put into the development of early detection methods, lung cancer is mostly diagnosed at advanced stages, resulting in poor prognosis of the patients (Herbst et al. 2008). However, lung cancer has not always been a widespread health problem. In 1878, malignant lung tumors accounted only for 1% of all cancers dissected in autopsy at the University of Dresden. During the 20th century the percentage of lung tumors has risen from 10% in 1918 to more than 14% in 1927 (Witschi 2001). According to the World Health Organization (WHO) lung cancer incidence rates are highest in the Western Pacific Region, followed by Europe and the Unitated States of America, while incidence rates in less developed countries including South-Central Asia, South America and Northern Africa are lower (http://www.who.int/, 2013). The American Cancer Society estimated 226 160 new lung cancer cases in the United States in 2012, which accounts for about 14% of all diagnosed cancers (American Cancer Society, 2012). In Austria, 2659 and 1483 new cases of lung cancer were registered in 2010 in men and women, respectively, accounting for 11% of all malignant tumors. As in other developed countries, lung cancer is the second most frequently diagnosed cancer after prostate cancer in men (20%) and after breast cancer in women (22%). Interestingly, in men the lung cancer incidence rate declined from 2818 cases in 1983 to 2659 cases in 2010, whereas the incidence rate in women increased from 753 cases in 1983 to 1518 cases in 2008. In 2010 the incidence rate in women declined to 1483 new lung cancer cases (Statistik Austria 2012).

Mortality rates show that lung cancer still accounts for more cancer-related deaths than any other cancer type in both men and women (shown in *Figure 1*). For the year 2012, 160 340 deaths are expected to occur in the United States caused by lung cancer, accounting for 28% of all cancer-related deaths (American Cancer Society 2012).

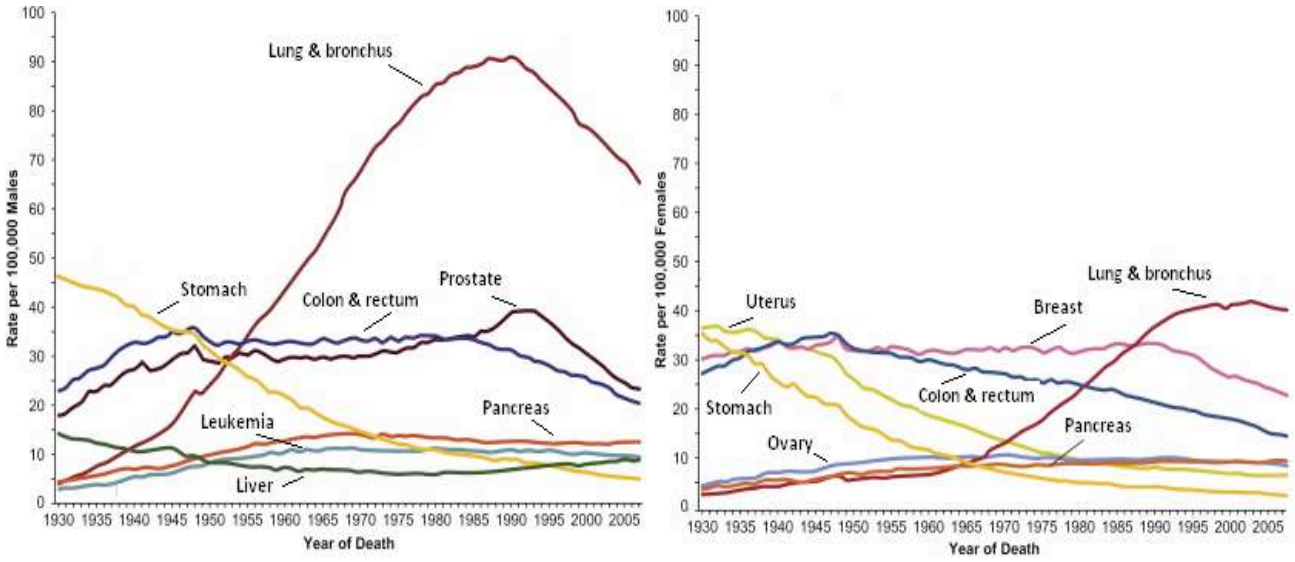

Figure 1. *Age-adjusted cancer death rates.*
Left panel: Graphs for cancer death rates per 100 000 males are age-adjusted to the 2000 US standard population.
Right panel: Graphs for cancer death rates per 100 000 females are age-adjusted to the 2000 US standard population. Uterus cancer death rates are for cervix and uterine corpus combined.
Adapted from American Cancer Society 2012.

In Austria, death rates declined in men from 2518 deaths in 1983 to 2386 deaths in 2010 correlating with declining incidence rates. On the other hand, in women death rates are still increasing from 649 deaths in 1983 to 1222 deaths in 2008 and 1266 deaths in 2010 reflecting historical differences between sexes in the uptake rate and abandoning of cigarette smoke. As mentioned earlier, most frequently diagnosed cancer types in Austria are prostate cancer in men (24%) and breast cancer in women (29%). However, due to early detection methods and efforts in treatment strategies the mortality rates for prostate cancer and breast cancer declined from 2000 to 2010 by 33% and 24%, respectively (Statistik Austria, 2012). Despite improvements in surgical techniques and new treatment strategies, the 5-year survival rate for lung cancer patients is still low with 6% for small cell lung cancer (SCLC) patients and 17% for non-small cell lung cancer (NSCLC) patients.

3.1.2. Histological classification of non-small cell lung cancers

Lung cancer is an epithelial cancer starting its development caused by molecular changes in histologically normal epithelial cells. During a long latent period over several years or even decades, progressional preneoplastic changes occur forming the basis of the multistage pathogenesis of lung cancer (Stratton et al. 2009, Gazdar et al. 2010). The two major types of lung cancer are SCLC, accounting for about 20% of all lung cancers, and NSCLC, accounting for about 80% of all lung cancers. Along with the two main types, other tumors can occur in the lungs e.g. carcinoid tumors, which account for fewer than 5% of lung tumors. The focus in this study was put on NSCLCs. Thus, only NSCLCs will be discussed in the following sections.

NSCLCs can be further divided into histological subtypes:

- Adenocarcinoma, ADC: predominant histological subtype accounting for 40% of NSCLCs; highly heterogenous with mixed patterns of different histological variants: acinar, papillary, bronichioloalveolar or solid with mucin formation.
- Squamous cell carcinoma, SCC: about 25% - 30% of NSCLCs (incidence decreased since availability of filter cigarettes); histological variants: papillary, small cell, clear cell and basaloid.
- Large-cell carcinoma, LCC: 10% - 15% of NSCLCs; different variants: basaloid, clear cell, lymphoepthelioma-like, rhabdoid phenotype.
  (Brambilla et al. 2001, Herbst et al. 2008, Travis 2011)

As the lung can be divided into central and peripheral compartments with different histological anatomies and functions, the major forms of lung cancer arise predominantly form either of these compartments. While SCCs arise predominantly in the central compartments of the lung, ADCs arise mainly peripherally (Gazdar 2010). The stages of disease will be classiefied according to the TNM staging system which describes the extent of the primary tumor (T), absence or presence of regional lymph node involvement (N) and absence or presence of distant metastases (M). Once the T, N and M stages are determined, disease stages from I to IV are assigned, with stage I being early disease stage and stage IV advanced disease stage (American Cancer Society 2012). Adequate disease staging is crucial to determine treatment strategies.

3.1.3. Lung cancer risk factors

The continuing decrease in age-adjusted lung cancer mortality in men in the recent years results almost entirely from a decrease in tobacco smoke uptake. 85 - 90% of lung cancer-related deaths are caused by tobacco smoking, thus tobacco smoke is the major risk factor for lung cancer (IARC 2004, Hecht 2012). Duration of smoking and number of smoked cigarettes are strong determinants for lung cancer and cessation of smoking prevents a further increase. However, compared to never smokers, the risk of former smokers for lung cancer remains at a high level for many years after cessation and declines only with a slow rate (Hecht 2006). Tobacco smoking increases the risk for all histological subtypes of lung cancer by great means. However, SCCs and SCLCs are most frequently diagnosed in heavy smokers (Herbst 2008). Tobacco smoke is not only a major risk factor for lung cancer but also for a variety of other diseases including renal cell carcinoma, cancer of the oral cavity, liver cancer and heart diseases (Hecht 2006, American Cancer Society 2012).

Nicotine is the major addictive substance in tobacco products, however, nicotine itself is not a carcinogen but a tumor promoter. Tobacco smoke is an aerosol consisting of a vapor and a particulate phase and contains a mixture of about 5000 compounds of which 72 are considered to be carcinogenic (Herbst 2012). Major components of the vapor phase are nitrogen, oxygen, carbon dioxide, carbon monoxide and water. Potentially carcinogenic components include nitrogen oxides (NO_x), isoprene, butadiene, benzene, styrene, formaldehyde and acetaldehyde. Major carcinogens of the particular phase are polycylic aromatic hydrocarbons (PAH), tobacco-specific *N*-nitrosamine (e.g. NNK and NNN), aromatic amines and metals such as nickel, chromium and cadmium (Hoffmann et al. 2001, Pfeifer et al. 2002, Hecht 2012). Due to their metabolic activation, there is strong evidence for the role of the carcinogens PAH and NNK in lung cancer.

The organism responds to carcinogen exposure similar to exposure to any other foreign compound through a “detoxification process” catalyzed by cytochrome P450 family members. During this process the compounds are metabolized, inactivated and finally excreted. Some of the metabolites are highly reactive molecules which can bind to DNA forming DNA adducts, which may lead to DNA damage during replication. The process of metabolic activation by which an unreactive carcinogen is converted to a form that binds to DNA is crucial in the carcinogenic process. If DNA damage cannot be repaired and persistent DNA adducts are bypassed incorrectly by a DNA polymerase, mutations may arise (Miller 1994, Hecht 1999, Pfeifer et al. 2002). Proto-oncogenes and tumor suppressor genes such as *KRAS* and *TP53* are

hotspots for mutations in cigarette smoke induced cancers (Denissenko et al. 1996, Pfeifer et al. 1998). Massion *et al*. (Massion et al. 2008) identified smoking-related genomic signatures in 75 NSCLC patients. Genomic regions mostly affected by cigarette smoke carcinogens were associated with genes that play major roles in cell cycle control, segregation of chromosomes and DNA methylation. Gene mutations and copy number alterations in such regions may cause loss of normal cellular growth control functions, genomic instability and uncontrolled cell proliferation resulting in the development of a malignant phenotype. *Figure 2* shows a schematic overview on how different mechanisms triggered by tobacco smoke may contribute to the development of cancer.

Furthermore, smoking-induced pulmonary inflammation may increase lung cancer risk by secretion of activated oxygen species, inflammatory mediators and proteolytic enzymes (Smith et al. 2006).

Other important risk factors for lung cancer are exposure to radon, asbestos, air pollution and genetic susceptibility to lung cancer (Alavanja 2002, Couraud et al. 2012, Ruosaari et al. 2008). Approximately 10 – 15% of lung cancer cases worldwide are associated with other factors rather than tobacco abuse (Sun et al. 2007). Adenocarcinomas of never- or light-smokers may show mutations within the tyrosine kinase domain of the epidermal growth factor receptor gene (*EGFR*) or *EML4-ALK* translocations (Thu et al. 2012).

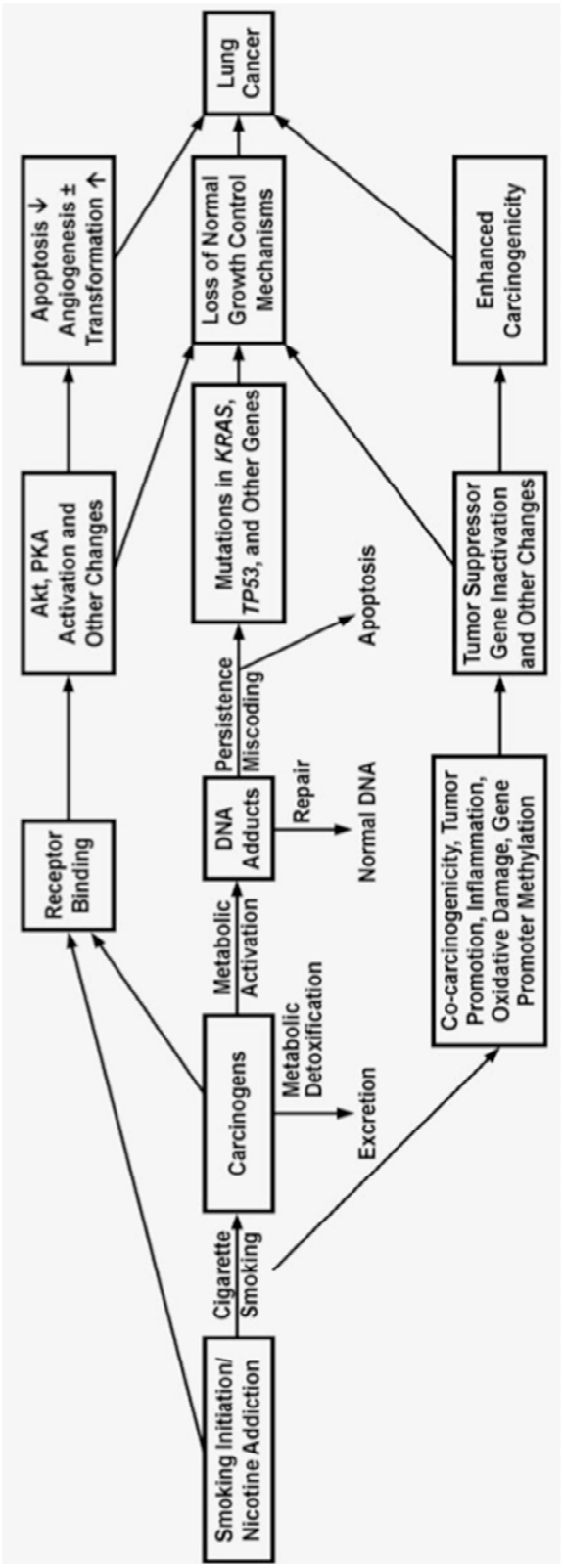

Figure 2. *Schematic overview of mechanisms linking tobacco smoke to cancer initiation.*
The central track involving exposure to carcinogens, the formation of covalent bonds between their metabolites and DNA producing DNA adducts and resulting genetic damage and mutations in critical genes of somatic cells displays the major pathway by which tobacco exposure causes cancer. As indicated in the top and bottom tracks, epigenetic pathways also contribute to development of cancer caused by smoking (Hecht 2006). Nicotine and nitrosamines bind to their cellular receptors leading to activation of protein kinases and a subsequent decreased apoptosis and increased angiogenesis and transformation (West et al. 2003). Aberrant methylation of promoter regions in tumor suppressor genes may result in gene silencing and uncontrolled cell proliferation (Belinsky 2005). Adapted from Hecht 2012.

3.1.4. Molecular pathogenesis of lung cancer

The multistep process of lung cancer development requires accumulation of various molecular changes including genetic and epigenetic alterations in proto-oncogenes and tumor suppressor genes. Mutations in the tumor suppressor gene *TP53* (p53) or the proto-oncogene *KRAS* (K-ras), aberrantly silenced expression of retinoblastoma tumor suppressor gene *Rb* (pRb), promoter hypermethylation of *CDKN2A* (p16) or loss of heterozygosity (LOH) in critical chromosomal regions are frequently occurring alterations in lung cancer, leading to clonal expansion of a cell having a competitive advantage and to malignant transformation (Nowell 1976, Mao 2001).

Since the first oncogene was discovered in the 1970s, a large number of oncogenes that are activated by mutation, translocation or amplification were discovered in various cancers. Upon activation, a proto-oncogene or its product can become a tumor-inducing oncogene. Proto-oncogenes encode mainly proteins that control cell proliferation, cell differentiation and apoptosis. They are often involved in signal transduction and mitogenic signaling. Frequently activated proto-oncogenes are *Ras, Wnt, Myc* and *Erk*, which were shown to be deregulated in lung cancer as well (Todd 1999, Croce 2008).

Another class of genes involved in cancer development are tumor suppressor genes (TSG) which were identified in the 1980s (Berger et al. 2011). TSGs were shown to inhibit cancer development by opposing oncogene function. A physiological balance between oncogenes and TSGs maintains homeostasis and controlled cell proliferation in normal cells. In cancer cells uncontrolled cell proliferation and deregulation of signaling pathways are caused by alterations in both oncogenes TSGs and, both holding crucial functions in regulation of cell cycle, proliferation, apoptosis and cell adhesion.

According to "Knudson`s two hit hypothesis", mutations in TSGs are recessive meaning that one functional copy of the gene is sufficient to inhibit cancer development. A second "hit" is necessary to inactivate the other allele either genetically or epigenetically (Knudson 1971, de Andrade, da Hora Barbosa et al. 2006, Plass and Soloway 2002) as shown in *Figure 3*. Alfred Knudson first described the "two-hit" mutation model in retinoblastoma cases. In sporadic retinoblastoma the two hits affecting the *Rb* gene are acquired in somatic cells, while in heritable retinoblastoma one mutated allele is inherited and the mutations on the other allele are generated somatically (Cavenee et al. 1985).

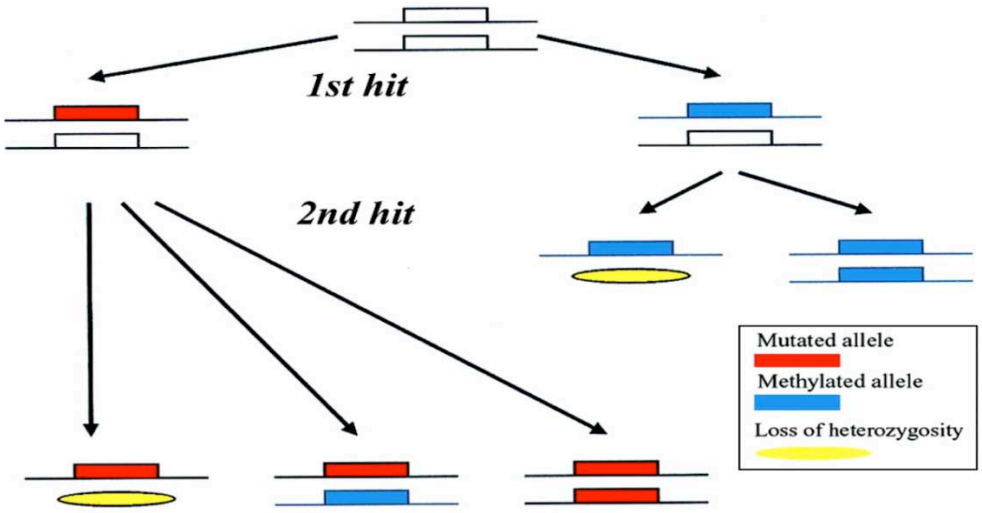

Figure 3. *Knudson`s Two Hit Hypothesis.*
If an individual carries two wildtype copies of a TSG, two independent "hits" are necessary for a loss of gene function. The first "hit" may be a mutation in the DNA sequence or aberrant promoter methylation. The second inactivating "hit" may be either LOH or an additional mutational or methylating event. Adapted from Wheeler et al. 2000.

Besides gene mutations, chromosomal aberrations such as deletions, translocations and amplifications in regions coding for TSGs or DNA repair genes are also frequently occurring events in cancer cells (Massion et al. 2003). LOH at chromosomal regions 3p, 9p and 17p were described as early events in the pathogenesis of lung cancer. These genomic regions harbor crucial TSGs including *RASSF1A*, *FUS1, FHIT, CDKN2A* and *TP53* (Sundaresan et al. 1995, Wistuba et al. 2002). LOH patterns differ in squamous cell carcinoma and adenocarcinomas as well as in smokers and former smokers (Mao et al. 1997). Other cytogenetic abnormalities involved in lung cancer development include deletions (e.g. chromosome 3p and 9p), amplifications (e.g. chromosome 1q and 3q) and rearrangements (e.g. transforming rearrangements of the *ALK* gene) which all lead to genomic instability, a fundamental characteristic of cancer initiation and progression (Massion et al. 2003, Solomon et al. 2009). Microsatellite instability with alterations in short-tandem DNA repeats as a result of deficiencies in DNA mismatch repair genes (e.g. *hMSH2* and *hMLH1*) was observed in both SCLCs and NSCLCs (Miozzo et al. 1996, Kouso et al. 2008).

Hanahan and Weinberg (Hanahan et al. 2000) defined six key events acquired during the multistep development of cancer which enable tumor growth and metastatic dissemination. These hallmarks include the ability to sustain chronic proliferation and to evade growth suppressors, different strategies to limit or even circumvent apoptosis, induction and perpetuation

of angiogenesis, activation of invasion and metastasis and a limitless replicative potential (Hanahan et al. 2000, Hanahan et al. 2011). Development of a neoplastic phenotype requires acquisition of all these characteristics which arise from both genetic and epigenetic alterations in oncogenes and TSGs.

While proliferation of normal cells is strictly regulated, cancer cells bypass normal cell cycle control by deregulating cell cycle checkpoints and various signaling pathways. Premalignant cells acquire the ability to avoid cell cycle checkpoints and to evade anti-proliferative signals through disruption of the *Rb* signaling pathway and inactivation of *CDKN2A* (Gazdar et al. 2010).

Silenced expression of the CDK inhibitor $p16^{INK4}$ in NSCLC is frequently caused by hypermethylation or homozygous deletions (Gazzeri et al. 1998). The "guardian of the genome" p53, encoded by the *TP53* gene, has many downstream target genes involved in cell cycle arrest, DNA repair and apoptosis as well as upstream regulatory genes such as $p14^{ARF}$ and *Mdm2* (Brambilla et al. 2009). With a mutation frequency of 60% *TP53* is the most frequently mutated TSG in human lung cancers having a strong relation to tobacco exposure (Denissenko et al. 1996). Mutation of *TP53* mostly affects its major downstream effector *Rb* and thereby control of G1 arrest. *ATM*, an upstream target gene of the p53/$p14^{ARF}$ pathway, which mediates response to DNA damage was recently also shown to be mutated in lung cancers (Ding et al. 2008). Furthermore, key players of the mitochondrial apopototic and the death receptor pathways, namely Bcl-2 and Bax, are regulated by p53. As both factors are highly deregulated in lung cancers, resistance to mitochondrial as well as to death receptor-induced apoptosis is possible (Brambilla and Gazdar 2009). Anti-apoptotic Bcl-2 is overexpressed in 10 – 35% of NSCLCs (Fong et al.1999).

One major element in regulation of proliferation, apoptosis, angiogenesis and invasion is the epidermal growth factor receptor *EGFR/HER1*, a member of the receptor tyrosine kinase family (Gazdar et al. 2010, Herbst et al. 2008). Activity of *EGFR* is frequently deregulated in NSCLCs by mutation and/or amplification and results in expression of almost all hallmarks of cancer (Brambilla et al. 2009). Somatic mutations in the kinase domain of *EGFR* were identified in approximately 10% of adenocarcinoma specimens from Caucasians patients and in 30 – 50% of adenocarcinoma specimens from patients in Asia. The majority of these mutations (~80%) are in-frame deletions within exon 19 or a specific point mutation, L858R, which occurs within exon 21 (Herbst et al. 2008). Via Ras/MAPK and PI3K/Akt signaling networks *EGFR* activation is linked to cell proliferation and survival (Sharma et al. 2007). Despite

EGFR and *KRAS* mutations (in about 20-40% of ADCs) other *EGFR* pathway genes were found to be deregulated in lung cancer including *PTEN*, *HER2/Neu/Erb-B-2*, *BRAF* and *PIK3CA* (Naoki et al. 2002, Samuels et al. 2004, Shigematsu et al. 2005, Sos et al. 2009).

Cellular growth and apoptosis are also regulated by nuclear phosphor-proteins c-myc, n-myc and l-myc. Overexpression and amplification of myc proteins results in deregulated cell proliferation of cancer cells. (Gazzeri et al. 1990, Gazzeri et al. 1994). *MYC* is amplified in approximately 5 – 10% of NSCLCs (Fong et at. 1999).

While normal cells have limited replicative capacity due to telomere shortening, the vast majority of tumors express the telomerase complex which maintains telomere length (Fernandez-Garcia et al. 2008). MRNA and/or protein expression levels of the catalytic subunit hTERT are significantly increased in preinvasive bronchial lesions according to severity of their grade (Lantuejoul et al. 2005).

During tumorigenesis, cancer cells must acquire the ability of inducing angiogenesis to supply the growing tumor with oxygen and nutrients. Formation of new vessels is induced by several factors including vascular endothelial growth factor (VEGF). VEGF and the VGF-R co-receptors Neuropilin 1 and 2 (Np1 and Np2) were found to be overexpressed in a large number of lung cancers enabling the tumor to sustain angiogenesis (Decaussin et al. 1999, Gazdar et al. 2010).

The ability to invade surrounding tissues and to metastasize is another major characteristic of malignant tumors. Cancer cells typically develop alterations in the attachment to other cells as well as to the extracellular matrix (ECM). Loss of E-cadherin expression, a cell-cell adhesion molecule, by down-regulation or mutational inactivation was frequently observed in various cancer types including NSCLC (Berx et al. 2009). E-cadherin expression was also found to be deregulated in NSCLCs by promoter hypermethylation (Wang et al. 2008).

Another important regulatory program by which transformed epithelial cells acquire the ability to invade and to disseminate is the “epithelial-mesenchymal transition” (EMT). Furthermore, invasive growth and metastasis requires crosstalk between cancer cells and cells of the neoplastic stroma (Hanahan et al. 2011).

Molecular pathogenesis of lung cancer requires accumulation of various genetic and epigenetic alterations, including deregulated signaling pathways which are potential targets for drug treatment. EGFR tyrosine kinase inhibitors (e.g. erlotinib and gefitinib) and angiogenesis inhibitors (e.g. bevacizumab) are in clinical use for treatment of NSCLC patients (Shepherd et al. 2005, Herbst et al. 2008).

3.2. Epigenetics

3.2.1. History and implication of epigenetics

Since the term "epigenetics" was first coined in 1942, its meaning and definition changed several times. While we currently understand epigenetics as "heritable changes in gene function and expression without changes in the underlying DNA sequence" (Jones et al. 2007), Conrad Hal Waddington used it to describe "the causal interactions between genes and their products, which bring the phenotype into being" (http://www.epigenome.eu/, 2012). He first introduced the term as a combination of the words *genetics* and *epigenesis,* but back then, the nature of genes and their role in hereditary was not clear yet (Waddington 2012). At this time the term was used to categorize all of the developmental events which lead to the final product, the mature organism. With the identification of DNA as the carrier of genetic information, it was necessary to find another definition for epigenetics to distinguish heritable changes which are caused by changes in the DNA sequence from those that are not caused by sequence changes (Allis 2007).

Many years later Russo *et al*. (Russo 1996) defined epigenetics as "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence", describing that epigenetic alterations are heritable, but not due to genetic mutations (illustrated in *Figure 4*). Aiming to unify and refine the existing definitions, A. Bird (Bird 2007) described epigenetic events as "the structural adaption of chromosomal regions so as to register, signal or perpetuate altered activity states".

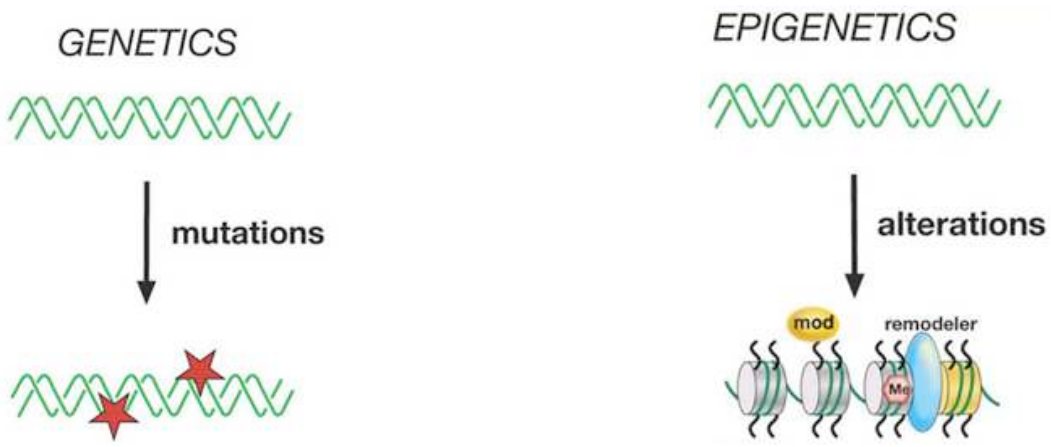

***Figure 4**. Genetisc vs. Epigenetics.*
Genetics: Mutations in the DNA sequence (indicated by red asterices) are heritbale somatically and through the germ line;Epigenetics: Alterations in chromatin structure such as histone modifications, chromatin remodeling or DNA methylation modulate the use of the genome. Marks on the chromatin template may be heritable through cell divisions. Adapted from Allis 2007.

Epigenetic mechanisms, namely DNA methylation, histone modifications, chromatin remodeling and RNA-associated gene silencing, interact to initiate and maintain gene silencing on the level of chromatin. Regulation of gene expression provides the basis for important cellular processes such as development, differentiation, imprinting and silencing of the female X-chromosome. DNA methylation also plays a major role in the suppression of parasitic sequence elements, such as transposons and endogenous retroviruses, and is responsible for host defense. Like any other biological process, epigenetic mechanisms can be disrupted leading to the development of diseases, such as cancer. (Bestor 1998, Egger et al. 2004, Jones et al. 2007).

3.2.2. Nucleosome structure regulates transcriptional activity

In eukaryotic cells, DNA is organized into nucleosomes, the basic functional units of chromatin. It contains 147 bp of DNA, which are wrapped around an octamer of core histone proteins H2A, H2B, H3 and H4. A linker DNA, 10-70 bp in length, connects the nucleosome cores (Kouzarides 2007, Grigoryev 2012). The N-terminal “histone tails” protrude from the surface of the nucleosome and are targets for a large number of modifications as described in section 3.2.3.

Chromatin can be categorized into two major regions: heterochromatin is highly condensed and contains transcriptional inactive genes whereas euchromatin is less densely packed and

therefore transcriptionally active (Dawson et al. 2012). Formation of heterochromatin respectively euchromatin is highly controlled and regulated by different mechanisms (shown in *Figure 5*). Epigenetic modifications such as DNA methylation do not happen alone, they occur in context with other modifications, such as histone modifications and nucleosome remodeling.

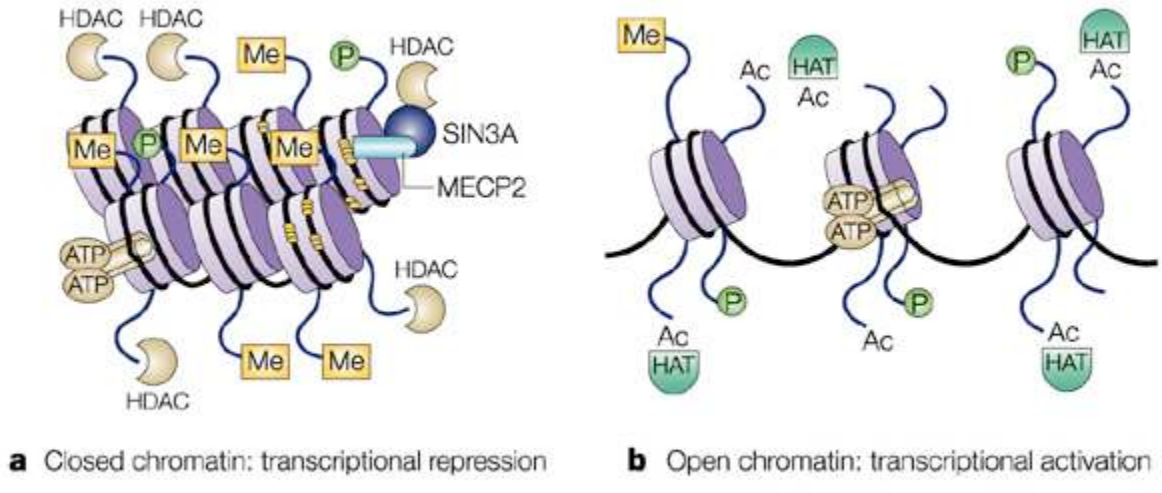

***Figure 5**. Interplay of epigenetic modifications.*
Nucleosome consist of DNA (indicated as black line) wrapped around histone octamers (indicated as purple cylinders). Nucleosome structure can be altered by post-translational histone tail modifications (methylation Me, phosphorylation P, acetylation Ac), the action of ATP-dependent chromatin remodellers (indicated ay yellow cylinders) and the opposing effects of histone acetylatransferases (HATs) and histone deacetlyases (HDACs).
A: Methyl-CpG binding proteins, such as MeCP2 target methylated DNA and recruit HDACs through Sin3A complex formation. Interplay of these mechanisms results in a closed chromatin configuration and transcriptional repression.
B: Demethylation of DNA and histone acetylation opens the chromatin and allows the transcription machinery to work. Adapted from Johnstone 2002.

3.2.3. DNA methylation

Methylation of DNA (referred to as methylation) is probably the best known and most widely studied epigenetic modification. DNA methylation happens through addition of a methyl group ($-CH_3$) to carbon 5 of a cytosine within a CpG dinucleotide (Bird 2002). This reaction is catalyzed by enzymes called DNA methyltransferases (DNMTs) which use S-adenosyl-methionine (SAM) as methyl group donor (Herman et al. 2003) (shown in *Figure 6*).

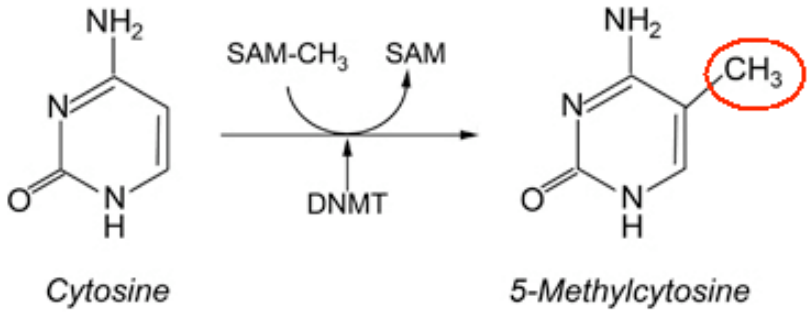

***Figure 6**. Conversion of cytosine to 5-methylcytosine by DNMTs.*
The transfer of a methyl group from SAM to the 5´carbon of a cytosine is catalyzed by DNMT. Adapted from Gibney et al 2010.

To transfer a methyl group to the 5´ carbon of a cytosine an electrophilic attack by SAM is necessary. As the 5´carbon is not nucleophilic itself, the methyltransferases must catalyze this process to increase nucleophilicity. A nucleophile from the enzyme attacks the position 6 of cytosine, so that position 5 is activated for an electrophilic attack by the cofactor SAM which transfers the methyl group. However, 6´ carbon can only be attacked after it is deprotonated by a DNA phosphate group. So DNA acts both as substrate and cofactor in the methylation reaction (Zangi et al. 2010). Not only DNA methylation, but also many other metabolic pathways require a transfer of a methyl group and use SAM as a methyl group donor which is produced and regenerated in the so called "SAM cycle" (Finkelstein et al. 2000).

The position of methylation relative to the transcription start site is crucial if altered DNA-protein interactions lead to either a decrease or increase in the transcription rate. While methylation of gene bodies correlates with increased transcription rate, methylation of promoters and enhancers correlates with transcriptional silencing (Chen et al. 2011).

To guarantee proper development and cellular differentiation, distinct methylation patterns must be established during mammalian development and maintained in adult somatic cells (Chen et al. 2011). DNA methylation is also the key player in genomic imprinting, X-chromosome inactivation and the suppression of repetitive elements. Through DNA methylation a large amount of noncoding DNA, inserted viral sequences and transposons are kept in a transcriptionally inert state (Herman et al. 2003).

3.2.3.1. DNMTs

Three major DNMTs are involved in mammalian DNA methylation (illustrated in *Figure 8*) which can be divided into two groups: *maintenance* methyltation by DNTM1and *de novo* methyltation by DNMT3A and DNMT3B (Klose et al. 2006, Chen et al. 2011). A fourth mammalian DNA methyltransferase, DNMT2, contains the amino acid motifs that are required for methylation of C5, but shows only weak methyltransferase activity in vitro (Hermann et al. 2003). DNMT3L, a member of *de novo* methyltransferases, shows no detectable DNA methyltransferase activity too, but is believed to regulate methylation of imprinted genes by interacting with DNMT3A and DNMT3B. DNMT3L was shown to increase the enzymatic activity of DNMT3A and DNMT3B not by recruiting DNMTs to the target DNA sequence but by direct effects on their catalytic activity (Hata et al. 2002, Suetake et al. 2004).

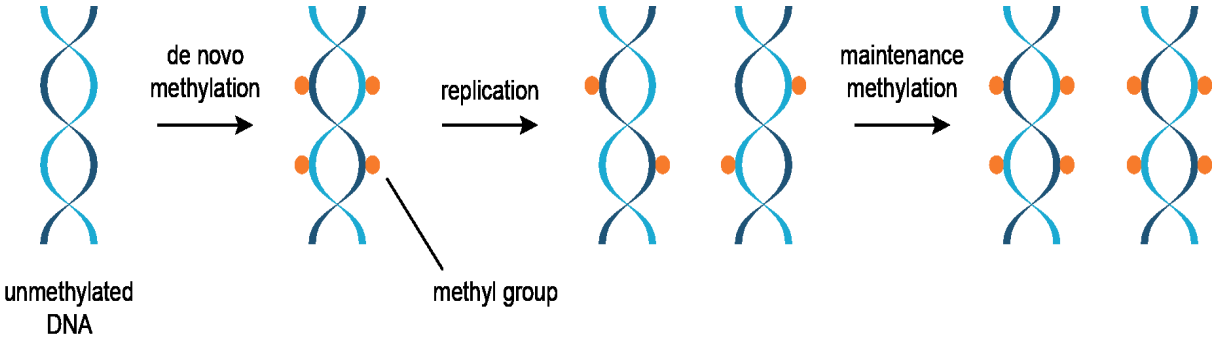

***Figure 7**. Two mechanisms of DNA methylation.*
De novo methylation of unmethylated DNA is carried out by DNMT3A and DNMT3B, whereas DNMT1 methylates hemimethylated DNA to maintain methylation patterns after replication. Adapted from http://www.atdbio.com, 2012.

Although methylation patterns are generally stable maintained through somatic cell divisions, at certain stages during mammalian development methylation patterns must be reprogrammed to generate totipotent cells. The epigenetic reprogramming of germ cells requires *de novo* methylation carried out by DNMT3A and DNMT3B (Reik et al. 2001). Okano *et al.* (Okano et al. 1999) showed that inactivation of both DNMT3A and DNMT3B is lethal for mice embryos which demonstrates that *de novo* methylation is essential for mammalian development. After cell differentiation, these enzymes are thought to be down-regulated but still expressed (Jones et al. 2009).

Newly established methylation patterns are then copied through cell divisions by DNMT1, the *maintenance* methyltransferase. DNMT1 is located at the replication fork and therefore it

can maintain the methylation pattern of newly synthesized DNA directly after replication (Hermann et al. 2004). DNMT1 has a higher affinity to hemi-methylated DNA (Fatemi et al. 2001). It is directed to its preferred substrate by the protein UHRF1 (Arita et al. 2008). With 183kDa DNMT1 is a very large protein which contains a number of functional domains at its N-terminus, e.g. a nuclear localization domain and a cysteine-rich region (Bestor 1992). Cysteine-rich regions are present in all mammalian metyhltransferases as well as in MBD and CpG binding proteins (Goll et al. 2005). Furthermore, DNMT1 interacts with the proliferating cell nuclear antigen (PCNA) which is involved in DNA replication and repair (Chuang et al. 1997).

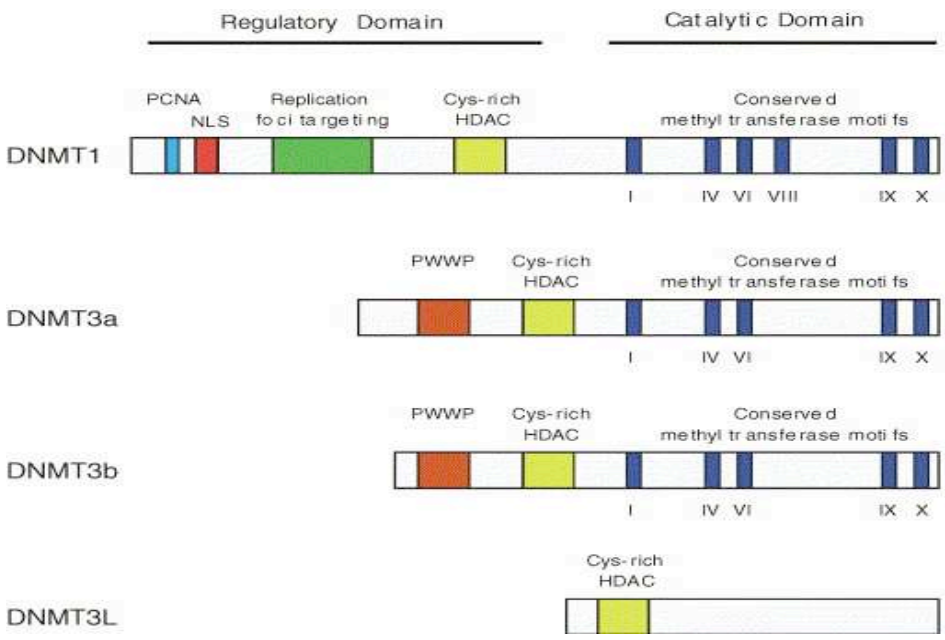

***Figure 8**. The family of mammalian DNMTs.*
Although the catalytic domains of DNMT1, DNTM3A and DNMT3B are conserved, their N-termini differ. The PCNA interacting domain is indicated as cyan box and the nuclear localization signal (NLS) as red box. The domain which is responsible for targeting DNMT1 is shown as green box. In all DNMTs conserved is the Cys-rich region, which is also the HDAC interacting domain, shown here as yellow boxes. DNMT3A and DNMT3B share the proline and tryptophane domain, indicated as brown boxes. DNMT3L completely lacks the catalytic domain. Adapted from Villa et al. 2004.

3.2.3.2. CpG islands

As mentioned above, the covalent addition of methyl groups to 5´carbon of cytosine rings occurs generally at cytosines within CpG dinucleotides. Nevertheless non-CpG methylation was found at a high level in human embryonic stem cells (Laurent et al. 2010).

Due to spontaneous deamination of methylcytosines to thymines, CpG dinucleotides are underrepresented in the human genome (Lander et al. 2001). The majority of CpG dinucleotides in the mammalian genome are found concentrated in clusters, called "CpG islands" (CGIs) (Illingworth et al. 2009). CGIs are characterized by a CG content higher than 50% and show a typical length of 0.4-5 kb. They are found in the promoter region of approximately 60-70% of human genes and may also extend into the 5´coding region of the gene (Gardiner-Garden et al. 1987, Larsen et al. 1992, Wang et al. 2004, Saxonov et al. 2006). While the majority (70-80%) of non-CGI CpGs in the human genome is methylated, CpGs within CGI promoters remain mostly unmethylated in normal cells (Razin et al. 1994, Kim et al. 2009). As CGIs remain umethylated with exception of imprinted genes, X-linked or tissue specific expressed genes, they are generally characterized by transcriptionally active chromatin state as shown in *Figure 9* (Takai et al. 2002, Deaton et al. 2011).

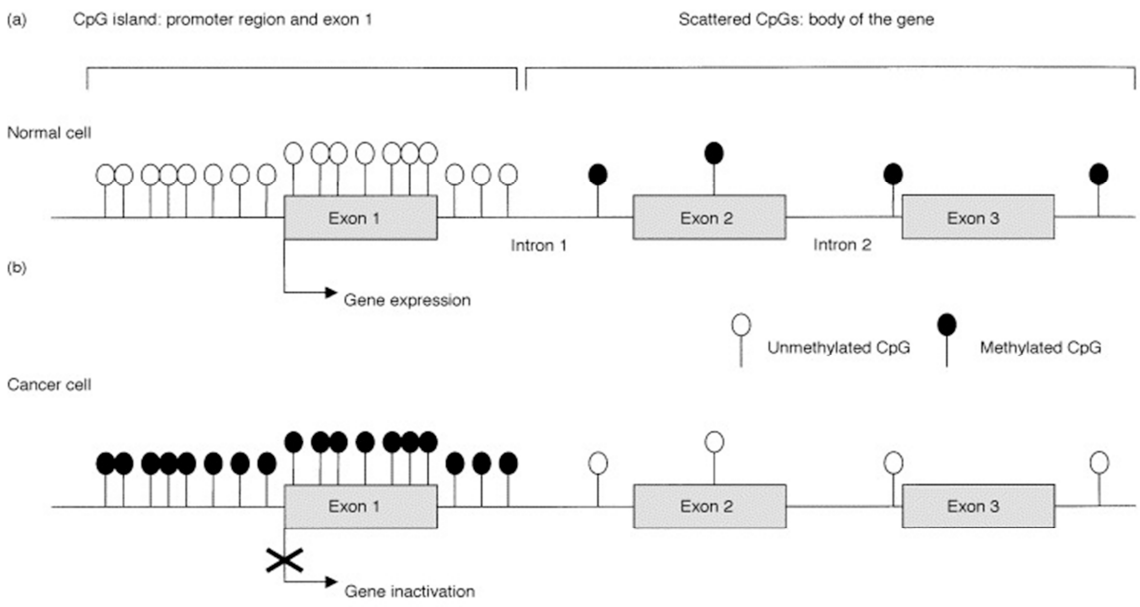

Figure 9. *CpGs in promoter regions and in the gene body.*
A: unmethylated CpGs within the promoter region are correlated with transcriptional activity of the target genes, as it is the case is housekeeping genes.
B: gene transcription is inactivated if methylation occurs at CpGs in promoter regions.
Adapted from Esteller 2000.

3.2.4. Histone modifications

Histone tails are targets for more than 100 different posttranslational modifications including acetylation, methylation, ubiquitination and sumolyation of lysines (K), phosphorylation of serines (S), tyrosines (Y) and threonines (T) and methylation of arginine (R) and lysines (K) (Bernstein et al. 2007, Kim et al. 2009). Histone modifications can act either by directly influencing the chromatin structure or by regulating the binding of effector molecules (Bannister et al. 2011). By regulating DNA accessibility covalent histone modifications play a major role during DNA transcription, replication, damage repair and other important cellular processes (Chi et al. 2010).

By transferring acetyl groups from acetyl coenzyme A onto specific lysine residues which are present in the amino-terminal tail of histone proteins, the acetylation reaction results in neutralization of positive charges of histones. This reaction is catalyzed by histone acetyltransferases, so called HATs (Brownell and Allis 1996). Because elimination of positive charges potentially weakens the electrostatic interactions between histone tails and the DNA backbone, acetylation is associated with transcriptionally active chromatin. On the other hand, deacetylation of histones, catalyzed by histone deacetylases (HDACs), removes the negative charge from histone proteins and thereby affinity between histones and DNA increases. Consequently, binding of transcription factors to more densely packed chromatin structure is no longer possible which results in transcriptional inactivity (Cress et al. 2000).

Just as acetylation, phosphorylation of histone tails is a dynamic process controlled by kinases and phosphatases. By transferring a phosphate group from ATP to the hydroxylgroup of the amino-acid side chain, the negative charge of histones is increased (Bannister et al. 2011). Although most of the phosphorylation sites are lying within the amino-terminal tails of histones, there are also sites which are located in the core regions; e.g. the non-receptor tyrosine kinase JAK2 phosphorylates Y41 on histone H3 (Dawson et al. 2009).

In contrast to acetylation and phosphorylation, histone methylation of lysines ard arginines does not change the charge of histone proteins (Bannister et al. 2011). Whereas methylation of H3K4, H3K36 and H3K79 are correlated with transcriptional activity, H3K9, H3K27 and H4K20 are connected to transcriptional repression. In order to act as either positive or negative epigenetic regulators of gene expression, histone tails can be mono-, di- or trimethylated. One major repressive histone mark is trimethylation of histone H3 at lysine 27 (H3K27me3)

which is catalyzed by the SET domain of histone methyltransferase EZH2 (Kouzarides 2007, Vrba et al. 2011).

EZH2 forms together with SUZ12 and EED the polycomb repressive complex 2 (PCR2). PCR2, as well as PCR1, harbor and may recruit other polycomb group proteins (PcGs), DNA methyltransferases and histone deacetylases all contributing to the formation of transcriptional repressive marks and to a higher degree of chromatin compaction (Allis 2007, Chase et al. 2011). While PcGs are responsible to maintain transcriptional silencing, proteins that are involved in maintaining a transcriptional active state are called trithorax group proteins (TrGs). Working in an antagonistically way, PcGs and TrGs regulate cellular memory via epigenetic mechanisms (Ringrose et al. 2007).

Similarly to methylation of lysines, methylation of arginines can be both, activating or repressing (Kouzarides 2007).

The dynamic transition between transcriptionally active or silent chromatin states is thus regulated by a large number of posttranslational modifications. These modifications regulate the binding of effector proteins in an either synergistic or antagonistic way and the interplay of all these modifications reveals the so called "histone code" (Jenuwein et al. 2001).

3.2.5. Chromatin-remodeling complexes

Another important mechanism to alter the chromatin structure is the recruitment of ATP-dependent "chromatin-remodeling complexes" (Allis 2007). They use energy of ATP hydrolysis to move nucleosomes along DNA, eject histones from DNA or to rearrange nucleosomes. By this way, these complexes change interactions between DNA and histones so that effector proteins gain access to their binding sites (Becker et al. 2002).

Remodeling complexes can be categorized based on their specific protein domains: the SWI/SNF family, the ISWI family, the NuRD/Mi-2/CHD family and the INO80 family. Although they all share the ATPase domain, their functions and recruited subunits differ (Eberharter et al. 2004, Wang et al. 2007).

Besides their importance in transcriptional regulation, ATP-dependent chromatin-remodeling complexes also play a key role in a wide range of cellular processes. Members of the ISWI family were shown to be implicated in chromatin assembly after DNA replication and members of the SWI/SNF family are implicated in DNA DSB repair (Chai et al. 2005).

3.2.6. DNA methylation regulates gene transcription

DNA methylation regulates gene expression via two major mechanisms (demonstrated in *Figure 10*). Firstly, the addition of methyl groups interferes with the binding of transcription factors (TFs) which interrupts the DNA-TF interaction. Several TFs recognize GC-rich sequences and are unable to bind DNA if these CpG sequences are methylated. This mode of action was first described by Bell and Felsenfeld (Bell et al. 2000) who studied the role of the CTCF protein in imprinting at the H19/Igf2 locus (Allis 2007).

The second mechanism attracts proteins that specifically recognize methylated CpG sequences and recruit co-repressors to silence transcription (Jones et al. 1998). The first of these proteins identified was MeCP2 (Meehan et al. 1992). Later several other proteins all sharing a methyl CpG binding domain (MBD) were identified. The MBD family of proteins consists of MeCP2, MBD1, MBD2, MBD3 and MBD4 (Wade 2001). Except of MBD3, which contains a MBD domain that does not recognize methylated DNA because of crucial amino acid changes in this motif, they all specifically recognize and bind to methylated CpG sequences (Klose et al. 2006).

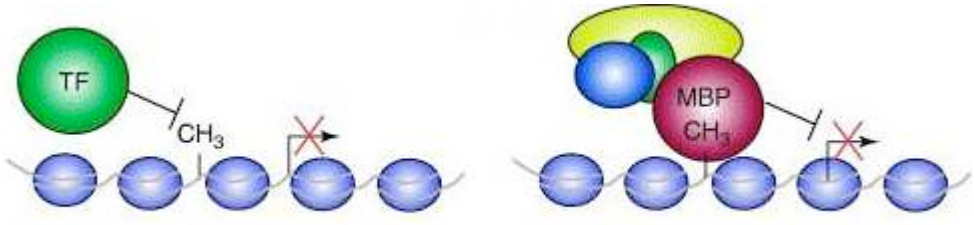

***Figure 10**. Mechanisms of silencing gene expression via DNA methylation.*
Left panel: DNA methylation prevents TFs from binding to DNA, thus transcriptional activation is inhibited. Right panel: methyl-CpG-binding proteins (MBPs) recognize and bind methylated DNA. Recruitment of co-repressors and modification of surrounding DNA silences gene transcription. Adapted from Klose et al. 2006.

Kaiso, a structurally unrelated protein, was also shown to bind methylated CpG sequences and repress transcription via a methylation-dependent mechanism, like members of the MBD protein family. However, unlike members of the MBD protein family, it recognizes methylated DNA via zinc-finger domains and exerts its ability to repress transcription of methylated genes through a POZ/BTB domain (Prokhortchouk et al. 2001, Filion et al. 2006). *Figure 11* shows structural characteristics of methyl-CpG binding proteins including MBD1-MBD4, MeCP2 and Kaiso.

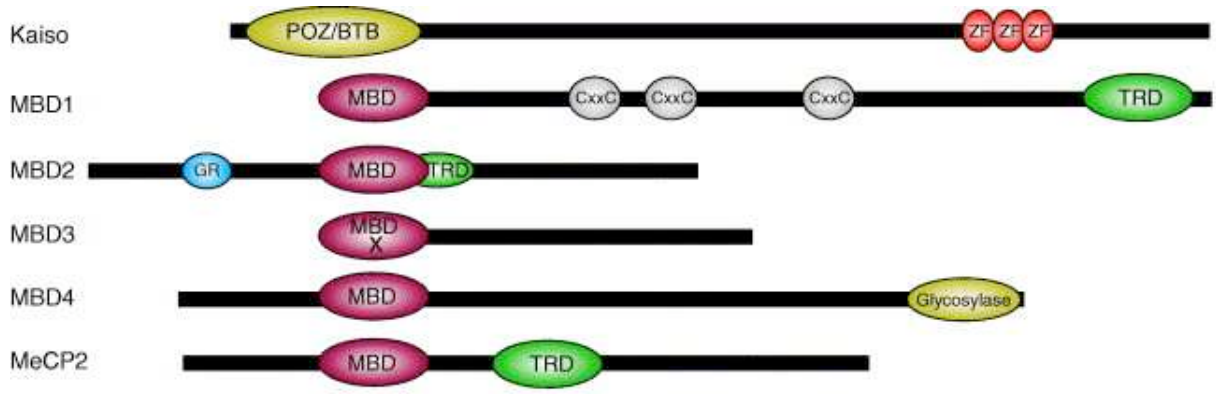

Figure 11*. Family of methyl-CpG binding protein (MBPs).*
Kaiso is atypical, because it recognizes methylated DNA via zinc-finger domains and contains a POZ/BTB domain to repress transcription. MBD1 contains a methyl-binding domain (MBD) and additionally contains three zinc-binding domains (CxxC) and a trancsiptional repression domain (TRD) on the C-terminus. The MBD and TRD of MBD2 overlap and it possesses an N-terminal GR repeat. The MBD of MBD3 is unable to bind methylated DNA due to amino acid changes. MBD4 binds methylated DNA via its MBD domain and additionally contains a glycosylase domain, which plays a role in DNA repair. MeCP2, which was the first member to be identified, possesses a conversed MBD domain and a TRD domain. Adapted from Klose et al. 2006.

MeCP2, the founder member of MBPs, recognizes methylated CpGs via its MBD domain. It binds to methylated DNA in the promoter region and recruits a chromatin-remodeling complex. This complex consist of Sin3A, BRM, a catalytic component of the SWI/SNF family and HDACs. Histone deacetlyation through HDACs leads to chromatin condensation resulting in transcriptional inactivity (Bird et al. 1999, Wade 2001, Georgel et al. 2003, Bienvenu et al. 2006). MBD2 was shown to interact with the nucleosomal remodeling complex NuRD and by binding to methylated DNA it directs the complex to the methylated CpG sequence. As HDAC1 and HDAC2 are components of the Sin3A and also of the NuRD complex, recruitment of NuRD to methylated DNA in promoter regions leads to transcriptional silencing. (Zhang et al. 1999). *Figure 12* illustrates the association between DNA methylation and histone deacetylation.

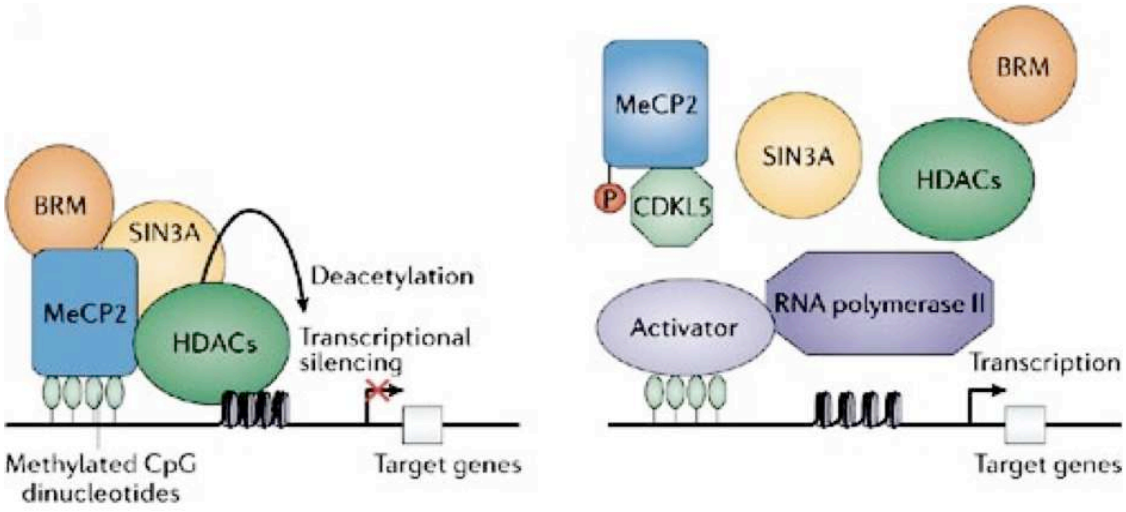

***Figure 12**. MeCP2 regulates gene expression.*
Left panel: MeCP2 recognizes and binds methylated CpGs in the promoter region, which results in the recruitment of a chromatin-remodeling complex. By removing acetyl groups, chromatin becomes more condensed and the transcription machinery can no longer work properly.
Right panel: If MeCP2 is not bound, the complex containing Sin3A, BRM and HDACs is not recruited and histones remain acetylated. The open conformation of chromatin allows the transcription machinery to bind and transcribe target genes. Adapted from Bienvenu et al. 2006.

DNA methylation, histone modification and nucleosome remodeling are all dynamically linked and act together to silence gene expression. Alterations in these processes which are essential for normal development and differentiation, may result in aberrant silencing of cancer-related genes. (Jones et al. 2007)

3.2.7. Techniques to analyse DNA methylation

For many years epigenetic research lagged behind due to technical limitations. The study of DNA methylation of specific sequences was restricted to the use of enzymes distinguishing between methylated and unmethylated recognition sites in the gene of interest. A major step in studying cancer epigenetics was the development of sodium bisulfite treatment of genomic DNA. By this procedure unmethylated cytosines are converted to uracils while methylated cytosines remain unchanged (shown in *Figure 13*).

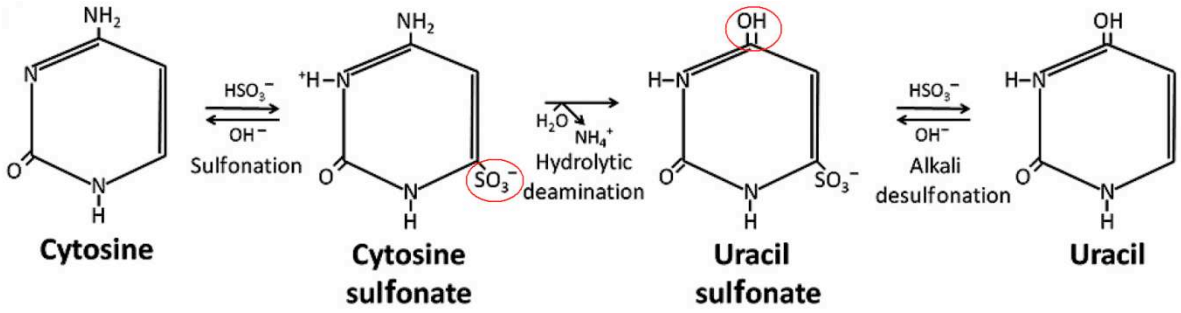

Figure 13. *Sodium bisulfite conversion of unmethylated cytosine.*
Single stranded DNA is converted with sodium bisulfite (HSO_3-) at low pH and high temperatures. First, sulfonation at C6 of cytosine happens, followed by an irreversible hydrolytic deamination at the C4 to generate uracil sulfonate. By subsequent desulfonation the uracil is generated. Methylation at C5 prevents sulfonation at the C6. Purification is necessary to remove bisulfite salts and other chemical compounds. Adapted from Kristensen et al. 2009.

Sodium bisulfite treatment of genomic DNA followed by methylation-specific PCR, sequencing or quantitative PCR-based approaches are the most frequently used techniques for detection of DNA methylation (Herman et al. 2001, Esteller 2007). Dependent on the initial methylation level of the gene of interest, sequences differ in their amounts of AT base pairs respectively CG base pairs after the PCR reaction. *Table 1* shows sequence differences after sodium bisulfite treatment and subsequent PCR reaction.

Table 1. *Sequence differences after sodium bisulfite treatment and subsequent PCR reaction*
Upon denaturation of DNA, unmethylated cytosines are converted to uracils while 5-methylcytosines are left intact. Modified DNA is then amplified using primers that allow specific amplification of the bisulfite converted DNA. During amplification uracils are replaced by thymines pairing with adenines whereas 5-methycytosines are replaced by cytosines pairing with guanines. Initially methylated regions thus show a higher CG content after PCR reaction.

	Original sequence	Sequence after treatment		PCR
Unmethylated DNA	**C**GGC**C**GACT**C**G G**C**CGG**C**TGAG**C**	**U**GGU**U**GAUT**U**G G**U**UGG**U**TGAG**U**	→	**T**GGT**T**GATT**T**G **A**CCA**A**CTAA**A**C C**A**ACC**A**ACTC**A** GTTGG**T**TGAG**T**
Methylated DNA	**C**GGC**C**GACT**C**G G**C**CGG**C**TGAG**C**	**C**GGU**C**GAUT**C**G G**C**UGG**C**TGAG**C**	→	**C**GGT**C**GATT**C**G **G**CCA**G**CTAA**G**C C**G**ACC**G**ACTC**G** G**C**TGG**C**TGAG**C**

A relatively new approach for analysis of DNA methylation is methylation-sensitve high resolution melting (MS-HRM). This method is based on the comparison of melting profiles of PCR products from sodium bisulfite treated genomic DNA. The base composition of sodium bisulfite treated templates is methylation dependent, thus methylated and unmethylated PCR products show different melting profiles after thermal denaturation (Wojdacz et al. 2008) (shown in *Figure 14*). While CG base pairs are bound by three H-bonds, AT base pairs are bound to each other with only two H-bonds. Therefore, CG base pairs melt at higher temperatures if subjected to thermal denaturation. The provided fluorescent dye EvaGreen actively binds to dsDNA during amplification and fluoresces as long as it is bound to dsDNA. Upon melting of dsDNA at the beginning of the HRM analysis it is released and the fluorescence declines. Changes in the fluorescence intensity were measured and plotted as a melting curve. Specific melting profiles provide information about the initial methylation level of the gene of interest. By using MS-HRM, rapid screening for methylation differences between samples at certain loci is possible and several genes were already found to be tumor-specifically methylated in NSCLC patients using MS-HRM analysis (Heller et al. 2012, Heller et al. 2012).

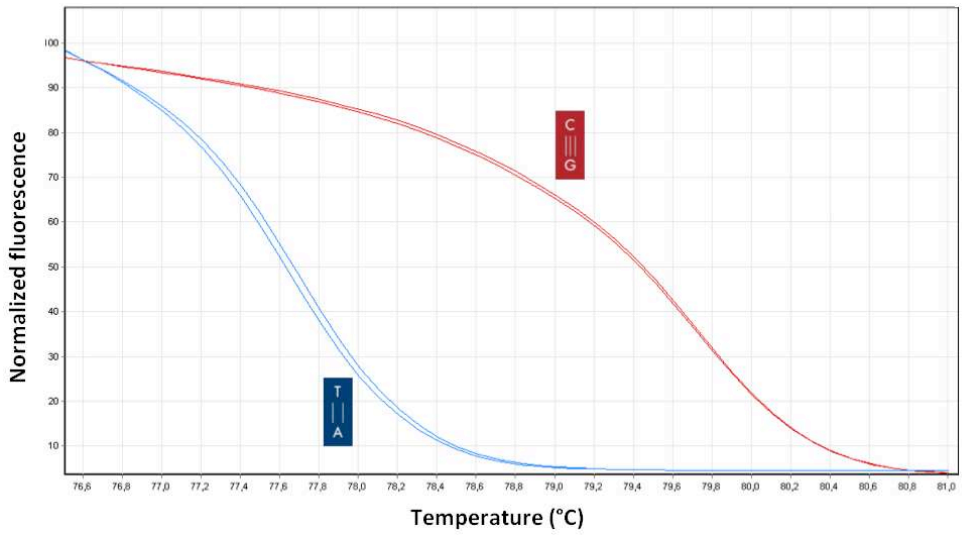

***Figure 14**. Melting curve profile of methylated sample compared to unmethylated sample. Provided fluorescent dye actively binds to dsDNA during amplification, changes in fluorescence are measured and plotted as a melting curve. Upon melting the fluorescence dye is released and fluorescence declines. Sequences with higher CG amount show a higher melting temperature than sequences with more AT base pairs. Differences in the melting profile provide information about the initial methylation status of the region of interest (Wojdacz et al. 2008).*

In recent years, interest increased to investigate not only methylation of single genes, but to determine methylation of the whole genome. The first described method for high throughput DNA methylation analysis was "Restriction landmark genomic sequencing" (RLGS) which is

based on radioactive labeling of methylation-specific cleavage sites and separation of fragments by two-dimensional electrophoresis (Costello et al. 2000). Other methods for profiling genome-wide methylation patterns are "Amplification of intermethylated sites" (AIMS), "Differential methylation hybridization" (DMH) or the analysis of expression microarrays of cells before and after treatment with DNMT inhibitors and/or HDAC inhibitors (pharmacological unmasking) (Esteller 2007, Heller et al. 2010). Weber *et al*. (Weber et al. 2005) established a technique that combines methylated DNA immunoprecipitation (MeDIP) and microarray analysis (MeDIP-chip). Methylated DNA fragments are isolated by immunoprecipitation using an anti-5-methylcytidine antibody and are subsequently hybridized to a microarray (Esteller 2007). The MeDIP procedure requires several steps, which are illustrated in *Figure 15*. To achieve efficient immunoprecipitation, genomic DNA is first randomly fragmented by sonication. Shearing efficiency should be verified by separation on an agarose gel and a smear between 200 and 1000bp is considered as "perfect sonication". To increase the affinity of the anti-5-methylcytidine antibody, sonicated DNA must be denatured. Enrichment of methylated DNA is performed by adding the antibody followed by overnight incubation. Immnuoprecipitated DNA is captured using either magnetic beads or salmon sperm DNA blocked agarose beads followed by standard phenol-chloroform extraction. As the amount of immunoprecipitated DNA from a single MeDIP reaction is low (~5-10 ng), samples should be amplified using PCR-based systems whose performance is not impaired by small fragments (e.g. whole genome amplification). To estimate the efficiency and specificity of the MeDIP procedure, immunoprecipitated DNA samples (IP) and input DNA samples should be subjected to quality assessment by qPCR using primer pairs detecting a known methylated (e.g. *REXO1L1*) and a known unmethylated (e.g. *GAPDH*) genomic region. In combination with high-throughput sequencing or microarray hybridization, MeDIP allows large-scale methylation analysis. To date, several different microarrays covering either the whole genome or only gene promoter regions or even different gene clusters at high resolution are commercially available. Most commonly used microarrays, especially for epigenomic mapping in cancer, are CGI or promoter regions arrays (Weber et al. 2005, Keshet et al. 2006, Pälmke et al. 2011). Moreover, custom designed microarrays for methylation detection of genomic regions of interest are available.

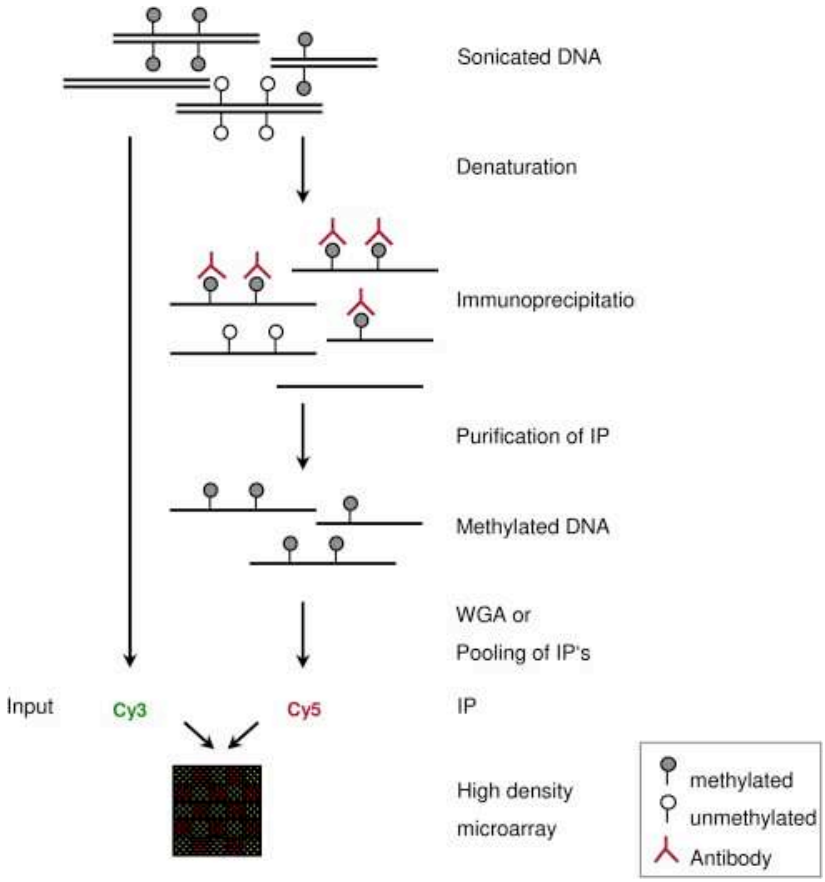

***Figure 15**. Principle of MeDIP-chip method.*
Genomic DNA is first randomly fragmented by sonication, heat-denatured and incubated with an anti-5mC antibody. Bound methylated DNA is then amplified using whole genome amplification. Methylated IP fraction and Input DNA are differentially labeled and co-hybridized on the same microarray to compare fold-enrichment. Adapted from Pälmke et al. 2011.

By using MeDIP for the first time, Weber *et al*. (Weber et al. 2005) confirmed that the inactive X-chromosome is hypermethylated at CGIs on a chromosome-wide level. Furthermore, it was shown that alterations in the DNA methylation pattern at CGIs of cancer cells primarily involve hypermethylation rather than hypomethyltion (Heller et al. 2012, Jacinto et al. 2008, Weber et al. 2005).

3.2.8. DNA methylation in NSCLCs

The epigenetic landscape of a cancer cell is profoundly deregulated. Alterations in DNA methylation contribute to tumorigenesis by silencing TSGs which regulate cell proliferation, DNA repair, apoptosis and other homeostatic functions and are now being recognized as a

hallmark of cancer. The aberrant epigenetic landscape of a cancer cell is characterized by global genomic hypomethylation, CGI promoter methylation and a specific histone modification pattern as well as deregulated expression profiles of chromatin modifying enzymes (Esteller 2007, Berdasco et al. 2010, Hatziapostolou et al. 2011, Sandoval et al. 2012).

In a normal cell, DNA methylation of non-CGI CpG dinucleotides prevents genomic instability by repressing parasitic DNA sequences and by silencing of transposable elements and non-coding DNA (Suzuki et al. 2008). Loss of DNA methylation at CpG dinucelotides was the first epigenetic alteration to be identified that distinguishes cancer cells from normal cells (Feinberg et al. 1983). DNA hypomethylation can contribute to tumorigenesis by different mechanisms; first, it causes genomic instability. Hypomethylation of repetitive DNA elements such as short and long interspersed nuclear elements (SINEs and LINEs) can cause deletions and translocations and can also promote chromosomal translocations (Eden et al. 2003, Esteller 2008). Hypomethylated retrotransposons might be activated and their translocation to other genomic regions further disrupts the genome (Hatziapostolou et al. 2011). A frequently occurring event in various cancer types including lung cancer is the hypomethylation of L1 retrotransposon (Wilson et al. 2007). Furthermore, demethylation of promoter regions leads to expression of previously silenced oncogenes in cancer cells (Feinberg et al. 2004). Aberrant hypomethylation followed by activated expression has also been suggested for the oncogenic *let-7a-3* miRNA gene in endometrial and colon cancer (Brueckner et al. 2007). Another mechanism by which loss of methyl groups contributes to tumorigenesis is loss of imprinting (LOH) (Berdasco et al. 2010, Hatziapostolou et al. 2011). LOH of the autocrine growth factor IGF2 increases susceptibility to colorectal cancer (Cui et al. 2003), Wilm`s tumor (Bjornsson et al. 2007) and is associated with the progression of lung cancer (Vu et al. 2010).

In contrast to hypomethylation, hypermethylation of CGIs in gene promoter regions (referred to as CGI promoter methylation) is associated with aberrant silencing of gene transcription and contributes to inactivation of TSGs in cancer cells (Ballestar et al. 2008, Esteller 2008, Baylin et al. 2011). Genes that acquire aberrant methylation in their promoter sequences are involved in various important cellular pathways. For example cell cycle-related genes, $p16^{INK4A}$ and $p15^{INK4A}$ are aberrantly silenced by methylation in many cancer types (Kulis et al. 2010). Genes associated with DNA repair mechanisms undergo methylation in cancer cells as well suggesting that epigenetic alterations may promote genetic alterations including mutations, which is the case for *MGMT* (Esteller et al. 2000). Defective DNA mismatch repair in

cancer cells is associated with methylation of *MLH1* and methylation of *BRCA1* affects DNA repair of double strand breaks in breast and ovarian cancer (Catteau et al. 2002, Fleisher et al. 2001). Genes involved in cell adhesion, for example, *CDH1* and *CDH13*, are also prone to methylation leading to invasion and/or metastasis (Kim et al. 2005). Other frequently methylated genes in cancer cells are connected with survival and apoptosis including *DAPK1* and *TMS1* (Gordian et al. 2009, Michie et al. 2010). In addition, methylation affects genes involved in signaling pathways (e.g. *RARB2* and *CRBP1*) and transcription factors including *GATA4* and *GATA5* (Esteller et al. 2002, Akiyama et al. 2003, Kulis et al. 2010). *Table 2* provides an overview about frequently methylated genes in NSCLC.

***Table 2**. Frequently methylated genes and their function in NSCLC*

Gene	Function
$P16^{INK4A}$	Cell cycle regulation
RASSF1A	Cell cycle regulation
FHIT	Cell cycle regulation, apoptosis
DAPK	Regulation of apoptosis
TIMP-3	Regulation of apoptosis
RUNX3	Regulation of apoptosis
MGMT	DNA repair
hMLH1	DNA repair
RARß	Cell growth, cell differentiation
APC	Cell adhesion, cell migration
ECAD	Cell-cell adhesion
DAL-1	Cell-cell adhesion
TSLC1	Cell-cell adhesion

(Heller et al. 2010, Cooper 2005, Kikuchi et al. 2005, Zöchbauer-Müller et al. 2002, Zöchbauer-Müller et al. 2001, Zöchbauer-Müller et al. 2005)

By methylation-specific PCR, Zöchbauer-Müller *et al.* (Zöchbauer-Müller et al. 2001) determined methylation of multiple genes in 107 resected primary NSCLC samples and 104 corresponding normal lung tissue samples. Tumor-specific methylation was detected for *RARβ, TIMP-3, $p16^{INK4A}$, MGMT, DAPK, ECAD, $p14^{ARF}$*, and for *GSTP1*. Subsequent studies revealed tumor-specific methylation of multiple other TSGs including *DAL-1, FHIT, TSLC1* and *RASSF1A* in NSCLCs as shown in *Figure 16* (Burbee et al. 2001, Zöchbauer-Müller et al. 2001, Zöchbauer-Müller et al. 2005, Heller et al. 2006).

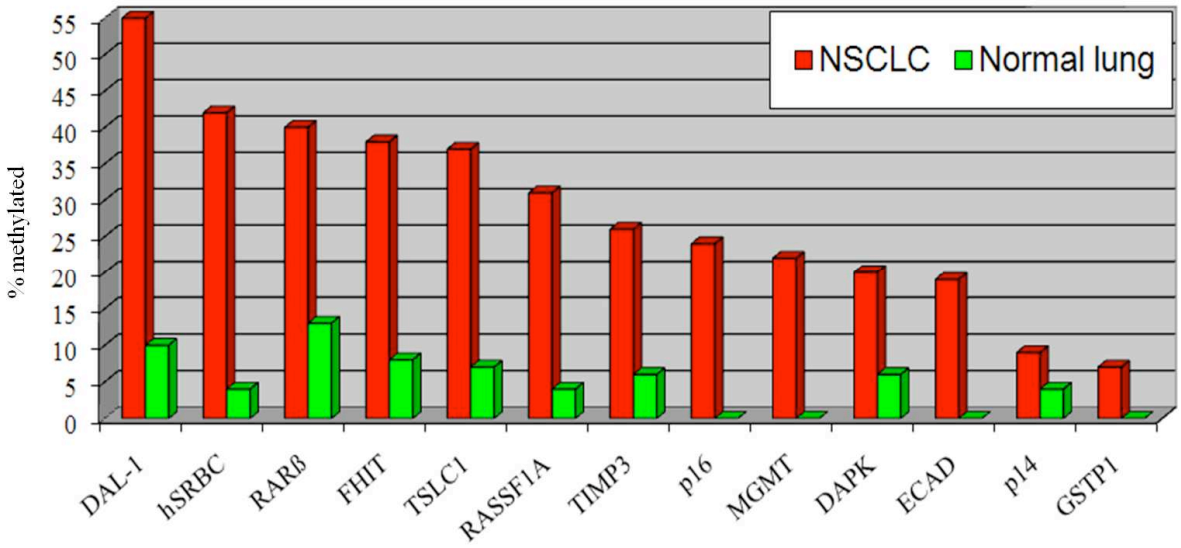

Figure 16. *Tumor-specific methylation of TSGs in NSCLC.*
Methylation of specific TSGs is represented as percentages of methylation in NSCLC samples compared to non-malignant lung tissue samples. Data were obtained by methylation specific PCR (MSP).
Adapted from Burbee et al. 2001, Zöchbauer-Müller et al. 2001, Zöchbauer-Müller et al. 2001, Zöchbauer-Müller et al. 2005, Heller et al. 2006.

Genome-wide screens for CGI promoter methylation identified a large number of targets for methylation in cancer. Given 29 000 CGIs in the human genome, 1400 CGIs are thought to be aberrantly methylated in the lung cancer genome (Risch et al. 2008). Using MeDIP-chip technique, Heller *et al*. (Heller et al. 2012) performed a genome-wide search for methylated CGIs in 101 stage I-III NSCLC patients. Of 2414 genomic positions which were identified to be differentially methylated in tumors and corresponding non-malignant lung tissue samples, 97% were found to be tumor-specifically methylated. A subsequent annotation of these genomic regions resulted in the identification of 477 tumor-specifically methylated genes. Many of them were found to be involved in regulation of gene transcription and in cell adhesion. Moreover, methylation of certain genes was associated with loss of protein expression and treatment of NSCLC cell lines with epigenetically active drugs resulted in upregulated expression of many tumor specifically methylated genes.

Several studies suggested that detection of methylated genes might serve as a powerful biomarker for prognosis of lung cancer patients and for disease recurrence as methylation cannot only be detected in primary tumors but also in blood samples, sputum and in bronchoalveolar lavage (BAL) samples (Toyooka et al. 2004, Shames et al. 2006, Heller et al. 2010). Furthermore, methylation of certain genes is strongly associated with lung cancer risk and can already be detected in samples of cancer-free smokers. By comparing methylation

results with clinico-pathological data of NSCLC patients, Heller *et al*. (Heller et al. 2012) identified methylation of *HOXA2* and *HOXA10* as prognostic parameters in SCC patients. Other frequently methylated genes in NSCLC with a potential unfavorable prognostic relevance include *APC, CDH1, DAPK, RASSF1A* and *p16* (Burbee et al. 2001, Heller et al. 2010, Tang et al. 2000, Toyooka et al. 2004). Thus, a panel of methylated genes may serve as prognostic tool for early detection of lung cancer and risk assessment (Belinsky et al. 2002).

In contrast to DNA methylation, less is known about tumor-specific histone modification patterns, however, it was shown that CGI promoter methylation of TSGs is associated with particular histone marks: deacetylation of histones H3 and H4, loss of H3K5 trimethylation and gain of H3K9 methylation and H3K27 trimethylation. These patterns are the opposite of that observed in normal cells expressing crucial TSGs (Jones et al. 2007, Berdasco et al. 2010).

3.2.9. Epigenetic changes are reversible

Whereas transcriptional silencing of tissue-specific expressed or X-linked genes by promoter methylation is part of normal development, aberrant methylation of promoter sequences is a frequent mechanism in the initiation and progression of various cancers (Belinsky et al. 2003, Baylin 2005). The fact that re-expression of previously silenced cancer-related genes may have profound anti-tumor effects promoted the development of a variety of epigenetically active drugs. Many agents with effects on DNA methylation patterns and on histone modifications were discovered and are being tested in clinical trials (Egger et al. 2004).

Initially, the prototypes of DNA methylation inhibitors, 5-azacytidine (5-aza-CR) and 5-aza-2´-deoxycytidine (Aza-dC), were synthesized in the 1960s as nucleoside antimetabolites with activity against hematopoietic neoplasms (Christman 2002, Herman et al. 2003). Only later it was discovered by Taylor and Jones (Jones et al. 1980) that the nucleoside analogs 5-aza-CR and Aza-dC had inhibitory effects on DNA methylation of newly synthesized strands and induced cell differentiation in cultured mouse embryo cells.

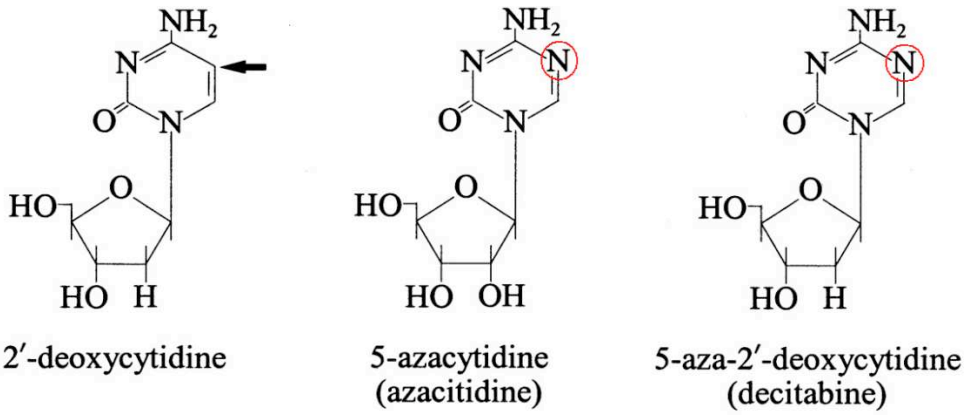

***Figure 17**. DNA methyltransferase inhibitors compared to the natural nucleoside deoxycytidine.*
Black arrow indicated carbon 5 of the the pyrimidine ring, which is replaced by nitrogen in the analogues azacitidine and decitabine. This modification inhibits active enzyme DNMT. Adapted from Goffin et al. 2002.

The uptake of nucleoside analogues into cells is mediated by nucleoside transporter proteins (hENT and hCNT), members of the proton oligopeptide cotransporter family (SLC15) and the SLC22 family of organic cation and anion transporters (Rius et al. 2012). After being reduced to deoxynucleoside triphosphates in S-phase cells, the analogues are incorporated into DNA instead of cytosines. Once incorporated, the modified bases covalently bind to all members of DNMTs which results in the depletion of active enzymes and subsequent demethylation of DNA after repeated replication (shown in *Figure 18*). Replication in the absence of DNMTs results in progressive hypomethylation and re-expression of previously silenced genes (Juttermann et al. 1994, Christman 2002, Goffin et al. 2002, Egger et al. 2004, Issa et al. 2005).

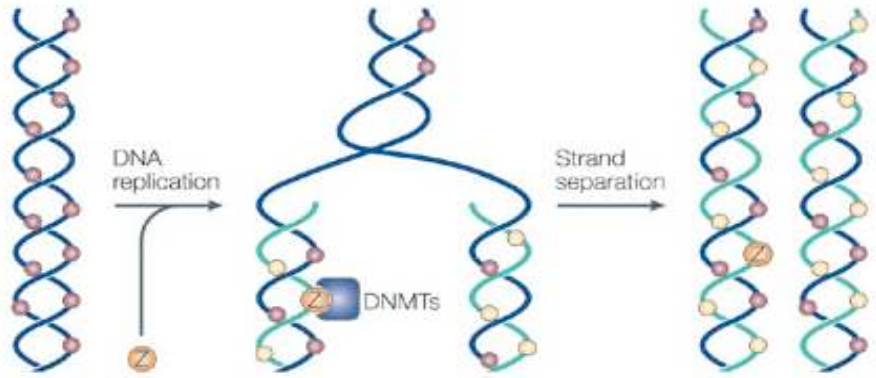

***Figure 18**. Mechanism of DNA methyltransferase inhibitors.*
Pink circles indicate methylated CpGs, yellow circles unmethylated CpGs; deoxy nucleoside analogues depicted by Z. After being converted into triphosphates, deoxynucleoside analogues are incorporated into DNA in S-phase cells. DNMTs are "trapped" on DNA containing modified bases and cells divide in the absence of DNMTs. Depletion of DNMTs results in decrease of DNA methylation after replication and reactivation of transcriptional silencing. Adapted from Issa et al. 2005.

Incorporation of deoxycytidine analogues into DNA and covalent attachment of DNMTs to DNA may also cause the major problems of epigenetically active drugs, the systemic toxic effects and the aberrant reactivation of transposable elements (Egger et al. 2004). Cytotoxicity exerted by these agents is observed especially at high doses (Michalowsky et al. 1987, Jackson-Grusby et al. 1997). However, administration of lower doses was shown to reduce the cytotoxic effects in clinical trials for the treatment of myelodysplastic syndrome (MDS) (Kornblith et al. 2002, Silverman et al. 2002).

The dynamic interaction of DNA methylation and histone deacetylation to ensure persistent gene activation encouraged the concept to target multiple components of the mechanism. Cameron *et al*. (Cameron et al. 1999) showed that administration of HDAC inhibitor Trichostatin A (TSA) reversed the formation of transcriptionally repressive chromatin on methylated promoter templates in vitro. However, hypermethylated genes cannot be reactivated by treatment with HDAC inhibitors when they are used alone in tumor cells but they do exert additive or synergistic effects in the presence of low doses Aza-dC.

Expression of various proto-oncogenes, including *MAF* and *FGFR3*, which are frequently overexpressed in multiple myeloma cell lines, was found to be down-regulated in response to TSA and Aza-dC/TSA treatment in three cell lines (Heller et al. 2008). In 2004, the methyltransferase inhibitor Vidaza™ (5-azacytidine) received approval by the U.S. Food and Drug Administration (FDA) for the treatment of MDS (Kaminskas et al. 2005). Furthermore, the HDAC inhibitors

SAHA and romidepsin were approved for the treatment of cutaneous T-cell lymphomas (Rius et al. 2012). The effects of DNA methyltransferase and HDAC inhibitors in the treatment of NSCLC patients are now being assessed in clinical trials.

3.3. The RNA machine

Although coding exons of protein-coding genes account only for 1.5% of the human genome, research focuses on these protein-coding genes for many years (Esteller 2011). Over the last years, it has become evident that the non-protein-coding part of the genome contains not only “junk DNA”, but is crucial for regulation of many biological processes. The Encyclopedia of DNA Elements (ENCODE) project, a decade long project to identify all functional elements in the human genome, revealed that “80% of the human genome serves some purpose, biochemically speaking” (Pennisi 2012). A large part of the genome is transcribed in a developmental- and tissue-regulating manner, producing a large number of long and short

non-coding RNAs (ncRNAs) (Taft et al. 2010, Mattick 2011). These non-coding RNAs include small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), large intergenic non-coding RNAs (lncRNAs), transcribed ultrconserved regions (T-UCRs), small interfering RNAs (siRNAs) and a class of small non-coding RNAs, the microRNAs (miRNA) (Mattick 2001, Esteller 2011). All of these ncRNAs fulfill critical roles in regulating gene expression on a transcriptional or post-transcriptional level and provide essential mechanisms for normal development (Taft et al. 2010). Thus, disruption or deregulation of ncRNAs may contribute to development of several diseases including cancer.

Now there is evidence that non-coding RNAs play also a crucial role in the control of epigenetic regulation, of dynamic changes to chromatin and nuclear architecture and of mechanisms for normal cellular differentiation and development. These mechanisms include dosage compensation to keep the balance between sex chromosomes via lincRNA Xist, imprinting of genes (which is frequently regulated by ncRNAs such as AIR and HOTAIR) and transcriptional gene silencing by RNA interference (RNAi) (Navarro et al. 2010, Nagano et al. 2008, Bernstein et al. 2005, Amaral et al. 2008, Mattick et al. 2009).

3.4. MicroRNAs (miRNAs)

The first miRNA to be identified was a 21-nucleotide RNA molecule in *C. elegans,* called *lin-4*. It was discovered accidently, when Lee and his colleagues studied stages of development in *C. elegans* and realized that mutations in this molecule lead to severe developmental defects. This miRNA was shown to regulate the expression of its target gene *lin-14* by base-pairing to its 3´-untranslated region (3´-UTR) (Lee et al. 1993). Since then a large number of miRNAs was identified in different organisms and their functions were studied extensively. To date, 2019 unique mature human miRNAs are annotated in the latest version of the miRNA database miRBase 19.

MiRNAs are single-stranded RNA molecules, 18-24 nucleotides in length, which regulate gene expression on a post-transcriptional level by targeting their complementary messenger RNA (Ambros 2004, Mendell 2005). MiRNAs are estimated to control the translation of mRNA of more than 60% of protein-coding genes. Through regulation of specific target genes, they regulate a variety of biological processes including proliferation, cellular differentiation, apoptosis and organismic development (Bartel 2004, He et al. 2004, Esteller 2011).

3.4.1. Genetics, biogenesis and mechanism of action

Except from the Y chromosome, miRNA genes can be found all over the human genome (Kim et al. 2006). However, most miRNA genes are clustered in the genome (Lagos-Quintana et al. 2002) and are transcribed as multi-cistronic primary transcripts (Lee et al. 2002). To identify the modes of transcription, Rodriguez *et al*. (Rodriguez et al. 2004) annotated the genomic positions of mammalian miRNA genes in the human and mouse genomes.

Basically, miRNA genes can be categorized by their genomic location: intronic miRNA genes in sense orientation in either protein-coding transcription units or in non-coding transcription units, exonic miRNA genes in non-coding transcription units (shown in *Figure 19*) and intergenic miRNAs genes. It was shown that the majority (70%) of 232 analyzed miRNA genes are located within defined transcription units of either protein-coding or non-coding transcription units and not as initially expected in intergenic regions (Lagos-Quintana et al. 2001, Lau et al. 2001). MiRNA genes in intergeneic regions, located far away from known protein-coding genes, are independent transcription units with specific core promoter elements (Kim et al. 2009). Intronic miRNA genes are transcribed either in parallel with their host gene or independently, but as they share the same promoter, expression profiles of miRNAs and the host transcript are usually very similar (Bartel 2004, Baskerville et al. 2005). Clustered miRNAs are transcribed as polycistronic units in single transcription units or, depending on splicing patterns of host genes, overlapped in the host transcripts in either introns or exons (Melo et al. 2011).

MiRNAs of similar sequences are distinguished by an additional letter e.g. *miR-193**b***. If a miRNA of identical mature sequence appears at several genomic loci with different precursor sequences, the different miRNA genes are distinguished by an additional number eg. *miR-9-**3*** (Lee et al. 2009).

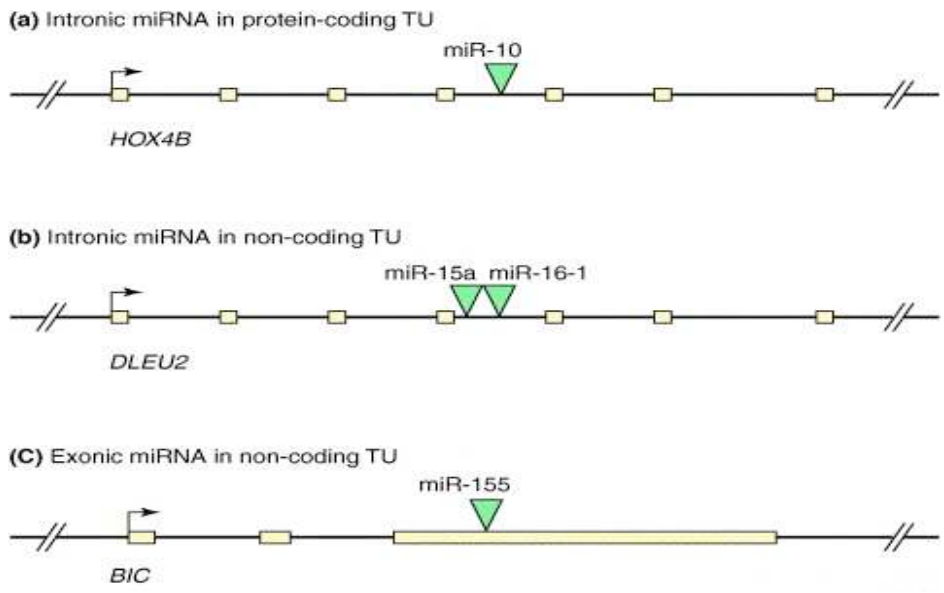

Figure 19. *Genomic positions of miRNA genes.*
Green triangles indicate the location of miRNA stem-loop structure and exons are indicated as yellow boxes.TU, transcription untis.
A: As example of an intronic miRNA gene in a protein-coding TU, miR-10 in HOX4B gene is shown.
B: MiR-15a~16-1 cluster is localized in the fourth intron of a previously defined non-coding RNA gene.
C: MiR-155is shown as example for the structure of an exonic miRNA gene in a non-coding transcript.
Adapted from Kim et al. 2006.

Clustered miRNAs from a single transcription unit are not all expressed at equal levels suggesting that miRNAs may also be regulated on a post-transcriptional level (Pillai 2005, Lee et al. 2009). Many mammalian miRNAs are expressed in a tissue- and/or developmental specific manner, which was attributed to transcriptional regulation until recently (Obernosterer et al. 2006). The heart-specific expression of *miR-1* for example is controlled by Pol II-associated transcription factors including MyoD and Mef2 (Zhao et al. 2005). Obernosterer *et al*. (Obernosterer et al. 2006) showed that miRNA expression is also regulated post-transcriptionally by different processing of precursor-miRNAs. They observed that e.g. *miR-138* is restricted to certain cell types, while its precursor, *miR-138-2* is ubiquitously expressed.

Furthermore, it was observed that a large number of miRNA genes are associated with CGIs suggesting that miRNA expression is regulated by DNA methylation of CGI associated promoters (Chuang et al. 2007). Analysis of several miRNA genes associated with CGIs revealed that miRNA gene methylation frequently occurs in both normal cells to ensure tissue-specific expression and aberrantly in malignant cells (Visone et al. 2009, Vrba et al. 2011). Recently, it was shown that miRNAs are not only targets for DNA methylation but also regu-

late epigenetic modifications. Fabbri *et al*. (Fabbri et al. 2007) showed that expression of *miR-29* is inversely correlated to DNMT3A and DNMT3B expression in lung cancer tissues and that *miR-29* regulates both DNMTs.

Biogenesis of miRNAs starts with the transcription of miRNA genes from different genomic regions, generally mediated by RNA polymerase II (Pol II) (Cai et al. 2004, Lee et al. 2004). However, some data indicate that a minor subset of miRNA genes may be transcribed by Pol III instead of Pol II (Borchert et al. 2006). Like other class II gene transcripts, the generated primary transcripts (pri-miRNAs) contain 7-methylguanosin caps as well as poly-(A) tails. Within these long precursor transcripts, the mature miRNA sequence can be found in a 60-80 nucleotide stem-loop structure (Mendell 2005). A microprocessor complex which harbors the RNase III enzyme Drosha and its partner DGCR8, also known as Pasha in *Drosophila* and *C. elegans*, recognizes the pri-miRNA in the nucleus and excises the hairpin structure to release the precursor of the miRNA (pre-miRNA) (Denli et al. 2004, Kim et al. 2006). For the next processing step, the pre-miRNA must be exported to the cytoplasm. Export is mediated by the RanGTP-dependent nuclear transport receptor Exportin-5 (Exp5) (Bohnsack et al. 2004, Lund et al. 2004). To manage the export through the nuclear pore, Exp5 binds cooperatively to its cargo, the pre-miRNA, and the cofactor Ran. By hydrolysis of GTP, the cargo is released into the cytoplasm (Yi et al. 2003, Melo et al. 2011). In the cytoplasm, the pre-miRNA is further maturated by another RNase III enzyme called Dicer. Dicer cuts off the loop motif of the stem-loop structure generating a double-stranded 18-24 nucleotide RNA molecule (Saito et al. 2005, Cowland et al. 2007). A helicase probably unwinds the double stranded RNA molecule and the miRNA strand is incorporated by a large protein complex known as the RNA-induced silencing complex, RISC, while the other strand is believed to be degraded. The result is a miRNA ribonucleoprotein (miRNP) complex which contains the argonaute protein, Ago, as a catalytic endonuclease component and a single-stranded miRNA which directs the sequence-specific binding to its target mRNA (Cowland et al. 2007, Mendell et al. 2012).

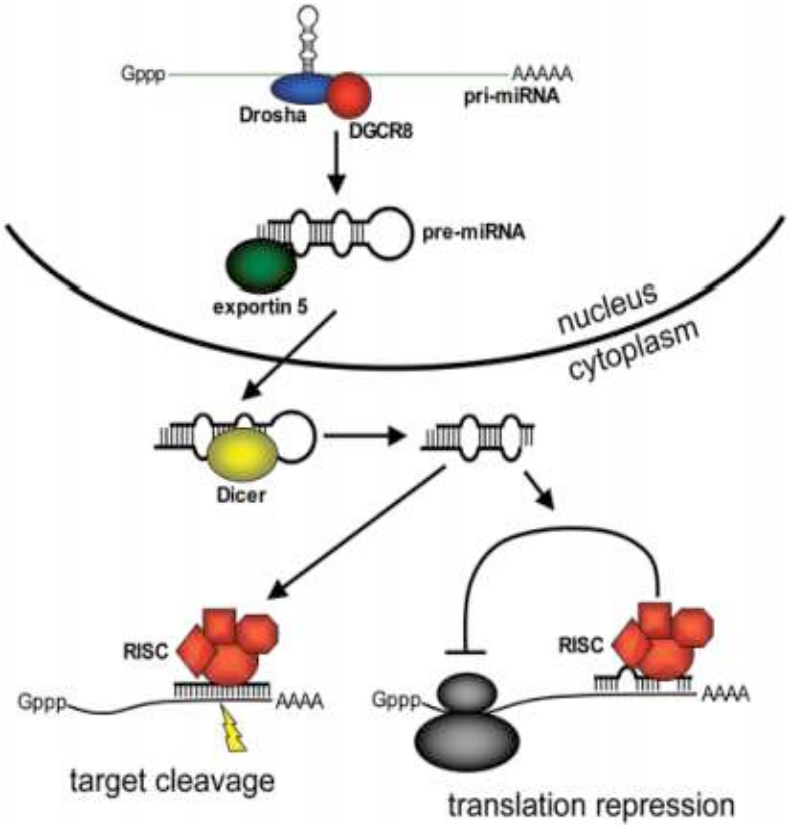

Figure 20. *MiRNA biogenesis.*
The primary transcripts are initially cleaved by Drosha and its cofactor DGCR8 to release the pre-miRNA which is transported to the cytoplasm by Exp5. Dicer generates an 18-24 nt RNA duplex, one of which is loaded onto RISC and guides mRNA silencing. Adapted from Mendell 2005.

MiRNAs are sequence-specific regulators of post-transcriptional gene expression. The precise mechanism how miRNAs recognize their binding sites of target mRNAs is not completely understood yet, however, it is suggested that target recognition mostly happens through interaction with the 3´-UTR of the transcript (Cowland et al. 2007, Filipowicz et al. 2008). Sequence comparison of miRNAs and their mRNA target sequences revealed that complementarity is usually restricted to the "seed region" which is located at nucleotides 2-8 at the 5´end of the miRNA. The 3´end region of the miRNA shows partial complementarity to the mRNA and contributes partly to formation of the miRNA-mRNA complex. Binding of the 3´ end region is required to stabilize the complex, if the seed region does not show complete complementarity (Brennecke et al. 2005, Tomari et al. 2005). If binding of a single miRNA is not enough to cause an effect, miRNAs which bind to the same target mRNA have to work in concert. Computational analysis of these binding requirements revealed that a single miRNA is able to target about 100 different mRNAs (Lewis et al. 2005, Cowland et al. 2007).

MiRNAs repress translation of mRNAs via two distinct pathways: mRNA cleavage or translational repression. The choice between these two mechanisms is determined by complementarity of the miRNA and the target mRNA. If complementarity is high the mRNA is cleaved in the miRNP complex. Low complementarity leads to repression of translation initiation (Bartel 2004). Most human miRNAs regulate gene expression mainly via translational repression. This mechanism exerts its effect on three different levels: repression of the initiation step of translation, repression of the elongation phase and degradation of transcripts through deadenylation and decapping (Cowland et al. 2007). MiRNA-silenced mRNAs are thought to accumulate in RNA processing bodies, so called P-bodies, where they are subjected to degradation by enzymes involved in deadenylation, decapping and degradation (Liu et al. 2005, Lee et al. 2009).

Whereas some miRNAs regulate expression of specific targets, other miRNAs regulate gene expression of hundred genes simultaneously. Thus, it is obvious that any alterations of miRNA expression, processing of miRNA precursors or mutations in either miRNA or mRNA sequences may have tremendous effects on normal cell physiology and may contribute to the development of malignant phenotypes (Cowland et al. 2007, Visone et al. 2009, Heller et al. 2012)

3.4.2. Oncogenic and tumor suppressive miRNAs

As discussed in detail in section 3.4.1., miRNAs regulate various biological processes. By regulating mRNA translation, some miRNAs were shown to promote cell proliferation and survival, while others diminish it. Compared to normal tissues, a general down-regulation of miRNAs can be observed in various cancers, including lung cancer (Lu et al. 2005). However, up-regulation of certain miRNAs is also a frequently occurring event in tumorigenesis and/or malignant progression. The different functions of miRNAs in cancer seem to be disrupted by the same mechanisms that disrupt expression of protein-coding genes including epigenetic alterations. However, miRNA genes are not only affected by epigenetic changes such as methylation of their CGI promoter region and accompanying histone modifications, components of the epigenetic machinery can be subjected to regulation by miRNAs as well (Sandoval et al. 2012). So called “epi-miRs” were shown to target DNMTs, HDACs and PRC genes (Valeri et al. 2009). Fabbri *et al*. (Fabbri et al. 2007) demonstrated that the *miR-29* family directly targets DNMT3A and DNMT3B in lung cancer cell lines.

Re-expression of *miR-29s* lead to global hypomethylation of cancer cells and previously silenced TSGs such as *FHIT* were re-expressed upon *miR-29s* treatment. Varambally *et al*. (Varambally et al. 2008) discovered a decreased expression of *miR-101* in prostate cancer cell lines and primary tumors, inversely correlating with an increase of *EZH2*.

MiRNAs that inhibit the translation of proto-oncogenes by targeting their 3´-ends are considered as "tumor suppressor miRNAs" (TS-miRs) and miRNAs enabling the downregulation of TSGs as "oncoMiRs" (Cowland et al. 2007). Disrupted miRNA expression and its contributions to tumorigenesis were first described when *miR-15* and *miR-16* coding sequences were linked to a region of deletion at chromosome 13q14. Expression and deletion analysis showed that both miRNA genes are located within a 30kb region of loss in chronic lymphocytic leukemia (CLL) and are deleted or down-regulated in 68% of CLL patients. *MiR-15* and *miR-16* were shown to act as TS-miRs by targetting the mRNA of anti-apoptotic *BCL-2*, which is frequently overexpressed in CLL and lung cancer (Calin et al. 2002, Ventura et al. 2009). Another major finding was the implication of miRNA *let-7* in lung cancer development. Johnson *et al*. (Johnson et al. 2005) revealed reduced levels of *let-7* and increased levels of *KRAS* oncogene in lung tissue samples compared to normal tissue, suggesting that *let-7* directly regulates Ras expression. Furthermore, reduced *let-7* expression was shown to be correlated with a shortened post-operative survival of lung cancer patients who underwent potentially curative resections (Takamizawa et al. 2004).

In contrast to TS-miRs, miRNAs with proliferative and anti-apoptotic activity are frequently up-regulated in cancers. The *miR-17-92* cluster is composed of six miRNAs including *miR-17, - 18a, -19a, -20a*, *-19b*-1 and *-92a-1* with the first miRNAs identified to be oncogenic (Lee et al. 2009). The cluster is located at chromosome 13q31.3 which is frequently amplified in various types of cancer. Forced expression of the *miR-17-92* cluster in a transgenic mouse model which overexpresses c-myc accelerated the development of B-cell lymphoma (He et al. 2005). Consistent to its oncogenic function the *miR-17-92* cluster was also found to be up-regulated in lung cancer (Hayashita et al. 2005). By producing a transgenic mouse model that overexpresses *miR-155* in B-cells, Costinean *et al*. (Costinean et al. 2006) demonstrated the contribution of *miR-155* to tumorigenesis.

MiRNAs can also contribute to tumorigenesis by affecting properties of the cancer cell, either in an oncogenic or tumor suppressive manner. *MiR-221* and *miR-222* were reported to target $p27^{kip1}$ $p57^{kip2}$ proteins, respectively, two important cell cycle regulators that bind to

CdK/cyclin complexes and prevent transition from G1 to S phase (Visone et al. 2007, Visone et al. 2009). Progression, invasion and metastasis were linked to miRNA regulation as well. *MiR-21* regulates the TSG *TPM1* which is involved in cell migration and *miR-10b* targets *HOXD10* resulting in higher levels of *RHOC* and cancer cell motility (Ma et al. 2007, Zhu et al. 2008). Other miRNAs contributing to tumorigenesis include miRNA *let-7*, *miR-9*, *miR-125a*, *miR-125b*, *miR-17-5p*, *miR-17-92* cluster, *miR-27a*, *miR-29a, b, c*, *miR-143* and *miR-145* (Takamizawa et al. 2004, Hayashita et al. 2005, Fabbri et al. 2007, Laneve et al. 2007, Matsubara et al. 2007, Mertens-Talcott et al. 2007).

3.4.3. Deregulation of miRNA expression in lung cancer

By performing high-throughput miRNA expression analysis, Yanaihara *et al*. (Yanaihara et al. 2006) identified 43 miRNAs to be differentially expressed in 104 primary NSCLC samples versus normal lung tissue samples. Up-regulated miRNAs include *miR-21, miR-191, miR-210, miR-155, miR-105, miR-212, miR-17-3p, miR-197, miR-192, miR-146* and *miR-203*. Examples of down-regulated miRNAs are *miR-126, miR-143, miR-30a-5p, miR-9*, *miR-124a-1, miR-95, miR-145miR-196b* and *miR-125a*. Furthermore, a decrease in expression of certain *let-7* family members and an increased expression of *miR-155* was linked with poor survival of NSCLC patients (Takamizawa et al. 2004).

Recently, by comparing micro array data of NSCLC A549 cells, Heller *et al*. (Heller et al. 2012) identified 33 miRNAs whose expression was upregulated after treatment with epigenetically active drugs and which are associated with a CGI. The vast majority of these miRNAs were found to be methylated in at least one of eleven NSCLC cell lines suggesting that methylation is an important mechanism for inactivation of certain miRNAs in NSCLC. Furthermore, *miR-9-3* and *miR-193a* are tumor-specifically methylated in NSCLC patients and *miR-9-3* methylation is of prognostic relevance in SCC patients. These results showed the importance of methylation for aberrant miRNA silencing in the pathogenesis of NSCLCs.

3.4.3.1. Mechanisms for deregulation of miRNA expression in lung cancer

MiRNA expression can be altered by different mechanisms in human cancer cells, including transcriptional regulation, genomic abnormalities, defects in the miRNA processing machinery and epigenetic modifications. Transcription factors can also induce miRNA expression by activating the transcription of pri-miRs. As transcription factors play crucial roles in fundamental cellular processes, they act as either TSGs or oncogenes. Major transcription factors regulating miRNA expression are p53, c-myc and E2F (Mendell 2005, Visone et al. 2009, Davalos et al. 2010).

I. Transcriptional regulation

Some studies put their focus on how miRNAs are regulated by TSGs, especially on miRNAs regulated by p53. Ventura *et al*. (Ventura et al. 2009) reported that the *miR-34* family is a direct target of p53. The *miR-34* family consists of *miR-34a* located on chromosome 1p36 and of the *miR-34b-34c* cluster located on chromosome 11q23. Both loci appear to be direct transcriptional targets of p53 through binding to conserved sites in the promoter sequences. Like p53 itself, expression of *mir-34* can induce cell-cycle arrest and promote apoptosis while loss of *miR-34* can impede p53-mediated effects on survival (He et al. 2007). Expression of *miR-34* was found to be dramatically reduced in a subset of NSCLC, especially in adenocarcinomas (Bommer et al. 2007). Global miRNA expression analysis identified other miRNAs to be p53-induced following DNA damage including *miR-30c, miR-103, miR-26a, miR-107* and *miR-182* (Chang et al. 2007, Melo et al. 2011).

M*iR-17-92* expression was found to be up-regulated either by amplification of the chromosomal region 13q31.3 or by transcriptional activation of the pri-miRNA by the oncogenic transcription factor c-myc which is frequently overexpressed in cancer cells (Visone et al. 2009). Another transcriptionally activated miRNA is *miR-21*. It is activated by the signal transducer and activator of transcription 3 (Stat3) in the IL-6 pathway and was shown to be up-regulated in many cancer types including lung cancer (Yanaihara et al. 2006, Löffler et al. 2007).

II. Genomic abnormalities

In silico analysis revealed that more than 50% of all miRNA encoding genes are located at chromosomal regions prone to alterations in cancer. These regions include either minimal

regions of deletions or LOH harboring a TSG, regions of amplification harboring an oncogene or fragile sites which are preferential sites for translocations, deletions, integration of plasmid DNA and viruses such as HPV (Calin et al. 2004, Visone et al. 2009). Well studied examples of miRNAs located in instable genomic regions are *miR-15* and *miR-16* at 13q14 and *miR-143* and *miR-145* at 5q33 (Calin et al. 2006).

Mutations and single nucleotide polymorphisms (SNP) can also deregulate miRNA expression if located in mature miRNA, pre-miRNA and in adjacent genomic regions (Visone et al. 2009). Hu *et al*. (Hu et al. 2008) demonstrated that a specific SNP in *miR-196-a-2* is associated with a significantly decreased survival in NSCLC patients.

III. Defects in the miRNA processing machinery

Because of an impairment of the processing mechanism reduced levels of mature miRNAs was observed in some cancers. Thus, defects in the processing machinery may also influence miRNA expression patterns. Expression levels of the enzymes Dicer and Drosha are altered in some cancers which could be responsible for deregulations in the processing machinery and thereby altered expression patterns. Furthermore, low expression levels of Drosha and Dicer were found to be associated with a poor clinical outcome in ovarian cancer (Merritt et al. 2008, Davalos et al. 2010). Moreover, Karube *et al*. (Karube et al. 2005) reported that expression levels of Dicer, but not Drosha, were reduced in NSCLC patients which correlated with reduced post-operative survival and poor tumor differentiation.

IV. Epigenetic regulation

It is now evident that many TSGs are aberrantly silenced by methylation of their CGI promoter regions. Extensive analysis revealed that a large number of miRNA genes are associated with CGIs suggesting that miRNAs may be targets for methylation as well (Saito et al. 2006, Lujambio et al. 2007, Berdasco et al. 2010, Hatziapostolou et al. 2011, Melo et al. 2011) (shown in *Figure 21*).

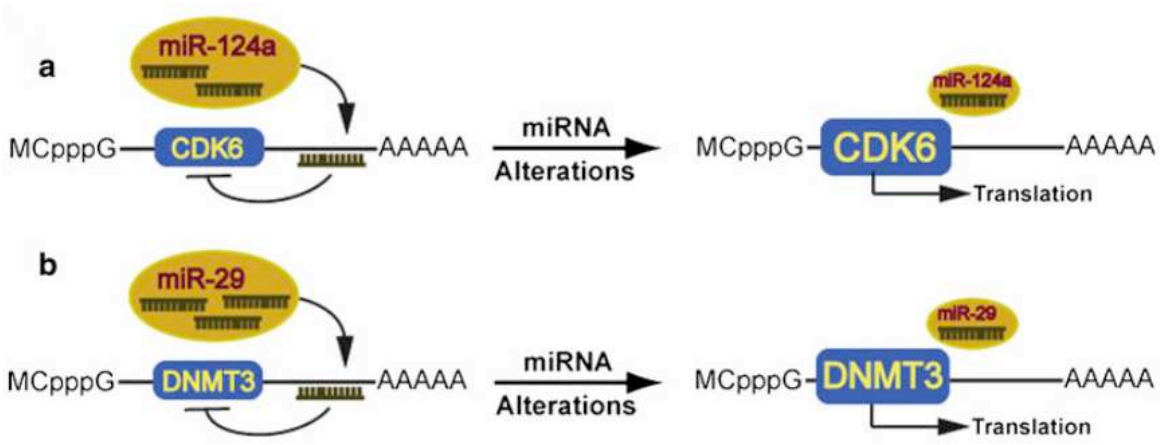

Figure 21. *Deregulated miRNA expression in cancer.*
A: Epigenetic silencing of miR-124a results in up-regulation of its target gene CDK6 in cancer cells.
B: miR-29 family members are down-regulated in cancer cells resulting in up-regulation of DNMTs and aberrant hypermethylation and silencing of TSGs. Adapted from Hatziapostolou et al. 2011.

An analysis of several miRNA-associated CGIs in different cell lines performed by Weber *et al*. (Weber et al. 2007) showed that miRNA methylation is detectable at high frequencies in both normal and malignant cells. Saito *et al*. (Saito et al. 2006) showed that *miR-127* expression was down-regulated by promoter methylation and additionally, that the potential target *BCL6* was translationally down-regulated after treatment with chromatin-modifying drugs. Similarly, Lujambio *et al*. (Lujambio et al. 2007) demonstrated that *miR-124a* is silenced by methylation and that loss of *miR-124a* is correlated with activation of CdK6 and phosphorylation of pRb. Recently it was shown that various miRNAs are methylated in NSCLC cell lines and in primary NSCLCs as described in detail in section 3.4.4. These miRNAs include *miR-7-2*, *miR-9-3*, *miR-34a, miR-99b, miR-124-2* and *miR-124-3*, *miR125a, miR-127, miR-152*, *miR-193a, miR-194, miR-200c, miR-212, miR-382, miR-432, miR-486, miR-494* and *miR-1203* (Lodygin et al. 2008, Incoronato et al. 2011, Kitano et al. 2011, Heller et al. 2012).

However, knowledge about epigenetic dysregulation of miRNAs in human cancer, especially in NSCLC, is still very limited. Thus, as described in detail in section 4, we were interested to gain further insight into methylation mediated miRNA silencing in NSCLC patients.

3.4.4. MiRNAs in cancer diagnostics and prognostics

Now there is increasing evidence that both miRNA expression profiles and DNA methylation may serve as potential biomarkers for diagnosis and prognosis of various cancer types. Firstly, malignant tissue samples can be discriminated from normal tissue samples by their

miRNA expression profiles with a general down-regulation of miRNAs in malignant tissue (Cowland et al. 2007). MiRNA expression profiles were identified for many cancer types including lung cancer (Yanaihara et al. 2006). Furthermore, Lu *et al*. (Lu et al. 2005) addressed the question whether global miRNA expression profiles could be useful to classify poorly differentiated tumors and demonstrated that miRNA signatures seem to be associated with their developmental origin. Finally, based on miRNA expression profiles it is possible to separate tumors of the same origin with different histology, e.g. pulmonary adenocarcinomas differ in their miRNome (global miRNA expression levels) from the miRNome of SCC (Yanaihara et al. 2006). These findings suggest that miRNAs could serve as good biomarkers in early and noninvasive detection of cancer.

Some miRNAs may be of prognostic relevance. It was shown that increased *miR-155* and decreased *let-7* levels are correlated with poor prognosis in lung cancer patients (Yanaihara et al. 2006). Recently, Heller *et al*. (Heller et al. 2012) observed a shorter disease free survival (DFS) and a shorter overall survival (OS) of *miR-9-3* methylated SCC patients compared to unmethylated patients.

Moreover, recent reports suggested miRNAs as potential predictors of the response to drug treatment (Meng et al. 2006). Inhibition of *miR-21* expression in malignant cholangiocytes and in patients with chronic lymphocytic leukemia increased the sensitivity to different drugs. In contrast, down-regulation of *let-7i* in patients with epithelial ovarian cancer was correlated to chemotherapy resistance (Deng et al. 2010, Hatziapostolou et al. 2011).

As discussed earlier, miRNAs were shown to be epigenetically regulated in at least some types of cancer and re-activation of aberrantly silenced miRNA genes by epigenetically active drugs was demonstrated in various cancer cell lines. Inhibition of onco-miRs and re-activation of TS-miRs are now being explored as new strategies in cancer therapy. For this purpose, modified RNA molecules with longer in vivo half-life and efficacy were developed, e.g. locked nucleic acid (LAN)- modified oligonucleotides, “anatagomiRs” and “agomiRs” (Hatziapostolou et al. 2011). An efficient and long-lasting silencing of endogenous miRNAs was achieved upon intravenous administration of antagomiRs in mice (Krutzfeldt et al. 2005). Another study in pancreatic cell lines showed that an agomiR targeting the 3´-UTR of oncogenic Gli-1 mRNA was able to inhibit cell proliferation (Tsuda et al. 2006).

Overall, miRNAs emerged as crucial regulators of important cellular processes and their deregulation is now being accepted as a hallmark of cancer which is at least in part an epigenetic disease. Their good conservation in tissue samples, the possibility to design targeted

therapies and the differences in miRNA signatures indicate that miRNAs may be attractive tools in diagnostics, prognostics and the development of new treatment strategies.

4. Aims of this diploma thesis

Despite enormous efforts in miRNA research during recent years, knowledge about epigenetic silencing of miRNA genes in lung cancer is still small. Very recently, we found that many miRNA genes are targets for methylation in NSCLC cell lines and that some of them are tumor-specifically methylated in NSCLC patients (Heller et al. 2012). Methylated miRNA genes in NSCLC cell lines were identified by comparing miRNA expression profiles of NSCLC cells before and after treatment with epigenetically active drugs. However, this approach is limited for analysis of cell lines and cannot be applied to analysis of tissue samples. To get a better insight into miRNA gene methylation in tissue samples of NSCLC patients, this diploma thesis is aimed to:

- develop a MeDIP-chip approach for a genome-wide screen of miRNA gene methylation
- analyse genome-wide miRNA methylation in primary tumor (TU) and corresponding non-malignant lung tissue (NL) samples of a large number of NSCLC patients
- identify tumor-specifically methylated miRNA genes by comparing miRNA gene methylation patterns of TU and NL samples
- develop MS-HRM assays for selected miRNA genes to verify results of our genome-wide approach
- correlate methylation results with clinico-pathological characterstics of the patients
- functionally classify miRNAs based on *in silico* target prediction.

Overall, we expect that the results of this project will contribute to a better understanding of the impact of miRNA gene methylation on the pathogenesis of NSCLCs. Furthermore, by identifying the clinical relevance of methylated miRNA genes in NSCLC patients, we hope to gain information about the potential use of miRNA genes as biomarkers and to influence future treatment strategies of NSCLC patients.

5. Materials and Methods

5.1. Tumor cell lines and tissue samples

Frozen primary TU and NL samples of stage I-III NSCLC patients who underwent surgical resection of the tumor were collected during the years 2000-2004. Tissue samples were stored at -80°C. For MeDIP-chip analyses 50 stage IA and IB NSCLC patients and matching normal lung tissue samples were selected. For subsequent MS-HRM analyses another cohort of 47 stage I-III NSCLC patients was selected (DNA of three patients was used for both analyses). Clinico-pathological characteristics including gender, age, histology, tumor stage, lymph node stage, disease stage, DFS and OS are listed in *Table 3*. NSCLC cell lines A549 and NCI-H2073 were purchased from the American Type Culture Collection (ATCC) and cultured as recommended. NSCLC cell lines NCI-H1650, NCI-H1975, Calu-6 and HCC827 were kindly provided by Prof. Dr. Walter Berger (Institute of Cancer Research, Medical University of Vienna). As negative controls for MS-HRM analyses, genomic DNA from normal human bronchial epithelial cells (NHBECs) was used. NHBECs cell pellets were purchased from PromoCell.

***Table 3**. Clinico-pathological characteristics of 47 NSCLC patients used for HRM analyses.*

Variables		N	Variables	Range	Median
Gender			Age	41-82	62
	Male	34			
	Female	13	DFS (months)	1-81	23
Histology					
	ADC	22	OS (months)	1-81	39
	SCC	23			
	LCC	1			
	NSCLC, NOS	1			
Disease stage					
	I	4			
	II	21			
	III	22			
T stage					
	T1	2			
	T2	24			
	T3	17			
	T4	4			
N stage					
	N0	13			
	N1	19			
	N2	15			
Disease recurrence					
	Yes	18			
	No	29			

ADC, adenocarcinomas; SCC, squamous cell carcinomas; LCC, large cell carcinoma; NSCLC, non-small cell lung cancer; NOS, not otherwise specified; T, tumor stage; N, lymph node stage; DFS, disease free survival; OS, overall survival

5.1.1. Cell culture

Materials:

Dulbecco`s Phosphate Buffered Saline (DPBS) (Lonza, #BE17-512F)

Dimethyl sulphoxide (DMSO) (Sigma, #D2650)

FCS (Gibco, #10500-056)

Refobacin (Merck, #112214)

RPMI 1640 Medium (Gibco, #61870-010)

Trypsin-EDTA (Gibco, #15400-054)

All cell lines were cultured in RPMI 1640 growth medium supplemented with 10% FCS and 1ml Refobacin at 37°C with 5% CO_2, 19.5% O_2 and 95% rH. Cell culture work was per-

formed in a sterile surrounding. Washing steps were performed by centrifugation for 10 min at 1000rpm.

For cell line cultivation, frozen cell pellets, which were stored at -196°C, were de-frosted at 37°C in a water bath. Immediately after thawing, the cell suspension was washed twice with 15ml growth medium and then resuspended in 5ml growth medium and transferred to a cell culture flask. Growth medium was changed on the next day and depending on the growth rate every three to four days. At ~ 80% confluency, cells were splitted into a new culture flask. In brief, growth medium was decanted, cells were washed with DPBS and detached using 1ml Trypsin-EDTA. After a few minutes of incubation streaking of cells was observed and cells were resuspended in 15ml fresh growth medium. Cell pellets were washed two times and the desired amount of cells was transferred into a new culture flask and cultured in the incubator.

5.1.2. Isolation of genomic DNA

Materials:

Chloroform:isoamyl alcohol (Sigma-Aldrich, #C-0549)
Ethanol absolute (Merck, #1070017)
Glycogen (Roche Applied Science, #109013930001)
5M NaCl (Ambion, #AM9759)
Mikro 22R (Hettich Zentrifugen)
Phenol Solution (Sigma-Aldrich, #P4557)
Proteinase K solution (Ambion, #AM2546)
Tris-EDTA buffer solution pH 7.4 (Fluka, #93302)
Trizma Hydrochloride Solution pH 6.1 (Sigma, #T2663)
Ultra Pure 0.5M EDTA pH 8 (Gibco, #15575-038)

Genomic DNA was isolated from NSCLC cell lines as well as from TU and NL samples by digestion with proteinase K, followed by standard phenol/chloroform isolation and subsequent ethanol precipitation.

Frozen tissue samples were homogenized to a fine powder using liquid nitrogen in a mortar followed by incubation with 500μl proteinase K buffer for one hour at 45°C. Afterwards, 500μl Phenol were added, mixed well by vortexing and centrifuged for 5 min at 14 000 rpm to separate proteins from DNA. The upper aqueous phase was transferred into a new tube and

500μl chloroform were added and again mixed and centrifuged. After the centrifugation step, 20μl NaCl and 1ml EtOH were added to adjust salt concentrations. Additionally, 1μl (20mg/ml) Glycogen was added to make precipitated DNA more visible. Samples were mixed thoroughly and incubated at -80°C for 15 min. After pelleting DNA by centrifugation for 15 min at maximum speed in a pre-cooled centrifuge, the pellet was washed with 70% EtOH. Air-dried DNA pellets were resuspended in TE buffer and DNA concentrations were measured using the NanoDrop 8000 Spectrophotometer.

5.1.3. RNAse treatment of extracted genomic DNA

Materials:

RNase (Invitrogen)

Thermomixer comfort (Eppendorf)

To eliminate RNA from extracted DNA, 1μl RNAse was added to each sample and incubated for one hour at 37°C. Afterwards, DNA was phenol/chloroform extracted and ethanol precipitated as described in section 5.1.2. RNAse treated DNA samples were stored at -20°C.

5.2. Methylated DNA immunoprecipitation (MeDIP)-chip analysis

5.2.1. MeDIP sample preparation

Materials:

Anti-5-Methylcytidine Monoclonal Antibody (Eurogentec, #080917)

Biorupter® (Diagenode)

Mikro22R (Hettich Zentrifugen)

Protein A agarose beads / Salmon sperm DNA (Milipore, #16-157)

Proteinase K solution (Ambion, #AM2546)

Tris-EDTA buffer solution pH 7.4 (Fluka, #93302)

Thermomixer comfort (Eppendorf)

5×IP buffer:

100mM Na-Phosphate, pH 7	50 ml
5M NaCl	14 ml
10% Triton X-100	2.5 ml
ddH_2O	33.5ml
Total	100 ml

Digestion Buffer:

1M Tris Hcl, pH 8	5 ml
0.5M EDTA	2 ml
10% SDS	5 ml
ddH_2O	88 ml
Total	100 ml

Buffers were filter-sterilized using 0.2μm filters and stored at 4°C

MeDIP sample preparation was performed according to NimbleGen`s MeDIP-chip protocol. Firstly, 2μg of genomic DNA were sonicated for 10min in intervals of 30 seconds to obtain 200bp-1000bp DNA fragments. To prevent DNA denaturation, DNA samples were cooled in ice-water during sonication. Fragmentation of DNA was confirmed by separation on a 2% agarose gel. After sonication, 1.25μg fragmented DNA were diluted to a final volume of 300μl in TE buffer and heat-denatured for 10 min at 95°C to produce single-stranded DNA (ssDNA). Immediately after the denaturation step, samples were put on ice for 5 min. 250ng of sonicated ssDNA were removed from each sample and stored at -20°C as Input DNA (referred to as input). To the remaining 240μl DNA 60μl 5×IP buffer and 1μl anti-5-methylcytidine antibody were added. The DNA-antibody mixture was incubated overnight on a rotating platform at 4°C. In the next step, the DNA-antibody mixture was bound to agarose beads. To pre-wash and block protein A agarose beads, 48μl beads were centrifuged at 1800 rpm for 1 min at 4°C and the supernatant was removed. Beads were resuspended in 600μl 0.1% PBS-BSA and incubated for 5 min on a rotating platform, followed by centrifugation for one minute. The washing step was repeated twice. Pre-washed beads were resuspended in 24μl 1×IP buffer to yield 50% slurry. To bind the DNA-antibody mixture, 48μl beads were

added to each sample and incubated on a rotating platform for 1.5h at 4°C. To remove all nonspecifically bound material, beads were washed 5 times with 1ml 1×IP buffer. After carefully removing all of the supernatant, methylated DNA bound beads were resuspended in 250μl Digestion buffer. Seven μl Proteinase K (10mg/ml) were added and incubated at 55°C for 1.5h on a thermomixer to separate proteins from DNA. Input samples were incubated with Proteinase K and Digestion buffer as well. Immunoprecipitated DNA (IP DNA) and Input DNA was isolated by standard phenol-chloroform extraction and ethanol precipitation as described in section 5.1.2. DNA pellets were resuspended in 15μl TE buffer.

5.2.2. Whole genome amplification (WGA)

Material:

GeneAmp® PCR System 9700 (Applied Biosystems)
GenomePlex Whole Genome Amplification (WGA) Kit (Sigma-Aldrich, #WGA2)
Microfuge® 18 Centrifuge (Beckman Coulter TM)

For NimbleGen microarray analyses, at least 2-4μg immunoprecipitated DNA were required. Thus, IP DNA and Input DNA were amplified using a whole genome amplification kit, which allows a ~ 500-fold amplification of genomic DNA, as recommended by the manufacturer. To achieve even higher yields of DNA amounts, each step was performed in dupilcates. This method is based on random fragmentation of genomic DNA, generation of PCR-amplifiable molecules flanked by a universal priming site and subsequent PCR amplification. The first step, fragmentation of genomic DNA was ignored, because DNA was already fragmented during the MeDIP sample preparation.

In brief, 10ng of each IP and Input DNA were diluted with WGA water and 2μl Library Preparation Buffer as well as 1μl Library Stabilization Solution were added. DNA was heat-denatured at 95°C for 2 min and then immediately put on ice to prevent re-ligation. One μl Library Preparation Enzyme was added to each sample. Samples were put in a thermal cycler and incubated as followed:

Temperature (°C)	Time (min)
16	20
24	20
37	20
75	5
4	Hold

During cycling a master mix with the following compounds was prepared:

7.5μl	10×Amplification Master Mix
47.5μl	WGA water
5μl	WGA DNA Polymerase

60μl master mix were added to each sample, mixed by pipetting and put again in a thermal cycler using the following cycling program to amplify DNA:

Step	Temperature (°C)	Time (min)	
Initial Denaturation	95	3	
Denature	94	15 sec	14 cycles
Anneal/Extend	65	5	
Hold	4	indefinite	

After amplification of DNA fragments using a universal primer, samples were maintained at 4°C or stored at -20°C until clean-up of amplified DNA.

5.2.3. Clean-up of DNA after WGA

Material:

GenElute PCR Clean-up Kit (Sigma-Aldrich, #NA1020)

Microfuge® 18 Centrifuge (Beckman Coulter TM)

To eliminate components of the PCR amplification such as excess primers, nucleotides and DNA polymerase, amplified DNA was purified using the GenElute PCR Clean-up kit from Sigma. Clean-up was performed on a separate desk to avoid any contaminations.

To maximize binding of DNA to the membrane, 500μl Column Preparation Solution were added to each mini spin column and centrifuged for 10 seconds. Duplicate WGA samples were pooled and 600μl Binding Solution were added, mixed well and transferred to the binding column. By centrifugation for one minute DNA at 13 000 rpm DNA bound to the column. The flow-through was discarded, 500μl Wash Solution were added to the column followed by centrifugation for one minute at 13 000 rpm. To remove any residual liquids, the column was centrifuged additional two minutes without any Wash Solution. Purified DNA was eluted into a new collection tube with 40μl Elution buffer after one minute incubation at room-temperature (RT). DNA concentration was measured using the NanoDrop 8000 Spectrophotometer and purified DNA was stored at -20°C.

5.2.4. Test of MeDIP efficiency by real-time PCR

Material:

QIAgility® (Qiagen)

StepOnePlus™ Real-Time PCR System (Applied Biosystems)

SYBR® Green Real-Time PCR Master Mix (Invitrogen, #4472908)

To test for enrichment of methylated DNA by MeDIP, Real-time PCR (RT-PCR) using primers for known methylated (*REXO1L1*) and known unmethylated (*GAPDH*) genomic regions was performed. Absolute fold enrichment of *REXO1L1* compared to *GAPDH* was calculated using cycle differences between IP and Input samples. Fc values ≥ 45 were considered as indicator for efficient MeDIP enrichment. Oligonucleotide sequences of selected primer pairs are listed in *Table 4*.

Table 4. *Oligonucleotide primer sequences used for RT-PCR.*

Gene	Forward primer (5´-3´)	Reverse primer (3´-5´)	Amplicon size (bp)	Annealing temp. (°C)
REXO1L1	CCATCCTGCTGAGCTTTTTC	TGGATGATCTGTGCCAGGTA	190	60
GAPDH	GCCCACACGCTCGGT	CCCTACTTTCTCCCCGCTTTT	71	60

For each gene a master mix was prepared using the QIAgility automation system:

SYBR Green Mastermix	12.5μl
Primer Mix	1.9μl
RNase-free water	9.6μl
Template DNA	1μl
Total volume per reaction	25μl

5.2.5. Microarray design, sample labeling, hybridization and analysis

Genomic sequences for microarray design were obtained from USCS genome browser (assembly "GRCh37/hg19", track "sno/miRNA"; http://www.genome.uscs.edu) and from miRBase database (version 18; http://www.mirbase.org/). Tiled regions spanned ~ 2 kb upstream and ~ 1 kb downstream of transcription start sites with 50 kb spacing. Additionally, 12 housekeeping genes were including on the microarray as negative controls. Overall, microarrays consisted of 720 000 probes covering 1552 miRNA genes and 390 snoRNA genes.

IP DNA and corresponding Input DNA of NSCLC cell lines A549 and H1993 and of TU and NL samples from 50 NSCLC patients were sent to Roche/NimbleGen service laboratories for quality control using the Agilent Bioanalyzer, sample labeling for two colour detection and microarray hybridization. IP DNA was labeled with Cyanine 5 (Cy5) while Input DNA was labeled with Cyanine 3 (Cy3). Arrays were scanned on a GenePix 4200A Scanner with GenePix 6.0 software and images were processed using NimbleScan 2.3 extraction software. The data were delivered as raw signal intensities (.pair files) and log2-ratios of IP/Input DNA for each probe on the microarray.

5.3. Development of methylation-sensitive high resolution melting (MS-HRM) analyses

Material:

Methyl Primer Express® v1.0 (Applied Biosystems)

To determine the methylation level of selected miRNA genes, MS-HRM assays were developed for *miR-10a, miR-10b, miR-129-2, miR-572* and *miR-615*. To amplify selected promoter regions, methylation independent primers were designed using the Methyl Primer Express® software. Using this software, the design of either methylation-specific primers (MSP) or bisulfite sequencing primers (BSP) is possible. The software searches for CGIs in the promoter region and simulates bisulfite modification of DNA.

Genomic sequences of miRNA genes were obtained from ENSEMBL database (http://www.ensemble.org, version 59). A defined genomic region of 700bp, located from -600bp to +100bp relative to the transcription start site was copied into the software and BSP primers were requested using the following settings:

PCR Amplicon Length: 90-160bp
Primer Length: 18-27bp
Tm reaction: 56-64°C
Tm forward-Tm reverse: < 7°C
Max # of primers to be designed: 10 pairs

Efficient amplification of methylated and unmethylated genomic regions is crucial for the subsequent HRM analysis, thus primer sequences had to be methylation independent. This was achieved by either setting primers into regions where no CpG dinucleotide was located or by random incorporation of either Cs or Ts in the forward primer and Gs or As in the reverse primer. For each promoter region two to three primer pairs designed. Lyophilized primers were diluted in ddH_2O to achieve a 100pmol/μl concentration and a 1:10 primer mix containing both forward and reverse primer was prepared. Each primer pair was tested for efficient differentiation of 0% and 100% methylated DNA (see section 6.2.1.).

Oligonucleotide sequences of selected primer pairs are listed in *Table 5*.

***Table 5**. Oligonucleotide primer sequences used for MS-HRM analyses.*

Gene	Forward primer (5´-3´)	Reverse primer (3´-5´)	Amplicon size (bp)	Annealing temp. (°C)
miR-10a	GAATGATTTTTTTTTTTTTTGTG	AATTACAAAATTTTCAATTCTCCT	159	55
miR-10b	TTTGGTAGAAGAATGAGGGAAT	CCCACCCAAAATAAAATACCTA	159	55
miR-129-2	GGAGATATTTTGGGTTGAAG	TTATATACAACAAACCCAAACC	95	55
miR-572	ATYGTGAAGTYGGTTTATTTTTT	CTTCCRAACCACRACCTC	129	55
miR-615	GGTTTTAGAGYGGGGATTTTT	TCCCRAATCRCTAAAATCC	100	55

Y in primers, random integration of C or T; R in primers, random integration of G or A.

5.3.1. Sodium bisulfite treatment of genomic DNA

Material:

EpiTect Bisulfite Kit (Qiagen, #59104)

GeneAmp® PCR System 9700 (Applied Biosystems)

Microfuge® 18 Centrifuge (Beckman Coulter TM)

To preserve information about DNA methylation during PCR amplification, genomic DNA was subjected to sodium bisulfite treatment using the EpiTect Bisulfite Kit from Qiagen.

Genomic DNA isolated from NSCLC cell lines as well as from TU and NL samples was modified according to the manufactory`s protocol. One μg genomic DNA diluted in 20μl RNase-free water was mixed with 85μl Sodium Bisulfite Mix and 35μl DNA Protect Buffer. The Protect Buffer prevents fragmentation of DNA and contains a dye, which indicates correct pH values. The conversion of unmethylated cytosines was performed in a thermal cycler using the following program:

Step	Time (min)	Temperature (°C)
Denaturation	5	95
Incubation	25	60
Denaturation	5	95
Incubation	85	60
Denaturation	5	95
Incubation	175	60
Hold	Indefinite	20

After cycling clean-up of the converted DNA was performed. The bisulfite reaction was transferred into a new tube and 560μl Buffer BL containing 10μg/ml carrier RNA was added, enabling the ssDNA to precipitate on the spin column membrane. After vortexing and centrifugation for one minute at 14 000 rpm, the solution was transferred to an EpiTect spin column. To bind DNA to the membrane, spin columns were centrifuged for one minute at 14 000 rpm and flow-through was discarded. Afterwards, a washing step using 500μl buffer BW and a desulfonation step using 500μl buffer BD were performed. To ensure complete elimination of sulfonyl groups, the reaction was incubated for 15 min at RT. Columns were centrifuged for one minute at 14 000 rpm and 500μl buffer BW were. This washing step was repeated twice. To remove any residual liquids, column were placed in a new tube and centrifuged again. Converted DNA was eluted with 20μl buffer EB which were added to the center of the membrane. After centrifugation for one minute, additional 20μl buffer EB were added. Concentration of DNA was measured using the NanoDrop 8000 Spectrophotometer and diluted DNA (30ng/μl) was stored at -20°C.

5.3.2. MS-HRM analyses

Material:

EpiTect HRM PCR Kit (Qiagen, #59445)

EpiTect methylated control DNA (Qiagen, #59665)

EpiTect unmethylated control DNA (Qiagen, #59655)

QIAgility® (Qiagen)

Rotor Gene Q® (Qiagen)

Strip Tubes and Caps, 0.1ml (Qiagen, #981103)

Sodium bisulfite treated DNA was subjected to PCR amplification using the following reaction:

EpiTect HRM PCR Mastermix	12.5μl
Primer Mix	1.9μl
RNase-free water	9.6μl
Template DNA	1μl
Total volume per reaction	25μl

Twenty-four μl were used for MS-HRM analysis using the Rotor Gene Q with the following program:

Step	Time (sec)	Temperature (°C)	
Initial PCR activation	300	95	
Denaturation	10	95	40 cycles
Annealing	30	55	
Extension	10	72	
HRM analysis	2	65-95 (0-1°C increments)	

Methylation status of the miRNA genes of interest was calculated using predefined methylation standards (100%, 75%, 50%, 25%, 10% and 0%) which were prepared by diluting 0% and 100% methylated control DNA. Melting curves were normalized by determination of two normalization regions before and after the major fluorescence decrease using RotorGene Q® software as reported previously (Balic et al. 2009). Fluorescence values were normalized against 0% methylation and maximum peak values of methylation standards were extrapolated resulting in a highly linear regression line. The linear equation was used to determine percentages of methylation of miRNA genes in NSCLC cell lines and in patient samples. Water blanks without DNA were used as negative controls, experiments were performed in duplicates.

5.3.3. Statistical analyses

Materials:

GraphPad Prism® 5

IBM SPSS Statistics 20

Microsoft Excel 2010

A miRNA gene was considered as methylated in MeDIP-chip experiments if the P-value resulting from Kolmogorov Smirnoff tests (computed by Roche/NimbleGen), which tests if the probe intensities of a specific gene are significantly larger than the overall intensities, reached a cut-off level of $-\log10\ P \geq 2$.

To validate MeDIP-chip data, Wilcoxon signed rank tests were used to determine methylation differences between TU and NL samples obtained by MS-HRM analyses. For each sample T/N methylation ratios of the different genes were calculated and patients who reached a cut-off level ≥ 1.5 were considered as methylated. Moreover, T/N ratios were used for comparison with clinico-pathological characteristics of NSCLC patients including gender, age, histology, tumor stage, lymph node stage, disease recurrence, disease stage, DFS and OS.

To determine differences between groups, Chi^2 testst and Fisher`s exact tests were used; to determine differences between means, t-test were used. Survival analyses of patients were performed using Kaplan-Meier`s log rank testing and the Cox proportional model was used for multivariate analyses on DFS and OS. Analyses were performed using the statistics software SPSS (version 20), GraphPad Prism 6 and Microsoft´s Excel.

6. Results

6.1. Genome-wide methylation analyses

6.1.1. Establishment of MeDIP-chip assay and microarray design

The major goal of this diploma thesis was to identify tumor-specifically methylated miRNA genes in NSCLC patients. Thus, we established a MeDIP approach in our lab according to Roche/NimbleGen´s sample preparation protocol. As first working step we sonicated genomic DNA to generate DNA fragments of 200bp - 1000bp in length. Using Diagenode´s Bioruptor, we observed highly efficient fragmentation of genomic DNA after sonication for 10 minutes in intervals of 30 seconds. Sonication efficiency was confirmed using agarose gel electrophoresis. Examples of sonicated DNA samples are shown in *Figure 22*.

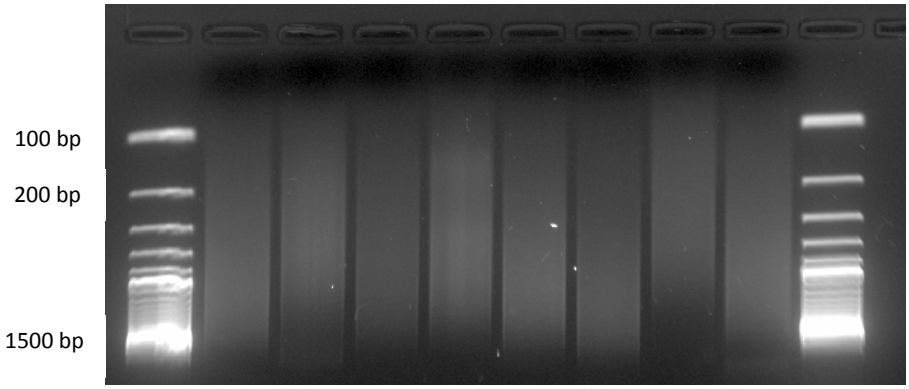

***Figure 22**. Agarose gel electrophoresis of genomic DNA after sonication.*
After sonication for 10 min in intervals of 30 seconds DNA fragments with a length of 150bp – 1000bp were mainly present. Outer lane: 100bp ladder (Invitrogen)

Next, methylated DNA fragments were captured using an anti-5-methylcytidine antibody and salmon sperm DNA blocked agarose beads. After amplification and purification of IP and Input DNA MeDIP efficacy was tested by Real-time PCR. For this purpose, we designed primer pairs to amplify the 5´ region of *REXO1L1* which is known to be ubiquitously methylated and of *GAPDH* which is known to be unmethylated. Quality of these assays was determined by melting curve analyses which are shown in *Figure 23*. Only one major peak was detected for both *REXO1L1* and *GAPDH* indicating that a single PCR product is amplified.

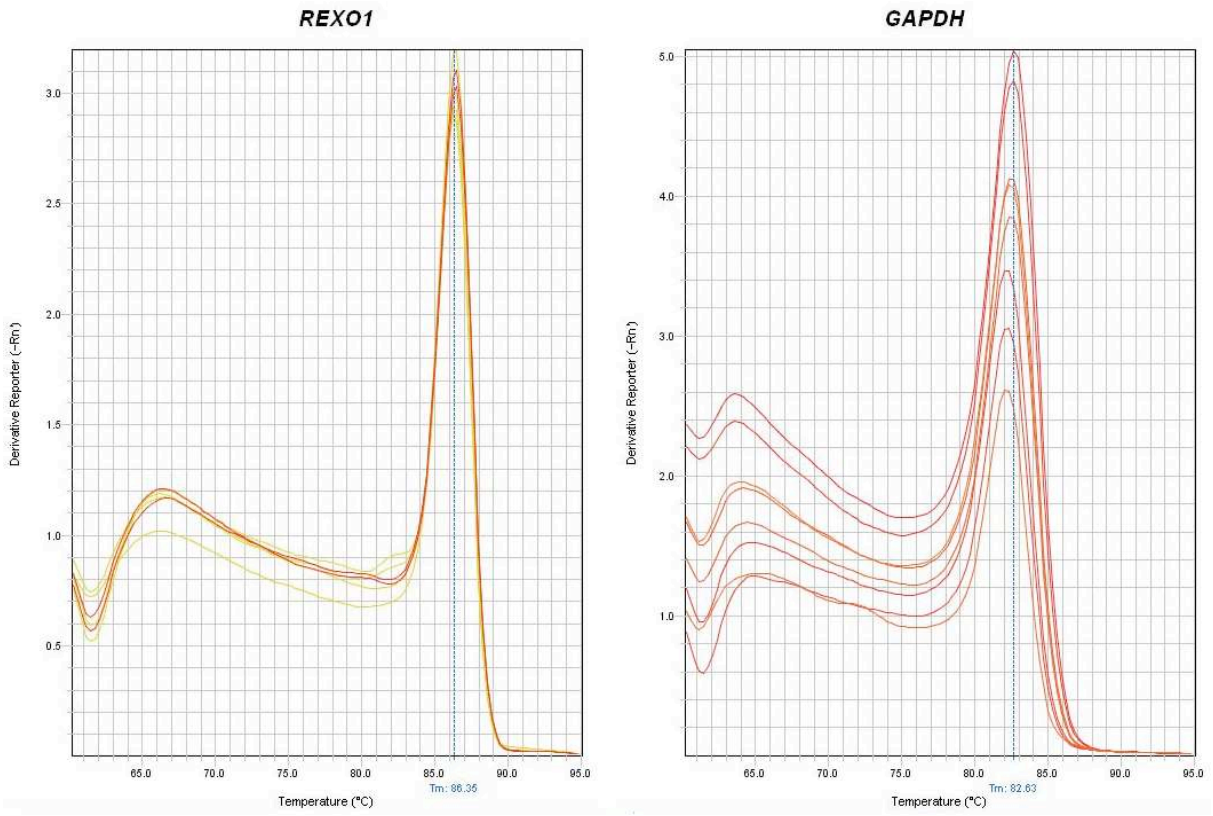

Figure 23. *PCR product melting curves of REXO1L1 and GAPDH.*
Each peak is formed from the degradation of a single PCR product. Contaminating DNA or primers would be detected as an additional peak.

Ct values of *REXO1L1* and *GAPDH* detected in IP and Input samples were used for calculation of enrichment of methylated DNA fragments. While *REXO1L1* was detected earlier in IP samples compared to Input samples, *GAPDH* was detected earlier in Input samples compared to IP samples indicating enrichment of methylated DNA in the IP sample. An example of enrichment verification using RT-PCR is shown in *Figure 24*.

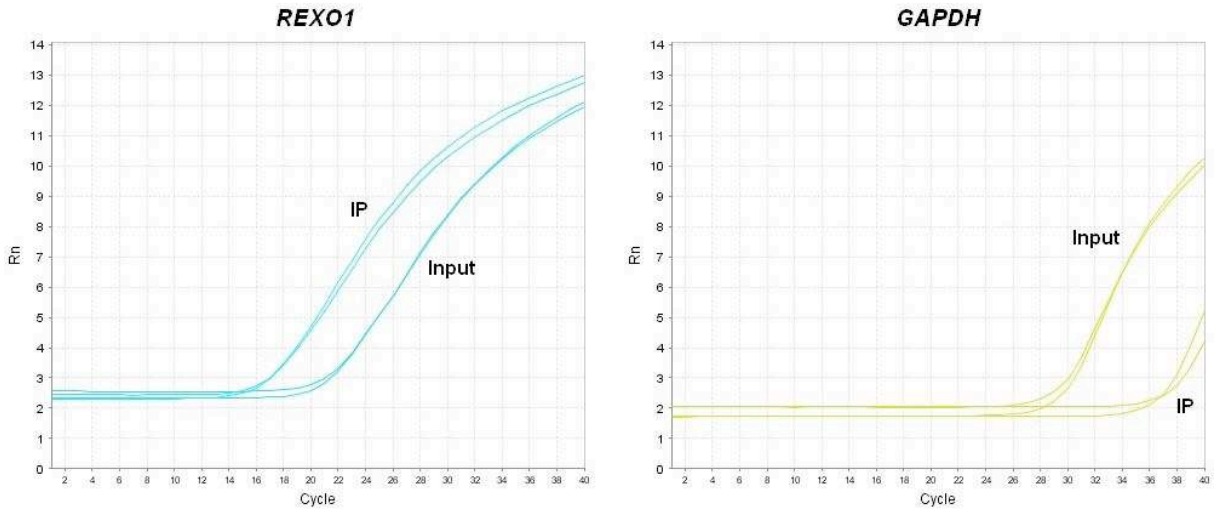

***Figure 24**. Amplification plots of RT-PCR analyses*
RT-PCR of REXO1L1 and GAPDH 5´ regions was performed to ensure enrichment of methylated DNA in the IP samples compared to Input samples. IP, immunoprecipitated DNA; Input, input DNA.
Left panel: amplification plot of REXO1L1.
Right panel: amplification plot of GAPDH providing information about unspecific antibody binding

Overall, these results demonstrate that our MeDIP assay efficiently enriches methylated DNA with minimal background caused by unspecific antibody binding.

After successful establishing the MeDIP technique, we designed in cooperation with Roche/NimbleGen a tiling microarray which covered 1552 miRNA genes and 390 snoRNA genes based on miRBase 18 and USCS genome browser (effective 04/2012). Six replicates of each probe were present on the microarray resulting in a total of 720 000 probes.

6.1.2. Genome-wide methylation analysis of clinical samples from NSCLC patients

To determine genome-wide methylation of miRNA genes in NSCLC patients, we performed MeDIP-chip analyses of TU and NL samples of 50 stage I NSCLC patients. Quality control of MeDIP samples, sample labeling, microarray hybridization and washing, image scanning and data extraction were performed by Roche/NimbleGen. Microarrays were statistically analysed by Roche/NimbleGen and a list of methylated genomic regions for each TU and NL sample was generated. These lists were used for identification of tumor-specifically methylated miRNAs. Overall, 543 small non-coding RNA genes including 417 miRNA genes and 126 snoRNA genes were found to be methylated in at least 1 TU sample. In addition, 495

small non-coding RNA genes including 382 miRNA genes and 113 snoRNA genes were found to be methylated in at least 1 NL sample. By comparing these groups of genes, we observed that > 80% of small non-coding RNA genes were methylated in both TU and NL samples as shown in *Figure 25*. These results suggest that a large number of small non-coding RNA genes is physiologically methylated in lung tissue. Moreover, 102 small non-coding RNA genes including 86 miRNA genes and 16 snoRNA genes were identified to be methylated exclusively in TU samples confirming our hypothesis about miRNA methylation in NSCLCs. Interestingly, we also found 51 miRNA genes and 3 snoRNA genes which were methylated exclusively in NL samples. Because our tissue samples were not microdissected, we cannot exclude contamination of NL samples with tumor cells. In addition, it was reported that methylation changes already occur in pre-malignant lung tissue of smokers (Belinsky et al. 2002, Guo et al. 2004). Thus, we hypothesize that methylation of some miRNA genes found to be methylated in both TU and NL samples may be pathologic rather than physiologic. To address this issue, we calculated T/N methylation ratios of these miRNA genes and considered miRNAs with a T/N ratio ≥ 3 as tumor-specifically methylated. Using this cut-off level, 52 miRNA genes and 12 snoRNA genes were found to be tumor-specifically methylated. Overall, 138 miRNA genes and 28 snoRNA genes were found to be tumor-specifically methylated in NSCLC patients. Because we were mainly interested in miRNA gene methylation in NSCLCs, snoRNA genes were excluded from subsequent analyses. Genomic sequences of tumor-specifically methylated miRNA genes were obtained from Ensembl database (release 69) and a CpG island search was performed using Methyl Primer Express software. Interstingly, only 25% of these genes were found to be associated with a CGI. Selected examples of these miRNA genes are listed in *Table 6*.

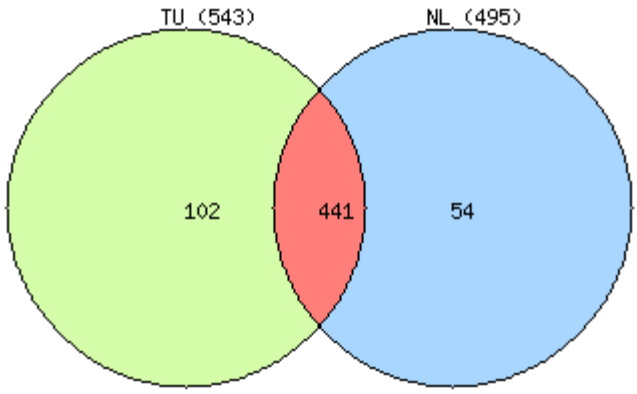

Figure 25. *Venn diagram of methylated small con-coding RNAs.*
The diagram summarizes the number of small non-coding RNA genes which are methylated in primary tumor samples, in non-malignant lung tissue samples and in both tissue samples.

Next, we addressed the question if some of the identified tumor-specifically methylated miRNA genes were already known to be methylated in various cancer types, especially in NSCLCs. By performing a PubMed search, we found that some of these genes are already known to be regulated by methylation in various tumor types (see *Table 6*). However, for the vast majority of the miRNA genes the finding that they are tumor-specifically methylated is new. Overall, we identified several miRNA genes whose epigenetic regulation in NSCLCs was unknown so far.

***Table 6**. Overview of 11 tumor-specifically methylated miRNA genes which are associated with a CGI, their chromosomal location, Ensembl ID and reported information about CGI promoter methylation in malignant diseases.*

Gene	Cytoband	Ensembl ID	CGI promoter methylation reported in
miR-10a	17q21.32	ENSG00000207777	HCC (Shen et al. 2012)
miR-10b	2q31.1	ENSG00000207744	Gastric cancer, HCC (Kim et al. 2011, Shen et al. 2012)
miR-124-1	8p23.1	ENSG00000208010	NSCLC, non-Hodgkin´s lymphoma, cervical cancer (Kitano et al. 2011, Wong et al. 2011, Wilting et al. 2010)
miR-124-2	8q12.3	ENSG00000207816	NSCLC, bladder cancer, cervical cancer (Kitano et al. 2012, Shimizu et al. 2012, Wilting et al. 2010)
miR-129-2	11p11.2	ENSG00000199077	Endometrial cancer, esophageal squamous cell carcinoma, colorectal cancer, gastric cancer (Bandres et al. 2009, Huang et al. 2009, Chen et al. 2012, Tsai et al. 2011)
miR-137	1p21.3	ENSG00000207958	Bladder cancer, NSCLC cell line A549, oral squamous cell carcinoma, colorectal cancer (Balaguer et al. 2010, Dang et al. 2012, Shimizu et al. 2012, Zhu et al. 2012)
miR-193a	17q11.2	ENSG00000207614	NSCLC, AML, oral cancer (Heller et al. 2012, Gao et al. 2011, Kozaki et al. 2008)
miR-200c	12p13.31	ENSG00000207713	NSCLC, NSCLC cell lines, breast cancer, bladder cancer (Ceppi et al. 2010, Tellez et al. 2011, Neves et al. 2010, Wiklund et al. 2011)
miR-615	12q13.13	ENSG00000207571	Myelogenous leukemia cell line K562 (Yang et al. 2012)
miR-9-2	5q14.3	ENSG00000207570	Gastric cancer (Tsai et al. 2011)
miR-9-3	15q26.1	ENSG00000207819	NSCLC, bladder cancer, gastric cancer, oral squamous cell carcinoma (Heller et al. 2012, Kitano et al. 2012, Minor et al. 2012, Tsai et al. 2012, Shimizu et al. 2012)

AML, acute myeloid leukemia; HCC, hepatocellular carcinoma; NSCLC, non-small cell lung cancer; n/a, not available

6.2. Single-gene methylation analyses

6.2.1. Development of MS-HRM assays for selected miRNA genes

To confirm results obtained by MeDIP-chip analyses, MS-HRM assays were developed to investigate methylation of selected miRNA genes in NSCLC cell lines and in TU and NL samples of 47 additional stage I-III NSCLC patients. Overall, MS-HRM assays were developed for 15 miRNA genes based on their exclusive methylation in TU samples or their high T/N methylation ratios. Prior to analysis of patient material MS-HRM assays were tested on methylation standards for efficient amplification and linearity of melting curves. Primer pairs which clearly differentiated 100% and 0% methylation standards were selected for subsequent MS-HRM analyses of NSCLC cell lines and of clinical specimens. As shown in *Figure 26*, MS-HRM assays for *miR-10a*, *miR-10b*, *miR129-2*, *miR-615* and *miR-572* were successfully established.

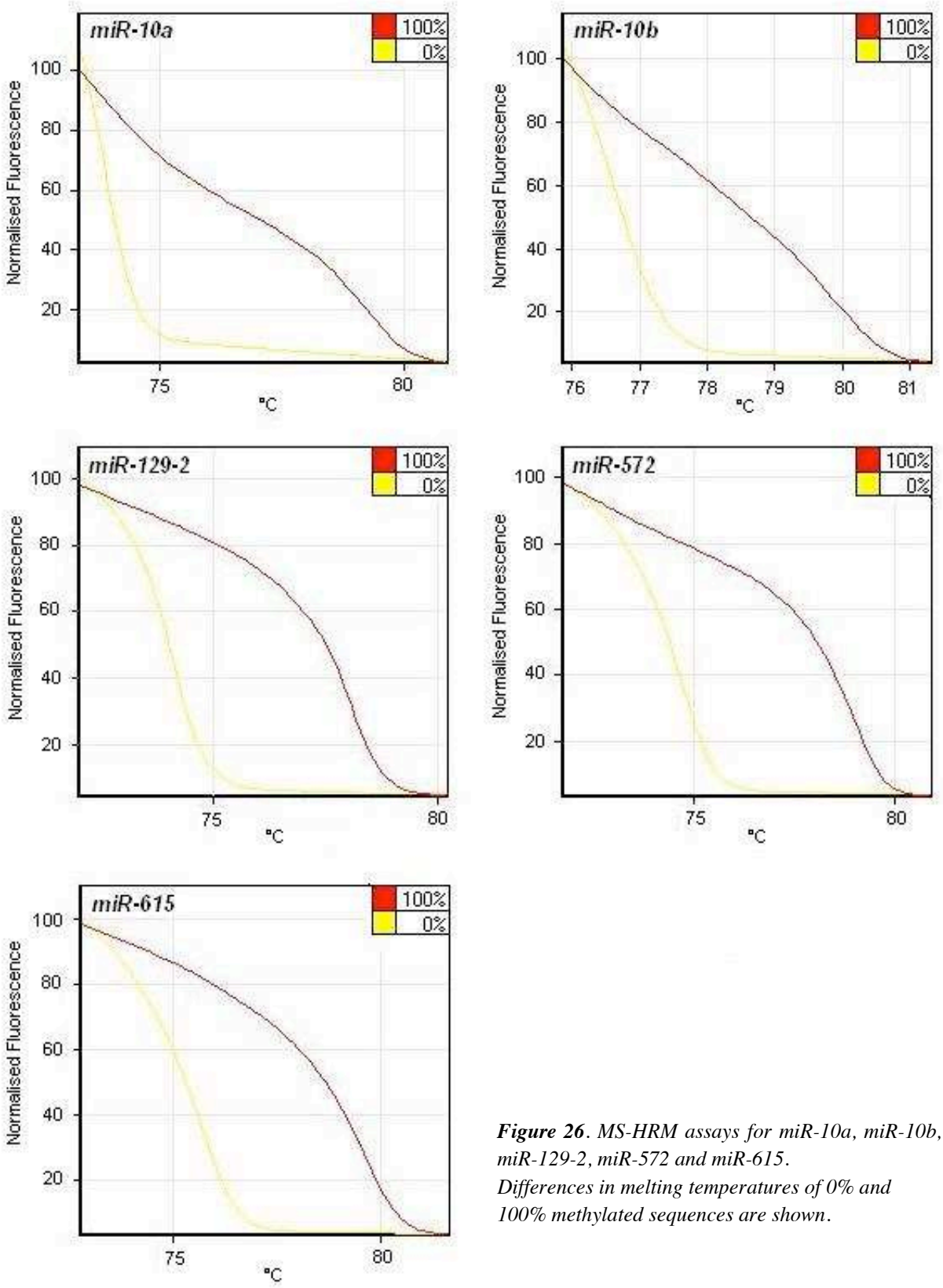

Figure 26. *MS-HRM assays for miR-10a, miR-10b, miR-129-2, miR-572 and miR-615.*
Differences in melting temperatures of 0% and 100% methylated sequences are shown.

6.2.2. MS-HRM analyses of 5 miRNA genes in NSCLC cell lines

After selection of efficient primer assays, miRNA methylation was determined by MS-HRM analyses in the NSCLC cell lines A549, Calu6, HCC827, NCI-H1650, NCI-H1975 and NCI-H2073. Normal human bronchial epithelial cells (NHBEC) were used as negative control, which were expected to show less methylation compared to NSCLC cell lines. Methylation values of the five miRNA genes in 6 NSCLC cell lines and in NHBECs are illustrated in *Figure 27*.

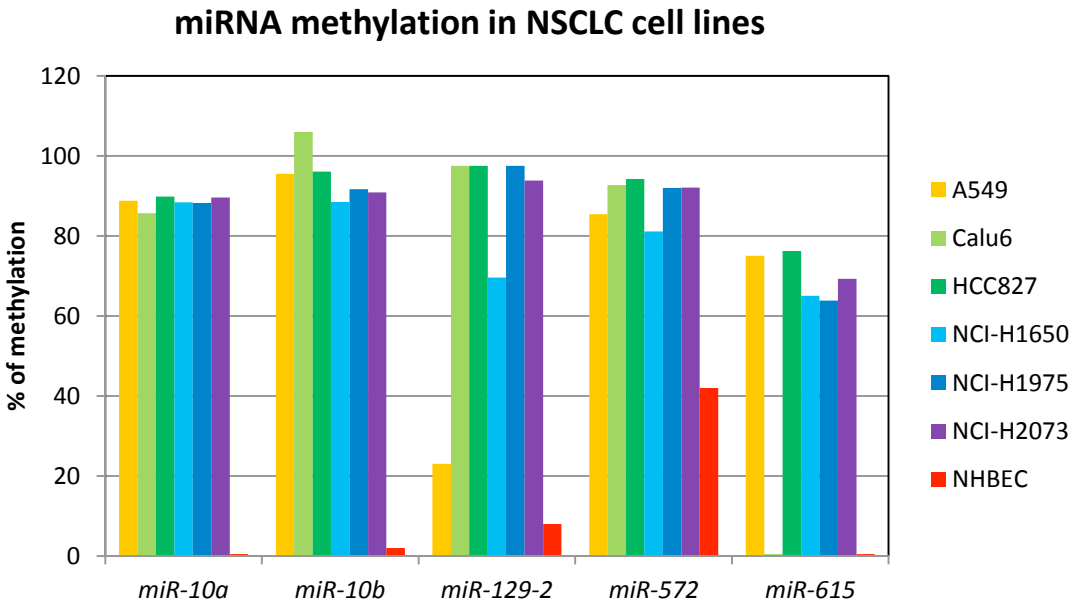

***Figure 27**. Methylation values of miR-10a, miR-10b, miR-129-2, miR-572 and miR-615 in NSCLC cell lines and NHBECs determined by MS-HRM.*

While *miR-10a* was found to be methylated in all NSCLC cell lines (mean methylation value of 88.42%), no *miR-10a* methylation was detected in NHBECs. Very similar results were found for *miR-10b* with a mean methylation value of 94.78% in NSCLC cell lines. *MiR-129-2* was weakly methylated in A549 cells (23%) and in NHBECs (8%). *MiR-572* was highly methylated in NSCLC cell lines (mean methylation value of 89.58%), however, methylation was also detected in NHBECs (mean methylation value of 42%). *MiR-615* was found to be methylated in all cell lines except of Calu6 and NHBECs. Overall, MS-HRM revealed that all selected miRNAs are highly methylated in NSCLC cell lines but weakly methylated in NHBECs. Moreover, these results confirmed that selected primer assay are suitable for subsequent MS-HRM analysis of clinical samples of NSCLC patients.

6.2.3. MS-HRM analysis of 5 miRNA genes in clinical samples of 47 NSCLC patients

After development and successful establishment of MS-HRM assays in NSCLC cell lines, methylation of *miR-10a, miR-10b, miR-129-2, miR-572* and *miR-615* was determined in TU samples and NL samples of 47 NSCLC patients. Descriptions of these MS-HRM assays in some clinical samples are shown in *Figure 29*. All samples were amplified with comparable Ct values while the negative water control was not amplified. Fluorescence values were normalized against 0% methylation standard and normalized fluorescence values were plotted against percentages of methylation of the other standards to generate a standard curve. The resulting linear equation was used to calculate methylation levels of miRNA genes in clinical samples. Mean R^2-values of 4 MS-HRM runs per gene performed to analyse the clinical samples are shown in *Figure 28*. The mean R^2 value of *miR-10a* was 0.9151 (range 0.901 – 0.931), for *miR-10b* it was 0.9924 (range 0.9896 – 0.9971), for *miR-192-2* 0.991 (range 0.9916 – 0.9953), for *miR-572* 0.9661 (range 0.9687 – 0.998) and for *miR-615* it was 0.9393 (range 0.9346 – 0.9427) indicating high linearity and reproducibility of the MS-HRM assays.

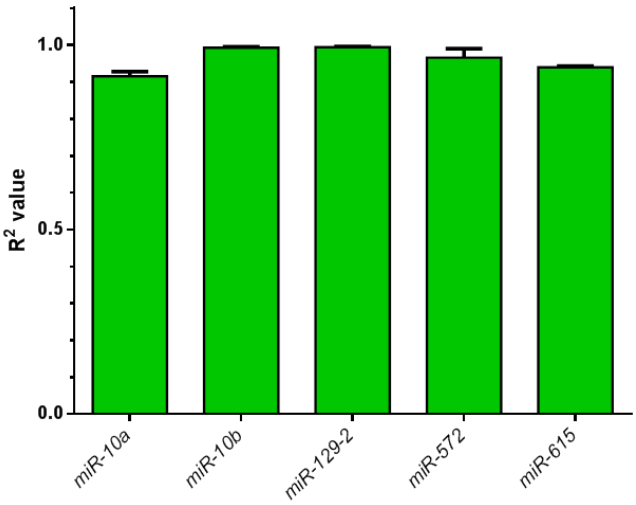

***Figure 28**. Mean R^2 values of four MS-HRM runs per gene.*
Mean R^2 values ranged from 0.9151 for miR-10a to 0.9961 for miR-10b.

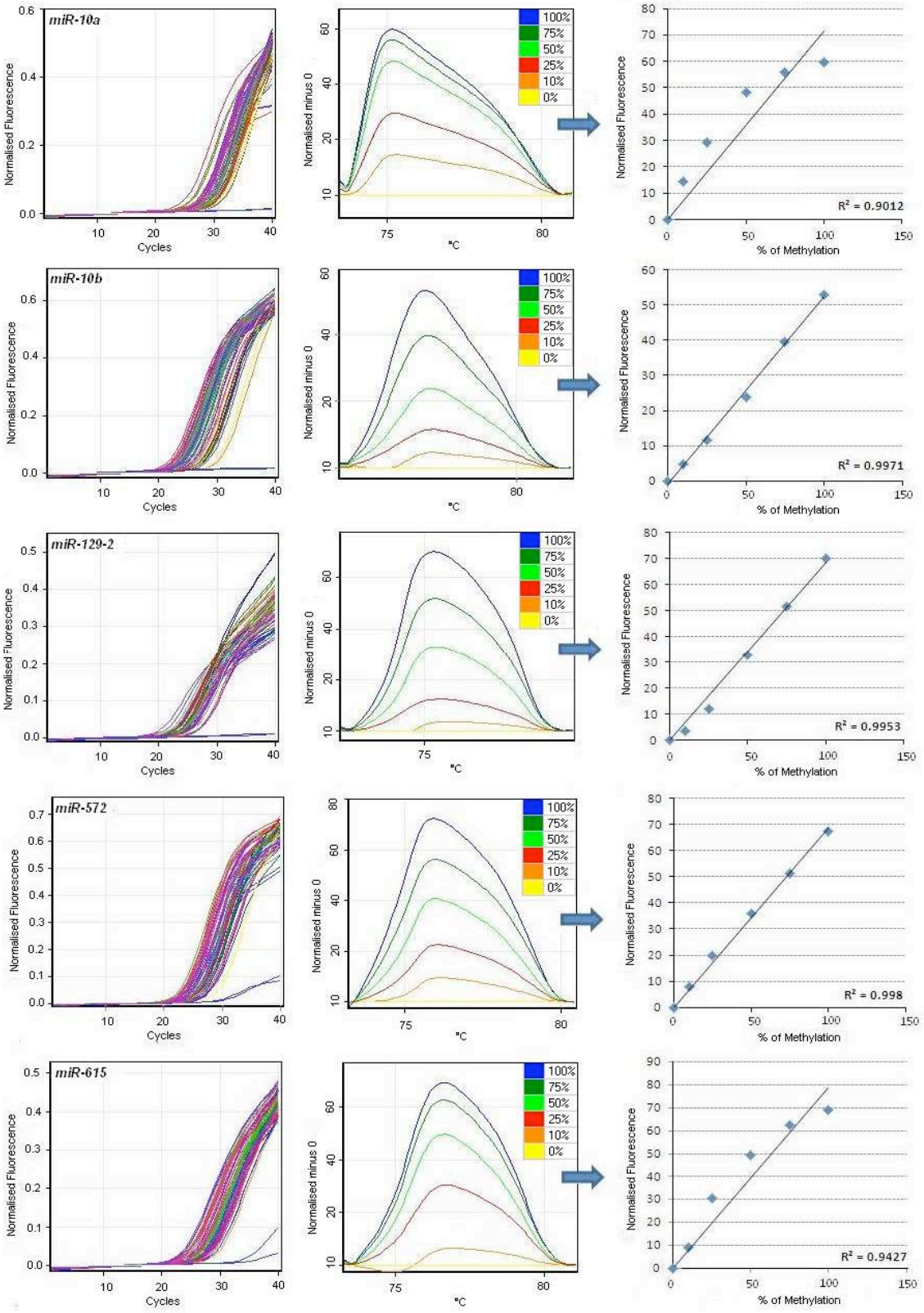

Figure 29. *MS-HRM analyses of miR-10a, miR-10b, miR-129-2, miR-572 and miR-615.*
Left panel: Amplification plot of methylation standards (100%, 75%, 50%, 25%, 10% and 0%) and some samples.
Middle panel: Differential graph showing maximum peak values of methylation standards. Curve separation depends on the percentage of methylation.
Right panel: Maximum peak values were extrapolated resulting in a linear regression line. Linear equation was used to determine methylation percentages of patient samples.

Percentages of methylation of miRNA genes were found to be higher in TU samples compared to NL samples. Wilcoxon signed rank tests were used to determine methylation differences between the two tissue types obtained by MS-HRM analyses. Tumor-specific methylation of *miR-10a, miR-10b, miR-129-2, miR-572* and *miR-615* and unadjusted p-values are shown in *Figure 30*.

After correction for multiple testing, all p-values except that of *miR-615* remained statistically significant. Consistent with our results obtained by MeDIP-chip anaylses, tumor-specific methylation was found for all five miRNA genes analysed.

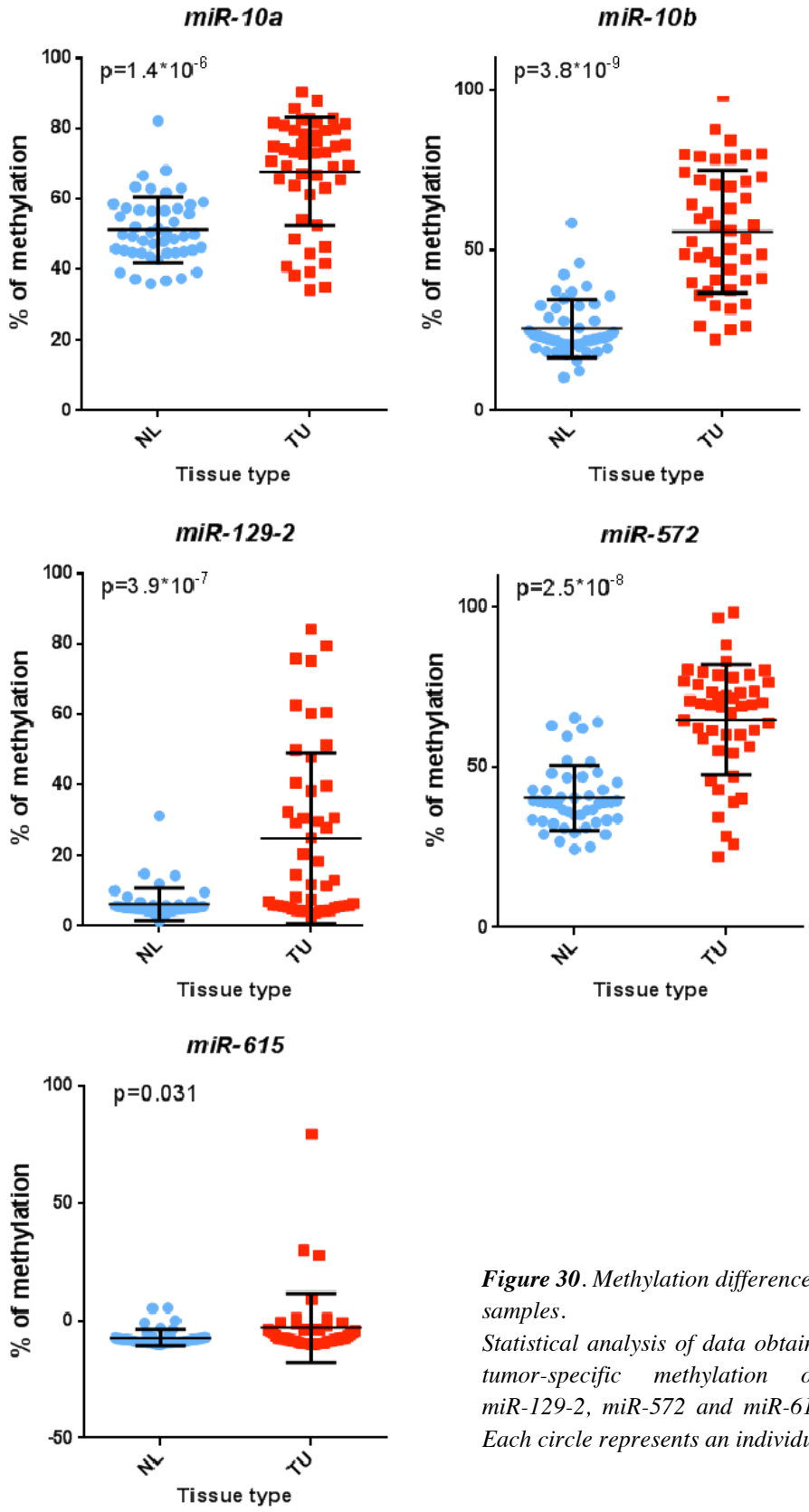

Figure 30. *Methylation differences between TU and NL samples.*
Statistical analysis of data obtained by MS-HRM revealed tumor-specific methylation of miR-10a, miR-10b, miR-129-2, miR-572 and miR-615 with P values < 0.05. Each circle represents an individual sample.

Moreover, T/N methylation ratios were calculated as percentage of methylation of a gene in a TU sample / percentage of methylation in the NL sample. Patients with a T/N ratio ≥ 1.5 were considered as methylated (Heller et al. 2012, Heller et al. 2012). The most frequently methylated gene was *miR-10b* (70%), followed by *miR-572* (54%), *miR-10a* (34%) and *miR-129-2* (34%). *MiR-615* was the less frequently methylated gene with 9% of samples reaching a T/N methylation ratio ≥ 1.5. ROC curve analyses revealed that methylation of *miR-10a, miR-10b, miR-129-2* and *miR-572* statistically significant distinguishes TU samples from NL samples. Areas under the curve ranged from 0.762 for *miR-129-2* to 0.934 for *miR-10b,* shown in *Figure 31*.

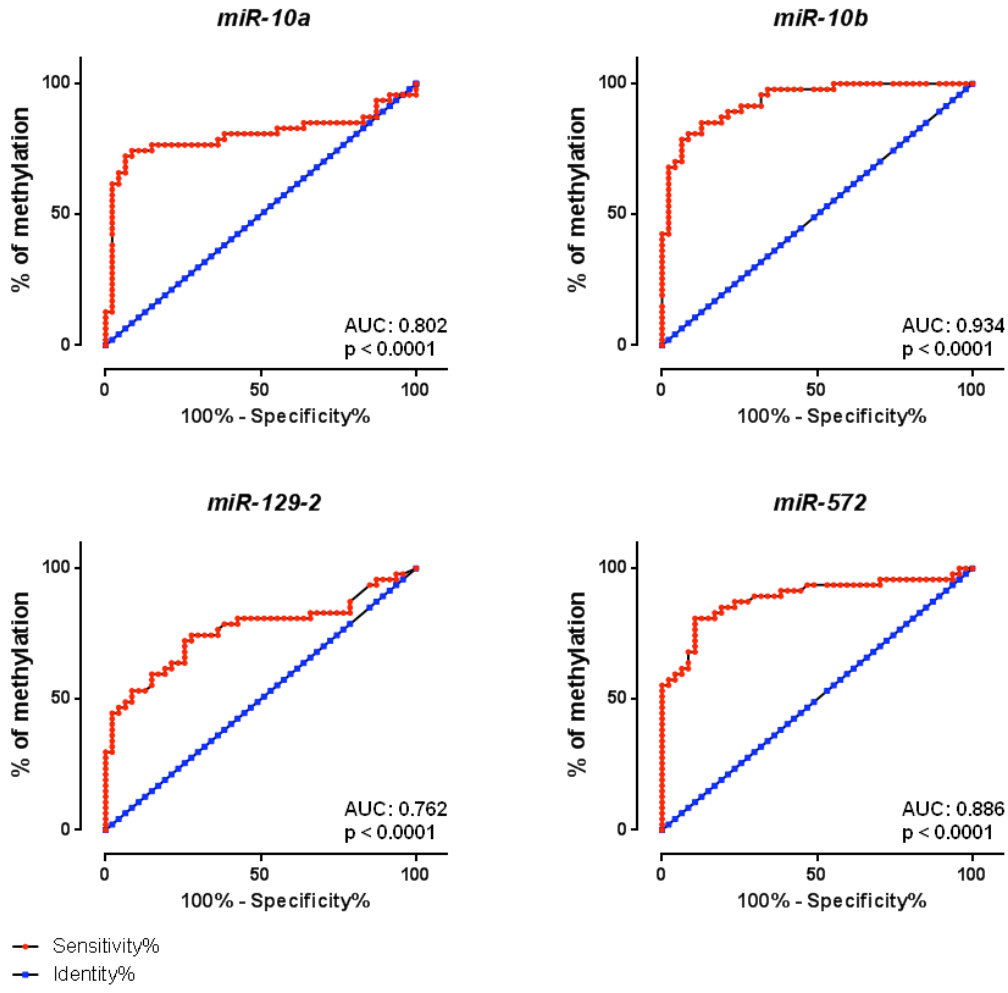

Figure 31. *ROC curve analyses of miR-10a, miR-10b, miR-129-2, miR-572 and miR-615 methylation determined by MS-HRM analyses of 47 NSCLC patients.*
Extent of methylation of these genes allows to distuingish between TU and NL samples. P-values and AUC values (Area under the curve) are shown. Blue line: reference line; red line: methylation of a particular gene (Heller et al. 2012).

6.3. Comparison of MS-HRM methylation results with clinico-pathological characteristics of NSCLC patients

T/N methylation ratios were used to compare methylation data obtained by MS-HRM analyses with clinico-pathological data of 47 NSCLC patients including gender, age, histology, tumor stage, lymph node stage, stage of disease, disease recurrence, DFS and OS.

Interestingly, *miR-10a* methylated NSCLC patients had a statistically significant shorter OS than *miR-10a* unmethylated patients (p = 0.036). The median OS was 31.4 months for methylated patients compared to 46.6 months for unmethylated patients. A similar finding was observed regarding the DFS and *miR-10a* methylation with a median survival of 38.2 months for methylated patients compared to 56.8 months for *miR-10a* unmethylated patients. However, this association did not reach statistical signifance (p = 0.103). Kaplan-Meier plots for DFS and OS in *miR-10a* methylated und unmethylated patients are shown in *Figure 32*. Multivariate analyses revealed that methylation of *miR-10a* is an independent prognostic factor for shorter OS in NSCLC patients (p = 0.012). Cofactors in the Cox proportional model included gender, age, tumor stage, lymph node stage, histology, disease recurrence and disease stage.

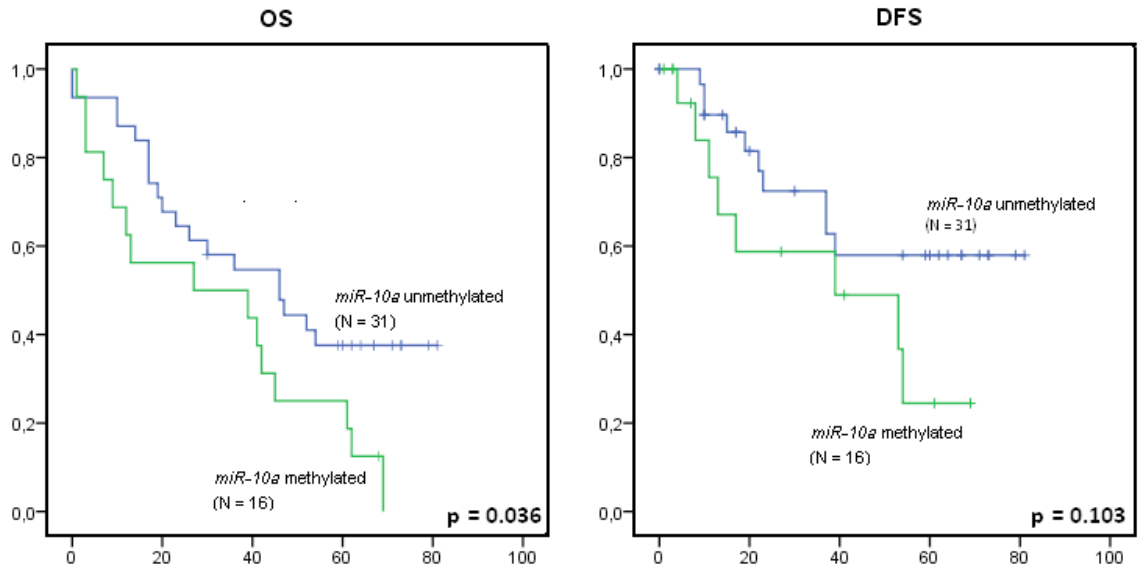

***Figure 32**. Impact of miR-10a methylation on OS and DFS in NSCLC patients.*
Kaplan-Meier plots of DFS and OS according to miR-10a methylation are shown. MiR-10a methylated patients were shown to have a statistical significant shorter OS. Association regarding DFS and miR-10a methylation did not reach statistical significance.

Moreover, *miR-10a* methylation was found more frequently in ADCs than in tumors of other histologies ($p = 0.031$). No statistically significant associations between methylation of the 5 miRNA genes and other clinico-pathological characteristics were observed.

6.4. MiRNA target prediction

To obtain information about potential targets of the selected miRNAs and how their deregulation may contribute to tumorigenesis, *in silico* miRNA target prediction using the open source program DIANA-microT-CDS 5.0 was performed (http://www.microrna.gr/microT-CDS.). The latest version of the DIANA-microT algorithm computes a miRNA-gene interaction score for predicted targets in the 3´-UTR and coding sequence transcript regions (Vlachos et al. 2012). The involvement of the five miRNAs in crucial pathways important for NSCLC pathogenesis was predicted using DIANA-mirPath prediction software DIANA-microT-4.0 (http://www.microrna.gr/miRPathv2). DIANA-miRPath is a web-based computational tool which was developed to identify molecular pathways potentially deregulated by the expression of single or multiple miRNAs. The software performs an enrichment analysis of miRNA targets comparing each set of targets with all KEGG pathways. The graphical output provides an overview of parts of the altered pathways (Papadopoulos et al. 2009).

Table 7 shows predicted miRNA targets whose deregulation may contribute to tumorigenesis and/or has already been reported in various cancer types. Predicted target genes include known oncogenes such as growth factors, receptor tyrosine kinases, serine/threonine kinases and transcription factors, as well as TSGs such as *BRCA1*, *DACH1* or *FOXO3*. These results suggest that some tumor-specifically methylated miRNAs directly regulate expression levels of certain proto-oncogenes thus function as tumor suppressor miRNAs.

***Table 7**. Selected miRNAs and their predicted target.*
Target prediction was performed using DIANA microT-CDS open source program. Targets include oncogenes whose over-expression has already been reported in various cancer types but also TSGs.

miRNA	Target	Function	Reference
miR-10a	*BCN2*	Controls nuclear processing of mRNA	(Vanhoutteghem et al. 2006)
	DACH1	Regulator of proliferation by repressing CdK inhibitors, Inhibitor of TGF-beta signaling	(Velasco-Velazquez et al. 2012, Liang et al. 2012)
	RUNX2	Bone-specific transcriptional regulator	(Akech et al. 2010)
	INO80D	Regulatory component of chromatin remodeling INO80 complex, involved in DNA repair and replication, checkpoint regulation and chromosome segregation	(Morrison et al. 2009)
	NCOR2	Part of multisubunit complex including HDACs, mediates transcriptional silencing	(Green et al. 2008) (Ghoshal et al. 2009)
	BCL2L11	Induces apoptosis	n/a
	HOXA3	TF regulating gene expression, morpho-genesis and differentiation	n/a
	HOXB3	TF involved in development	(Palakurthy et al. 2009)
	GATA6	Zinc-finger TF regulating cellular differentiation and organogenesis	(Zhong et al. 2011) (Belaguli et al. 2010)
miR-10b	*RAB4A*	Member of RAS oncogene family, regulates procathepsin L secretion	(Barbarin et al. 2011)
	ERBB4	Receptor tyrosin kinase regulating Epithelial differentiation, proapoptotic protein	(Vidal et al. 2007) (Sundvall et al. 2008)
	FOXO3	TF regulating apoptosis, post-transcriptional Regulation of MYC	(Karube et al. 2011)
miR-129-2	*MAP3K1*	Serine/threonine kinase regulating major pathways	(Rebbeck et al. 2009)
	STX6	Regulates intracellular vesicle trafficking	(Zhang et al. 2008)
	DOK4	Insulin-receptor substrate family involved In cellular growth, signaling and survival	(Gray et al. 2008)
miR-572	*ACVR2B*	Growth and differentiation factor, belonging to TGF-beta superfamily	(Senanayake et al. 2012)
	MAP3K14	Involved in activation of NF-kappa-B	
miR-615	*MEF2A*	TF involved in activation of growth factor-and stress induced genes	n/a
	MAPK7	Involved in proliferation, differentiation, cell survival	(Tesser-Gamba et al. 2012)
	BRCA1	Nuclear phosphoprotein maintaining genomic stability	(Rosen et al. 2003)
	FASN	Fatty acid synthetase catalyzes formation of long-chain fatty acids	(Flavin et al. 2010)

n/a, not available.

Next, we were interested to get further information about molecular pathways which are deregulated through methylation associated silencing of certain miRNAs and might therefore

contribute to tumorigenesis. Results of DIANA-miRPath analysis are shown in *Figure 33*. Pathways involved in tumorigenesis include TGF-beta, Wnt and Erb-B signaling and the formation of adherens junctions, protein complexes occurring at cell-cell junctions, and thight junctions, holding cells together and preventing passage of molecules and ions.

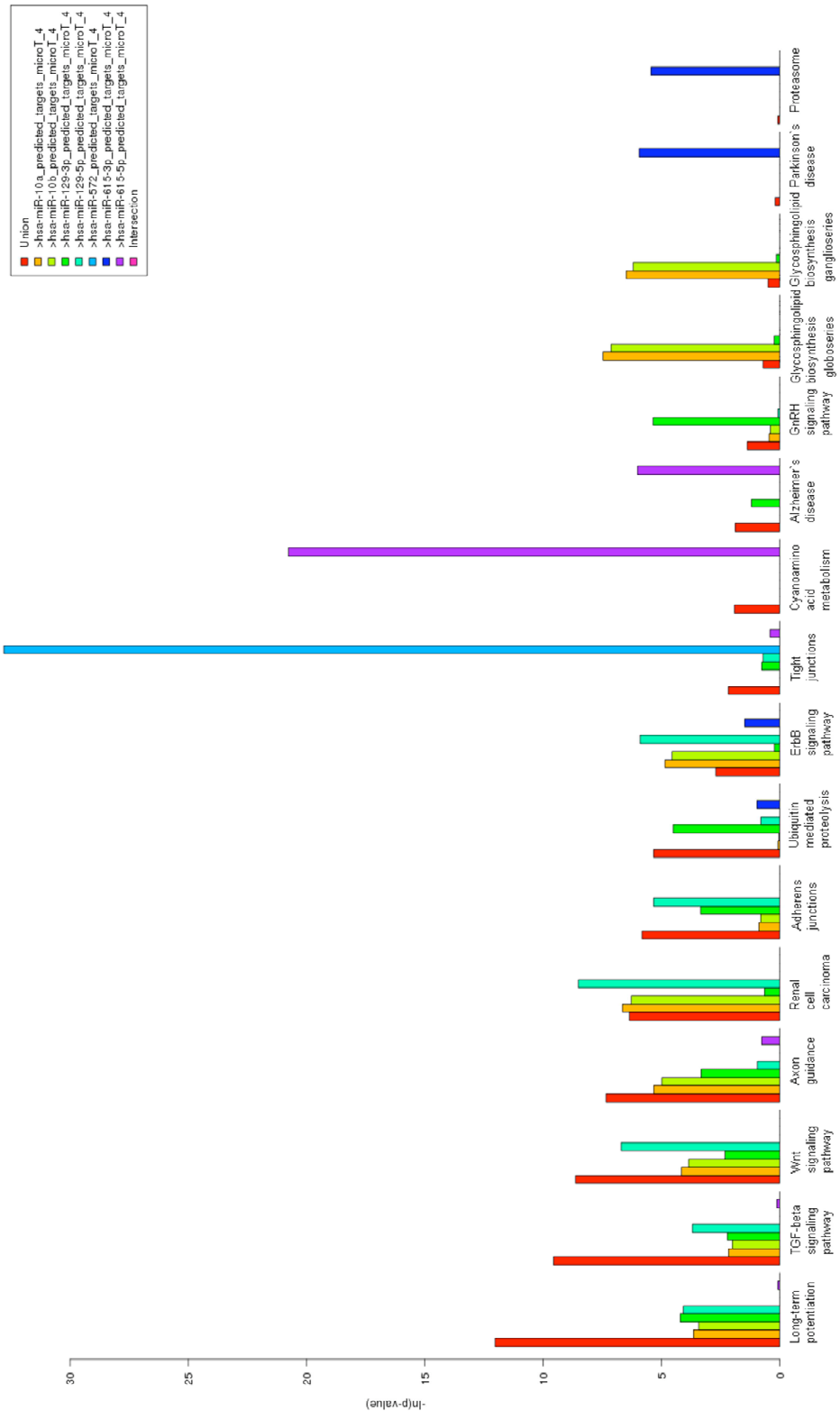

Figure 33. *Pathways which are affected by loss of selected miRNAs were identified with DIANA-miRPath prediction software. Adapted from Papadopoulos et al. 2009.*

As shown in *Figure 34*, possible targets of the five miRNAs are *PIK3R1, PIK3CA, STK4, MAPL3, RB1, RASSF5, CDK6* and *RXRB*. Deregulation of these factors might contribute to NSCLC pathogenesis affecting tumor progression, G1/S progression, cell proliferation and apoptosis *(Papadopoulos et al. 2009)*.

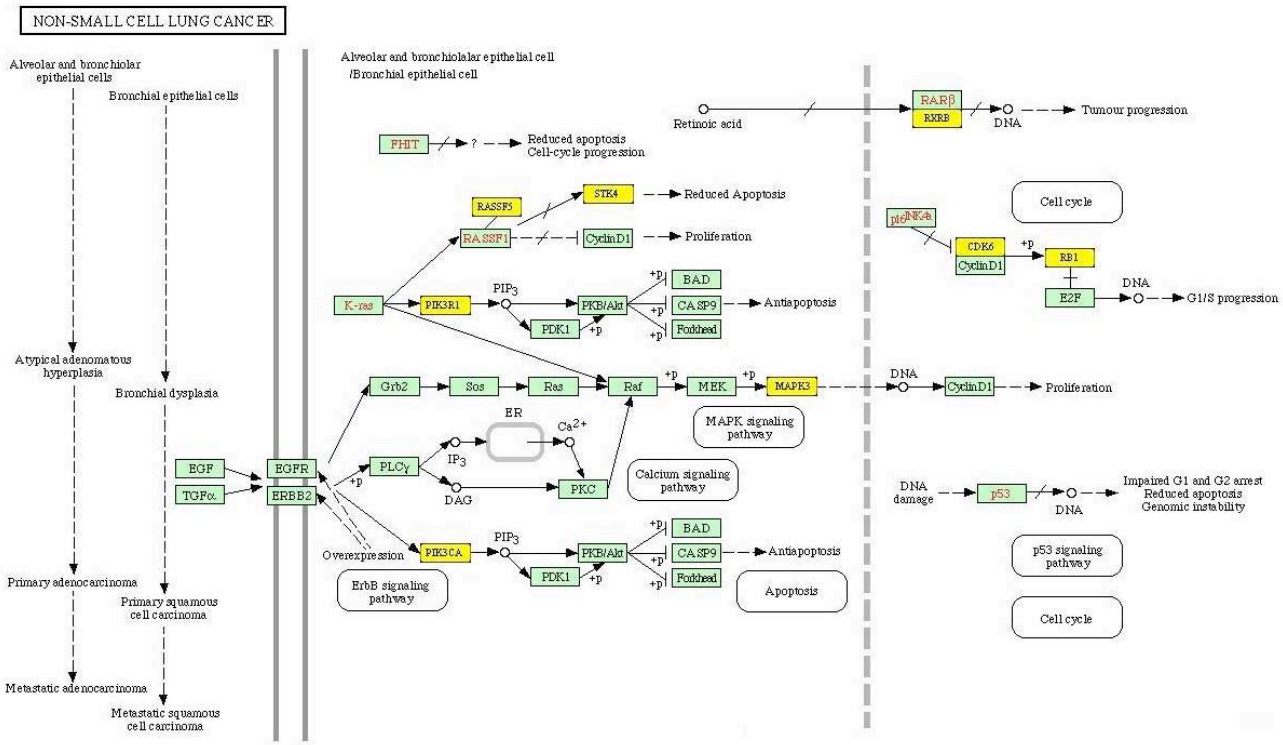

Figure 34. *Altered KEGG pathways showing implications of deregulated target genes.*
Affected targets are marked in yellow. DIANA-microT 4 program predicted possible targets of selected miRNAs and provides information how loss of these targets may contribute to pathogenesis of NSCLC. Adapted from Papadopoulos et al. 2009.

7. Discussion

Lung cancer is a heterogeneous disease whose development requires accumulation of multiple genetic and epigenetic alterations. Continued exposure to tobacco smoke, the major risk factor for lung cancer, results in accumulation of alterations in proto-oncogenes and TSGs which may lead to clonal expansion and malignant transformation (Mao 2001). A large number of genetic alterations such as mutations in the *p53* TSG and *KRAS* proto-oncogene or loss of heterozygosity in critical chromosomal regions were identified as frequently occurring events in human lung cancer. Over the last years, the role of epigenetic modifications including DNA methylation and histone modifications, their deregulation in various cancer types and the epigenome of cancer cells were extensively studied. Epigenetic aberrations in cancer cells include transcriptional silencing by CGI promoter methylation and histone deacteylation, global genomic hypomethylation, loss of imprinting and a lack of repression of intragenomic parasitic sequences (Esteller et al. 2002). Epigenetic silencing by CGI promoter methylation was demonstrated to affect important TSGs, cell-cycle genes, DNA repair genes and genes involved in invasion and metastasis and is now being recognized as a hallmark of cancer (Brown et al. 2002).

Recently, a so far relatively new abnormality was observed in various cancer types including lung cancer: the deregulated expression of miRNA genes. By posttranscriptional regulation of target gene expression, miRNAs regulate a variety of biological processes including cellular differentiation, proliferation and apoptosis (He et al. 2004). Thus, deregulated expression of certain miRNAs genes results in alterations of these processes and may contribute to the development of a malignant phenotype. Recent data suggest that abnormal expression of miRNA genes plays an important role in the pathogenesis of lung cancer (Calin et al. 2006). By performing high-throughput miRNA gene expression analyses in 104 pairs of primary NSCLC samples and corresponding non-malignant lung tissue samples, Yanaihara *et al.* (Yanaihara et al. 2006) demonstrated tumor-specific up- and down-regulation of various miRNA genes. Different mechanisms were proposed to be responsible for deregulated miRNA expression in cancer cells. One of these mechanisms is methylation mediated silencing of miRNA genes. Examples for methylated miRNAs in NSCLC analysed by MSP, COBRA and MR-HRM are members of the *let-7* family*, miR-34a, miR-34b, miR-124a*, *miR-200c*, *miR-196a*, *miR-9-3* and *miR-193a* (Lujambio et al. 2007, Gallardo et al. 2009, Ceppi et al. 2010, Wang et al. 2011, Heller et al. 2012). Some of these miRNAs target major oncogenes including *RAS* and *MYC*, while others are part of molecular pathways whose depletion may contrib-

ute to the development of a malignant phenotype. However, knowledge about miRNA methylation in lung cancer and its contributions to NSCLC pathogenesis is still limited.

To obtain more insight into this topic, we performed a high-throughput screen for methylated miRNA genes in TU and NL samples of 50 stage I NSCLC patients using the MeDIP-chip technology. This approach provides sensitive detection of genome-wide methylation and is based on isolation of methylated DNA fragments by immunoprecipitation using an anti-5´-methylcytidine antibody and was frequently used to analyse methylation of protein encoding genes in various cancer types including NSCLC (Heller et al. 2012, Heller et al. 2012, Weber et al. 2005, Wilson et al. 2006).

Based on miRBase 18 and USCS genome browser, we designed a tiling microarray containing probes for promoter regions of all known human miRNA genes. By comparing methylated miRNA genes in TU and in NL samples, we identified 138 tumor-specifically methylated miRNA genes. Some of these miRNA genes were already known to be methylated in NSCLCs. Recently, using miRNA microarray expression analyses of NSCLC cells before and after treatment with epigenetically active drugs, we identified 30 miRNAs genes which are targets for methylation in NSCLC cells (Heller et al. 2012). Moreover, *miR-9-3* and *miR-193a* were found to be tumor-specifically methylated in NSCLC patients. Both miRNA genes were also found to be tumor-specifically methylated using our MeDIP-chip approach indicating high reproducibility of our data. *MiR-124-2* which was found to be tumor-specifically methylated in our study was previously reported to be methylated in NSCLC by Kitano *et al*. (Kitano et al. 2011). Similar results were found for *miR-152* (Kitano et al. 2011). More importantly, we identified a large number of miRNA genes whose methylation status in NSCLC was unknown so far. A search for CGIs in their promoter regions revealed that only 25% of these genes are associated with a CGI. This observation was surprising, however, we suggest that methylation changes in these genomic regions may also affect non-CGI CpG dinucleotides, which was reported previously (Han et al. 2011). In addition, high enrichment of methylated DNA fragments detected by RT-PCR indicated that MeDIP procedure efficiently immunoprecipitated methylated DNA and that the false positive rate of MeDIP-chip results is low. The biologic relevance of non-CGI CpG dinucleotide methylation changes needs to be determined in additional studies.

Besides miRNA genes also snoRNAs and scaRNAs were found to be tumor-specifically methylated in NSCLC patients. ScaRNAs are a class of snoRNAs, small non-coding RNA molecules primarily guiding chemical modifications including methylation and pseudouridylation of other RNAs (Matera et al. 2007). Recent reports revealed that snoRNAs can be

processed into a class of small RNAs with miRNA-like functions (Ender et al. 2008, Taft et al. 2009).

To further investigate methylation levels of tumor-specifically methylated miRNA genes (*miR-10a*, *miR-10b, miR-129-2*, *miR-572* and *miR-615*) in NSCLC cell lines and in clinical samples of NSCLC patients, MS-HRM assays were developed. Using MS-HRM, cost efficient methylation analyses of a large number of samples is possible. To avoid unnecessary loss of clinical sample DNA, MS-HRM assays were established using DNA of NSCLC cell lines and NHBECs. As we expected, all miRNA genes were highly methylated in NSCLC cell lines but weakly methylated in NHBECs. After successful establishment of MS-HRM assays, methylation of the five miRNA genes was determined in clinical samples of 47 stage I-III NSCLC patients. Highly significant tumor-specific methylation was observed for the miRNA genes *miR-10a*, *miR-10b, miR-129-2* and *miR-572* confirming our MeDIP-chip data. Furthermore, ROC curve analyses of MS-HRM results of these genes statistically significant distinguished between TU and NL samples indicating that miRNA gene methylation might have a potential role in diagnosing NSCLCs.

So far, there are no reports about epigenetic silencing of these miRNAs in NSCLCs and possible contributions to its pathogenesis. *MiR-10a* was shown to be down-regulated by aberrant CGI hypermethylation in hepatocellular carcinomas and to be involved in metastatic processes by regulating epithelial-mesenchymal transition and cell adhesion in hepatoma cells and in gastric cancer cells (Chen et al. 2012, Shen et al. 2012, Yan et al. 2012). Recently, it was reported that *miR-10b* is down-regulated in metastatic tissue samples of renal clear cell carcinomas and glioblastomas where it is also believed to regulate angiogenesis and local invasion (Lin et al. 2012, Wotschofsky et al. 2012). Oncogenic function of *miR-10b* was reported in colorectal cancer, oral cancer and in gastric cancer (Liu et al. 2012, Lu et al. 2012, Nishida et al. 2012). Epigenetic regulation of *miR-129-2* was demonstrated in gastric cancer, esophageal squamous cell carcinoma and in endometrial cancer where repression of *miR-129-2* results in over-expression of *SOX4* oncogene (Chen et al. 2012, Huang et al. 2009, Tsai et al. 2011). So far, there are no reports about epigenetic regulation of *miR-572*, however, serum levels of *miR-572* were shown to be up-regulated in patients with chronic hepatitis B (Zhang et al. 2012). Furthermore, expression of *miR-572* is down-regulated in basal cell carcinoma (Sand et al. 2012).

Adequate staging of NSCLC patients is mandatory for an efficient treatment. However, staging based on clinical and pathological characteristics is often insufficient to predict the prognosis of NSCLC patients. Thus, eligible molecular parameters are needed to more pre-

cisely predict the prognosis of NSCLC patients. To investigate the clinical relevance of our results, MS-HRM results were compared with clinico-pathological characteristics of 47 NSCLC patients including gender, age, histology, tumor stage, lymph node stage, stage of disease, disease recurrence, DFS and OS. We observed a significantly shorter OS of *miR-10a* methylated patients compared to unmethylated patients and *miR-10a* methylation was identified as an independent prognostic factor for shorter OS in NSCLC patients. A similar association was observed regarding *miR-10a* methylation and DFS. However, these results did not reach statistical significance. Moreover, *miR-10a* methylation was found more frequently in adenocarcinomas than in tumors of other histologies. Overall, our results demonstrate a potential prognostic impact of *miR-10a* methylation on survival of NSCLC patients. However, the number of NSCLC patients used in this study was low and our results need to be confirmed in a larger study population.

It was shown that some miRNAs directly regulate expression levels of certain proto-oncogenes thereby acting as tumor suppressors (Shenouda et al. 2009). To determine if the five selected tumor-specifically methylated miRNAs target proto-oncogenes, *in silico* target prediction was performed. Predicted targets include crucial proto-oncogenes such as growth factors, receptor tyrosine kinases, serine/threonine kinases and transcription factors. Aberrant silencing of miRNA genes by DNA methylation may lead to deregulated expression of these oncogenes finally resulting in alterations of major pathways and the development of a malignant phenotype. However, these results need to be further investigated and confirmed in additional functional studies.

Furthermore, target prediction revealed that the five selected miRNAs play major roles in regulating crucial signaling pathways including Wnt pathway, Erb-B pathway and TGF-beta pathway. Potential targets in the canonical Wnt signaling pathway are *CTNNBIP1* and *MAP3K7* by *miR-10a* and *miR-10b*, *F2D10* and *SMAD3* by *miR-129-3p* and *CXXC4*, *APC* and *PPP2CB* by *miR-129-5p* having effects on the cell cycle and proteolysis. Targets in the Planar cell Polarity and Wnt/Calcium pathway include *F2D10*, *VANGL2, DVL3, DAAM1, CAMK2D, PPP3CA* and TF *NFATC2*. Alterations in the non-canonical Wnt signaling may result in changes in the cytoskeleton and in intracellular calcium concentrations having effects on cell adhesion, migration and invasion (Jauliac et al. 2002). With effects on proliferation, survival, angiogenesis, adhesion, migration, invasion and differentiation, excessive Erb-B signaling is also associated with the development of a variety of tumor types (Hynes et al. 2009). Targets of selected miRNAs are different MAP kinases, namely *MAP2K4* and *MAPK3*, PI kinases *PIK3CA* and *PIK3R1*, serine/threonine kinase *PAK7*, proto-oncogene

CRK and *NRG2*, which interacts with ErbB receptors. The TGF-beta signaling pathway is involved in major cellular processes including cell growth, cell differentiation and apoptosis with both positive and negative influences on cancer development (Derynck et al. 2001). *MiR-10a* and *miR-10b* target the ligand inhibitor *LTBP1*, *ACVR2A*, the activin type II receptor and *ID4* which encodes a DNA-binding protein inhibitor. *ID4* is also a potential target of *miR-615*. Targets of *miR-129* in the TGF-beta pathway are *FST,* which directly binds to activin, *SMAD3* and *SMAD6,* the intracellular effectors of TGF-beta signaling regulating transcription and negative feedback, *MAPK3,* TF *E2F5,* histone acetyltransferase *EP300* and TF *SP1,* which interacts with *EP300*. Depending on which target is deregulated by methylation mediated silencing of miRNA genes, altered TGF-beta signaling may exert either cancer promoting or repressing effects.

In conclusion, using MeDIP-chip technique we identified several tumor-specifically methylated miRNA genes in NSCLC patients. Epigenetic changes of most of these genes have not been studied so far. Selected miRNAs were further investigated by gene-specific approach using MS-HRM analyses to make sure that MeDIP-chip technique produces reliable results. Using this approach, we confirmed our MeDIP-chip data suggesting that this method is a powerful tool to analyse miRNA methylation on a genome-wide level. Moreover, our data suggest that *miR-10a* methylation is an independent prognostic factor for NSCLC patients. These findings could be of potential importance for a more personalized treatment and for early detection systems using methylated miRNAs as potential biomarker. By *in silico* target prediction, we found that DNA methylation mediated deregulation of certain miRNAs may lead to altered expression levels of target oncogenes, thereby contributing to the development of a malignant phenotype. Taken together, our results strenghten the importance of miRNA gene methylation in the pathogenesis of NSCLCs.

8. Table of figures

9. References

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10. Curriculum vitae

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Education

2009 - 2013	Master Studies Microbiology & Genetics; Major: Molecular Genetics and Pathology
02/2009	First Diploma in Biology
2006 - 2009	Master Studies Biology
1998 - 2006	High School: Öffentliches Gymnasium der Stiftung Theresianische Akademie Wien, Degree: Matura

Work experience

09/2011 - 09/2012	Diploma thesis "Genome-wide analysis of miRNA gene methylation in non-small cell lung cancer patients", supervised by Univ. Prof. Dr. Zöchbauer-Müller, Division of Oncology, Department of Medicine I, Medical University of Vienna
02/2011	Internship "Epigenetic studies on NPM-ALK positive anaplastic large cell lymphomas"; supervised by Univ. Prof. Dr. Gerda Egger, Division of Pathology, Department of Medicine I, Medical University of Vienna
10/2010	Internship "The role of peroxisomal HMG CoA Reductase in the Isoprenoid Pathway and Detection of the endogenous HMG

	CoA Reductase"; supervised by Univ. Prof. Dr. Johannes Berger, Center of Brain Research, Medical University of Vienna
08 - 09/2008	Volunteering in National Parks in Costa Rica, South America
06/2004	Holiday internship at "Mostly Mozart", 1010 Vienna
06/2003	Holiday internship at "Alles steuerbar" Steuerberaterkanzlei, 1010 Vienna

Lab skills

Epigenetic research techniques: MeDIP-chip, ChIP, methylation-sensitive high-resolution melting analyses (MS-HRM), MSP primer design, MSP, sodium-bisufite treatment of genomic DNA, genomic bisulfite sequencing (BGS), COBRA
Others: isolation of nucleic acids and proteins, PCR, Real-time PCR, western blotting, cell culture, cloning and transformation.

Language skills

German	Mother Tongue
English	Fluent, good presentation skills
French	Eight school years
Latin	Five school years
Russian	Three school years
Spanish	Basic knowledge

Additional skills

MS Office 95-2010, IBM SPSS, GraphPad Prism
Drivers license class B since 2005

Personal interests

Traveling, sports (Pilates, diving, jogging, horse back riding), music, arts.