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Methylation-sensitive high resolution melt analyses of 5 genes in non-small cell lung cancers

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Vielen Dank auch an meine Freunde!

4 Abbreviation

AAH	Atypical adenomatous hyperplasia
AGO	Argonaute protein
ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leukemia
ATCC	American type culture collection
Aza-dC	5-aza-2´-deoxycytidine
BAC	Bronchioloalveolar carcinoma
BAH	Bromo-adjacent homology domain
BAL	Bronchioloalveolar lavage
BGS	Bisulfite genomic sequencing
CCA	Extrahepatic cholangiocarcinoma
CGI	CpG island
CTCF	CTC binding factor with zinc finger motif
CXXC	Cysteine-rich domain
CYP	Cytochrome P450
DFS	Disease-free state
DIPNECH	Diffuse idiopathic pulmonary endocrine cell hyperplasia
DNMT1	DNA methyltransferase 1
DNMT2	DNA methyltransferase 2
DNMT3a	DNA methyltransferase 3a
DNMT3b	DNA methyltransferase 3b
dsDNA	Double stranded DNA
H-bond	Hydrogen bond
HDAC	Histone deacetylase
ICE	Imprinting control element
LCNEC	Large cell neuroendocrine carcinoma
MDB 1 – 4	Methyl-CpG-binding-protein 1-4
miRNA	Micro RNA
mRNA	Messenger RNA

MS-HRM	Methylation-sensitive high resolution melt analyses
MTase	Cytosine DNA methyltransferase
NL	Corresponding non-malignant lung tissue
NLS	Nuclear localization signal
NNK	Nicotine-derived Nitrosoaminoketon
	4-(*N*-Nitrosomethylamino)-1-(3-Pyridyl)-1-Butanone
NNN	N´-Nitrosonornicotine
NSCLC	Non-small cell lung cancer
OS	Overall survival
OSCC	Oral squamous cell carcinoma
*O*2-pobdC	*O*2-[4-(3-Pyridyl)-4-Oxobut-1-yl]-2´-Deoxycytosine
*O*2-pobdT	*O*2-[4-(3-Pyridyl)-4-Oxobut-1-yl]-2´-Deoxythymidine
O4-mT	O4-Methylthymidine
O6-mG	O6-Methylguanine
*O*6-pobdG	*O*6-[4-(3-Pyridyl)-4-Oxobut-1-yl]-2´-Dexoyguanosine
PAH	Polynuclear aromatic hydrocarbon
PCR	Polymerase chain reaction
PWWP	Proline-tryptophan-tryptophan-proline-domain
RAB14	Ras related protein 14
RFT	Replication foci-targeting domain
RISC	RNA induced silencing complex
ROC	Receiver operating characteristic
RT	Room temperature
ssDNA	Single stranded DNA
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SDS	Sodium dodecyl sulphate
SCNEC	Small cell neuroendocrine carcinoma
TOPO I	Topoisomerase I
TRD	Transcriptional repression domain
TSG	Tumour suppressor gene
TSA	Trichostatin A

TSNA	Tobacco specific n-nitrosamine
TU	Primary tumour
UPD	Uniparental disomy
WHO	World health organization
Xic	X-inactivation centre
4-ABP	4-Aminobiphenyl
7-mG	7-Metyhlguanine
7-pobdG	7-[4-(3-Pyridyl)-4-Oxobut-1-yl]-2´-Deoxyguanosine

5 Abstract

Lung cancer is one of the leading causes of cancer deaths in the world causing over 1 million deaths each year. Numerous genetic (including gene mutation and chromosomal aberration) and epigenetic abnormalities (including mainly DNA methylation and histone acetylation) have already been identified to be involved in the pathogenesis of lung cancer. These changes affect expression of both protein encoding genes and microRNA (miRNA) encoding genes. This diploma thesis is based on the results of 2 genome-wide approaches to detect DNA methylation in non-small cell lung cancer (NSCLC) patients and NSCLC cell lines. The first approach combined immunoprecipitation of methylated DNA fragments and microarray analyses and was used to identify tumour-specifically methylated genes in a large number of NSCLC patients. In the second approach NSCLC cells were treated with epigenetically active drugs followed by miRNA expression microarray analyses to identify epigenetically regulated miRNAs. Overall, 298 protein encoding genes and 33 miRNA encoding genes were identified to be targets for methylation in NSCLC. The genes *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* and *miRNA-193a* were selected for gene-specific DNA methylation analyses by methylation-sensitive high resolution melting (MS-HRM) and bisulfite genomic sequencing (BGS) in 5 NSCLC cell lines and in primary tumour (TU) and corresponding non-malignant lung tissue samples (NL) of 97 NSCLC patients, respectively. All genes were found to be methylated in nearly all NSCLC cell lines analysed (Section 12.1). While also TU were found to be methylated for these genes, only weak methylation was observed in the NL samples. These results suggest that methylation of *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* and *miRNA-193a* is tumour-specific. Some of these results were confirmed by BGS. By comparing our methylation results with clinico-pathological characteristics of the patients, we found that methylation of *HOXA2* and *miRNA-9-3* may be of prognostic relevance in squamous cell carcinoma (SCC) patients. In conclusion, our data suggest that methylation of *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* and *miRNA-193a* is an important event in the pathogenesis of NSCLC and that *HOXA2* and *miRNA-9-3* methylation may serve as prognostic parameters in SCC patients.

6 Zusammenfassung

Lungenkrebs zählt zu den führenden Todesursachen und fordert jedes Jahr über eine Million Todesopfer. Zahlreiche genetische (Genmutationen und Chromosomen-Abberationen) und epigenetische Abnormalitäten (vorallem DNA Methylierung und Histon Acetylierung) konnten als Einflussfaktoren der Pathogenese des Lungenkarzinoms identifiziert werden. Diese Veränderungen betreffen sowohl Protein-kodierende Gene als auch miRNA kodierende Gene.

Diese Diplomarbeit basiert auf Ergebnissen zweier genom-weiter Projekte, die sich mit der Detektion der DNA Methylierung in Patienten mit nicht-kleinzelligem Lungenkarzinom (NSCLC) und in NSCLC Zelllinien beschäftigten. Der erste Ansatz kombinierte Immunopräzipitation methylierter DNA Fragmente und „Microarray“ Analysen um tumor-spezifisch methylierte Gene in einer großen Anzahl von NSCLC Patienten zu identifizieren. Im zweiten Ansatz wurden NSCLC Zellen mit epigenetisch aktiven Pharmaka behandelt um mit darauf folgenden miRNA „Microarray“ Analysen epigenetisch regulierte miRNAs zu identifizieren. Insgesamt konnten 298 Protein-kodierende Gene und 33 miRNA kodierende Gene als Ziel für die DNA Methylierung identifiziert werden. Die Gene *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* und *miRNA-193a* wurden für gen-spezifische Methylierungsanalysen ausgewählt. Die Durchführung erfolgte mittels methylierungs-sensitiver hoch auflösender Schmelzkurvenanalyse und Bisulfid genomischer Sequenzierung in 5 NSCLC Zellinien und in Proben primärer Tumoren und korrespondierendem nicht-malignem Lungengewebe von 97 NSCLC Patienten. Die Methylierung der Gene konnte in allen Zelllinien mit unterschiedlicher prozentueller Auspägung bestätigt werden. Während die Methylierung dieser Gene in primären Tumoren festgestellt werden konnte, waren korrespondierende nicht-maligne Proben lediglich zu einem geringen Prozentsatz methyliert. Die Ergebnisse deuten darauf hin, dass die Methylierung der Gene *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* und *miRNA-193a* tumor-spezifisch ist. Einige dieser Ergebnisse wurden mittels Bisulfid genomischer Sequenzierung bestätigt. Durch Vergleich der Methylierungsergebnisse mit klinisch-pathologischen Charakteristika der Patienten konnten wir die Methylierung von *HOXA2* und *miRNA-9-3* als mögliche prognostische Relevanz für Patienten mit Plattenepithel-Karzinomen finden.

Schlussfolgernd deuten unsere Daten darauf hin, dass Methylierung von *HOXA9*, *SHOX2*, *TAL1*, *miRNA-9-3* und *miRNA-193a* ein wichtiges Ereignis in der Pathogenese von NSCLC ist und dass *HOXA2* und *miRNA-9-3* Methylierung als potentieller prognostischer Parameter bei Patienten mit Plattenepithel-Karzinomen dienen kann.

7 Publications

Parts of this diploma thesis were submitted for publication or have already been published:

“Genome-wide CpG island methylation analysis in non-small cell lung cancer patients”, Gerwin Heller, Valerie N. Babinsky, Barbara Ziegler, Marlene Weinzierl, **Christian Noll,** Corinna Altenberger, Leonhard Müllauer, Gerhard Dekan, Yuliya Grin, Gyorgy Lang, Adelheid End-Pfutzenreuter, Irene Womastek, Sonja Zehetmayer, Balazs Döme, Britt-Madeleine Arns, Kwun M. Fong, Casey M. Wright, Ian A. Yang, Rayleen V. Bowman, Walter Klepetko, Martin Posch, Christoph C. Zielinski, Sabine Zöchbauer-Müller, submitted for publication.

„Genome-wide miRNA expression profiling identifies miRNA-9-3 and miRNA-193a as targets for DNA methylation in non-small cell lung cancers”, Gerwin Heller, Marlene Weinzierl, **Christian Noll**, Valerie N. Babinsky, Barbara Ziegler, Altenberger C, Minichsdorfer C, Lang G, Döme B, End-Pfützenreuter A, Arns BM, Grin Y, Klepetko W, Zielinski CC, Zöchbauer-Müller CC, Clin Cancer Res. 2012 Mar 15;18(6):1619-29. Epub 2012 Jan 26.

8 Basis for this diploma thesis

DNA methylation is part of the epigenetic gene regulation complex which is relevant for the pathogenesis of non-small cell lung cancers (NSCLC). Ao. Univ. Prof. Dr. Sabine Zöchbauer-Müller and her colleagues performed a genome-wide search for methylated CpG islands (CGI) in TU and NL samples of 101 stage I-III NSCLC patients by combining methylated DNA immunoprecipitation (MeDIP) and microarray analysis (MeDIP-chip) using NimbleGen´s 385K Human CpG Island plus Promoter arrays. They identified 298 unique tumour-specifically methylated genes. Gene Ontology analyses revealed that about half of the tumour-specifically methylated genes are involved in regulation of gene expression and cell adhesion. Based on these results, several genes were selected for further gene-specific DNA methylation analysis in NSCLC cell lines and in clinical samples of NSCLC patients during this diploma thesis.
In a second research project, Ao. Univ. Prof. Dr. Sabine Zöchbauer-Müller and colleagues performed a genome-wide screen for methylated miRNA encoding genes in NSCLCs. In brief, they investigated expression of 856 miRNAs before and after treatment of NSCLC cells (A549) with the DNA methyltransferase inhibitor 5-aza-2´-deoxycytidine (Aza-dC) and/or the histone deacetylase inhibitor trichostatin A (TSA). Overall, 66 miRNAs were identified whose expression was up-regulated after drug treatment. 33 of these miRNA genes are associated with a CGI and 2 of these genes were further analysed for methylation in clinical samples of NSCLC patients during this diploma thesis.

9 Introduction

9.1 Epidemiological overview of lung cancer

During the last decades lung cancer became the leading cause of cancer deaths with more than a million deaths a year worldwide (Jemal et al 2010, Wen et al 2011).

In the year 2008 lung cancer was the most frequent cancer worldwide in men resulting in 1.3 Million lung cancer deaths (Ferlay J 2008). Compared with the main occurring cancer types in men, lung cancer had an incidence of 16.5% (Figure 1). The mortality rate of lung cancer was 22.5% (Figure 2).

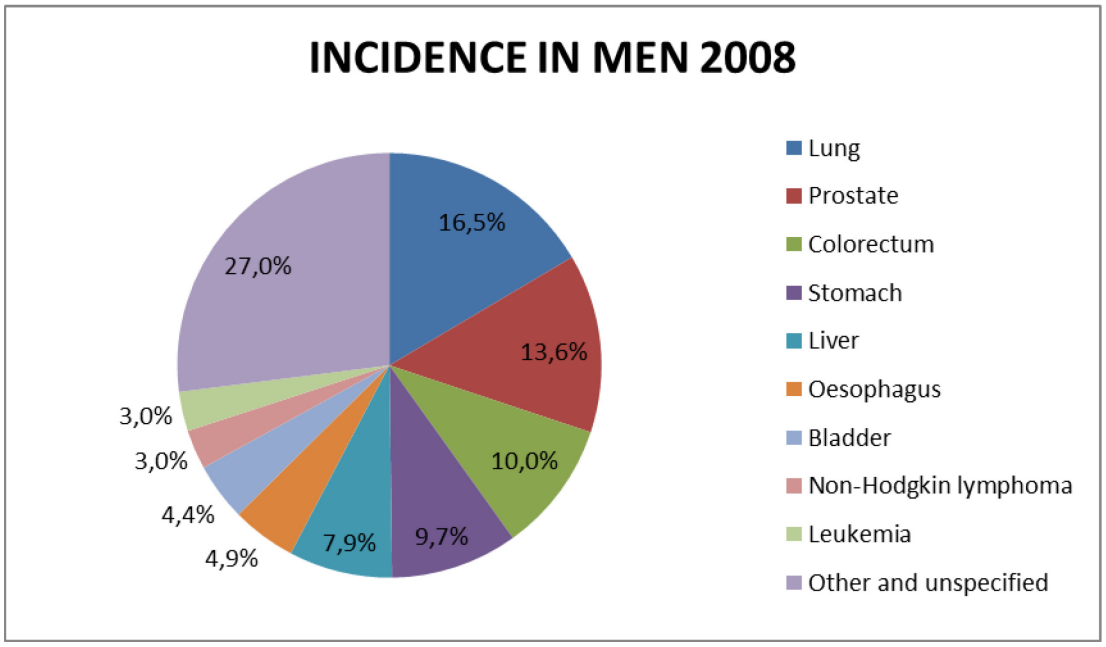


Figure 1. Incidence of cancer types in men in the year 2008. Lung (16.5%), Prostate (13.6%), Colorectum (10%), Stomach (9.7%), Liver (7.9%), Oesophagus (4.9%), Bladder (4.4%), Non-Hodgkin lymphoma (3.0%), Leukemia (3.0), other and unspecified cancer types (27%) Figure adapted from reference (Ferlay J 2008).

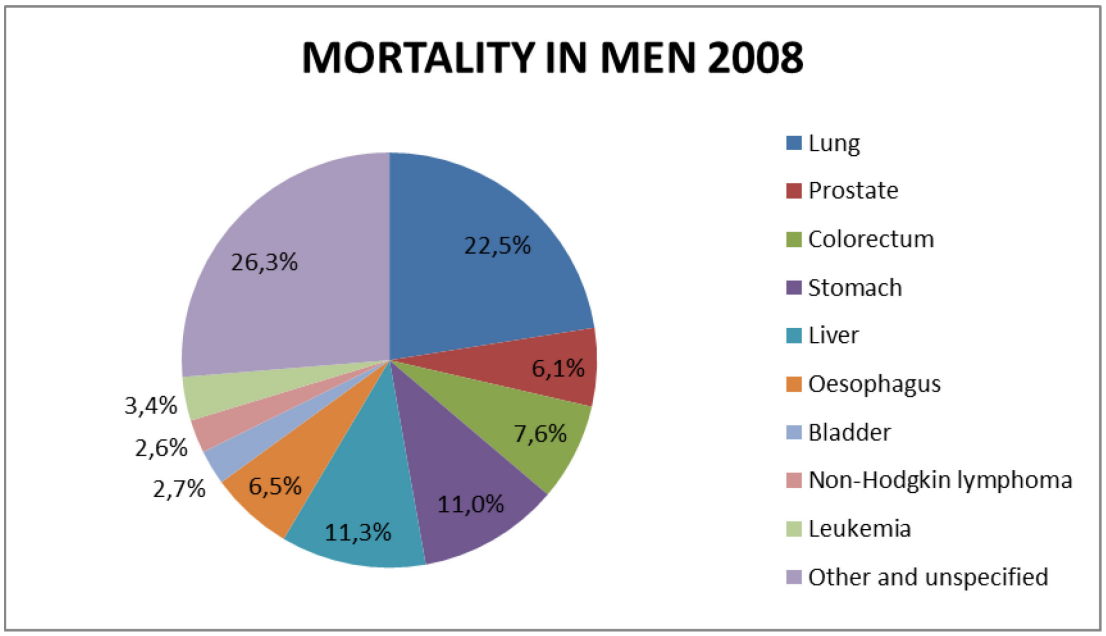


Figure 2. Mortality of cancer types in men in the year 2008. Lung (22.5%), Prostate (6.1%), Colorectum (7.6%), Stomach (11%), Liver (11.3%), Oesophagus (6.5%), Bladder (2.7%), Non-Hodgkin lymphoma (2.6%), Leukemia (3.4%), other and unspecified (26.3%) Figure adapted from reference (Ferlay J 2008).

In the year 2008 lung cancer was one of the most frequently occurring cancer type in women worldwide resulting in 420.000 lung cancer deaths (Ferlay J 2008). Comparing with the main occurring cancer types in women, lung cancer had an incidence of 8.5% (Figure 3). The mortality rate of lung cancer in women was 12.8% (Figure 4).

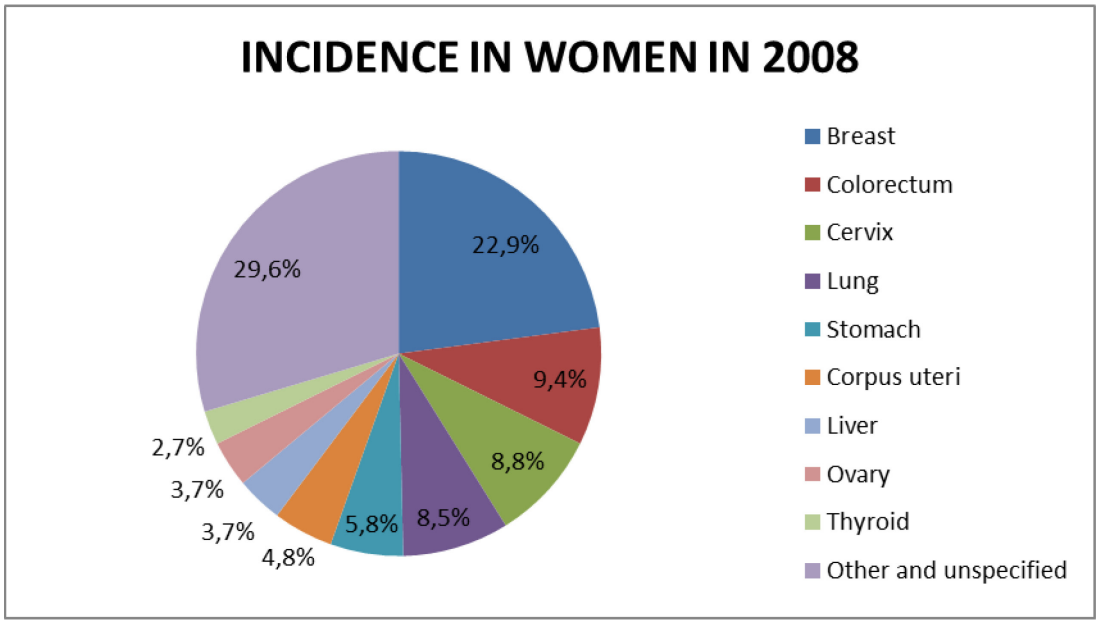


Figure 3. Incidence of cancer types in women in the year 2008. Breast (22.9%), Colorectum (9.4%), Cervix (8.8%), Lung (8.5%), Stomach (5.8%), Corpus uteri (4.8%), Liver (3.7%), Ovary (3.7%), Thyroid (2.7%), other and unspecified (29.6%) Figure adapted from reference (Ferlay J 2008).

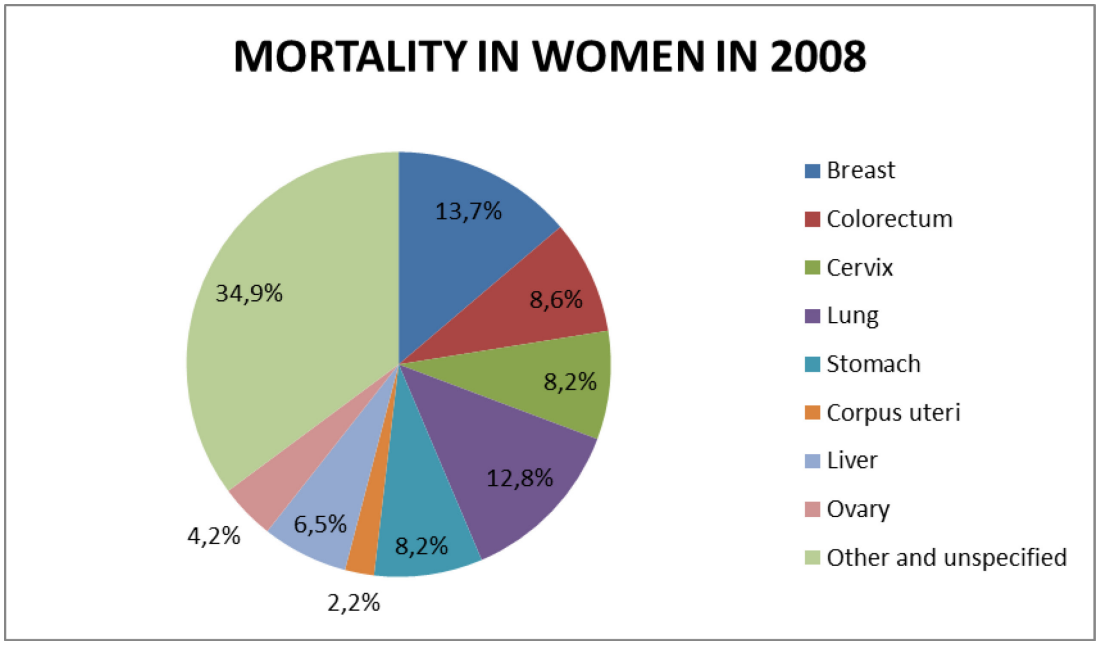


Figure 4. Mortality of cancer types in women in the year 2008. Breast (13.7%), Colorectum (8.6%), Cervix (8.2%), Lung (12.8%), Stomach (8.2%), Corpus uteri (2.2%), Liver (6.5%), Ovary (4.2%), other and unspecified (34.9%) Figure adapted from reference (Ferlay J 2008).

The statistics for Europe reported similar results for men and women in the year 2008. Comparing with the main cancer types in men, lung cancer had an incidence of 16.8% and a mortality rate of 26.6% (Ferlay J 2008). The incidence in women was 6.8% and the mortality rate was 11.5% (Ferlay J 2008).

Very similar data were shown for Austria. Lung cancer was the leading cause of death in men and the second cause of death in women. The incidence in men was 13.6% and the mortality rate was 22.8% (Ferlay J 2008). The incidence in women was 8.4% and the mortality rate was 13.3% (Ferlay J 2008).

It is predicted that lung cancer increases about 45% from 2007 to 2030.

9.2 Risk factors of lung cancer

The main risk factor for the development of lung cancer, is tobacco smoking both active as well as second hand smoking (Alberg et al 2005, Alberg et al 2007, Boffetta 2006, Chen et al 2004, Cooter 2000, Doll and Hill 2004, Doll et al 2005, Evans 1962, Hackshaw et al 1997, Henley et al 2004, Vineis et al 2005, White 1990, Whitrow et al 2003, Wynder and Graham 1950).
The second leading cause of lung cancer is the naturally occurring gas radon (Alberg et al 2007, Boffetta 2004, Chen 2005, Puskin 1992, Warner et al 1996).
Additional factors which may increase the risk of lung cancer development are air pollution, arsenic, arsenic containing compounds, asbestos, silica and chromium (Alberg et al 2007, Boffetta 2004, Boffetta 2006, Hughes et al 2011, Whitrow et al 2003).

9.3 Tobacco and lung cancer

Tobacco smoking was confirmed to be the predominant risk factor for lung cancer development (Bartal 2001, Boyle 1993, Boyle 1997, Doll et al 1994, Doll et al 2004, Gan et al 2007, Pukkala et al 2009, Ray et al 2010, Roychowdhury et al 2005). It was shown that 80% to 90% of US-lung-cancer patients are smokers. Tobacco smoke includes 3996 different components (WHO 2004).

69 of these components have been identified as carcinogens including 10 variants of polynuclear aromatic hydrocarbons (PAHs), 6 heterocyclic hydrocarbons, 4 volatile hydrocarbons, 3 nitrohydrocarbons, 4 aromatic amines, 8 N-heterocyclic amines, 10 N-nitrosamines, 2 aldehydes, 10 miscellaneous organic compounds, 9 inorganic compounds and 10 phenolic compounds (Hecht 1999, Hoffmann et al 2001, Peterson 2010, Wen et al 2011, Wogan et al 2004). Furthermore it has been reported that 11 compounds which are listed as “IARC Group 1 human carcinogens”, are included in tobacco smoke. These carcinogens are: 2-naphthylamine, 4-aminobiphenyl, benzene, vinyl chloride, ethylene oxide, arsenic, beryllium, nickel compounds, chromium, cadmium and polonium-210 (Hecht 1999, Hoffmann et al 2001, Kavvadias et al 2009a, WHO 1987, WHO 1994, WHO 2004, Wogan et al 2004). Some

substances were under further investigation because of their observed carcinogenicity. These substances are: benzo-[a]pyrene which is a surrogate for PAHs, tobacco-specific N-nitrosamines (TSNA), especially N′-nitrosonornicotine (NNN) and 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) and aromatic amines, especially 4-aminobiphenyl (4-ABP) (Hecht 1998, Hecht 2002, Hecht et al 2004, Kavvadias et al 2009a, Vineis and Pirastu 1997, WHO 2004, Wogan et al 2004). Putative precursors of NNN and NNK are nicotine and nitrate (Peterson 2010, Upadhyaya et al 2006, WHO 2004, Wogan et al 2004).
The exchange of the methyl group of nicotine with a nitroso group results in formation of NNN (Hecht 1998, Hecht 2003, Upadhyaya et al 2006). The chemical structures of Nicotine, NNN and the related NNK are shown in figure 5.

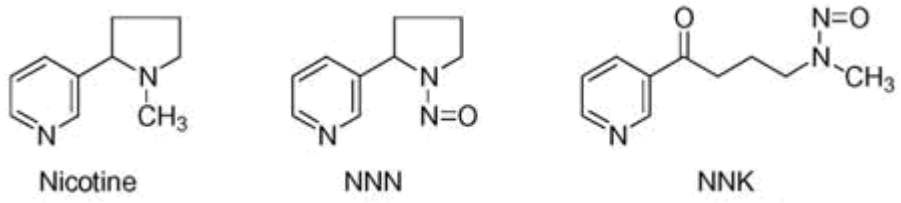


Figure 5. Chemical structures of tobacco smoke components with addictive and carcinogenic effects. Nicotine, the structurally related N´nitrosonornicotine (NNN) and nitrosamine 4 (methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Figure adapted from reference (Upadhyaya et al 2006).

Benzo[a]pyrene and nicotine-derived nitrosoaminoketone (NNK) are aromatic hydrocarbons and members of the most important carcinogens (Hecht 1998, Hecht 1999, Peterson 2010, Sun et al 2007, Wen et al 2011, WHO 2004). These carcinogens and accordingly their metabolites, which are produced during smoking, are able to cause cell proliferation and initiate survival signals. In addition, these signals are able to result in preneoplastic changes in bronchial epithelial cells and thus can lead to lung cancer in laboratory animals (Peterson 2010, Sun et al 2007, Wen et al 2011). It has been reported that the carcinogens NNN and NNK can not only induce tumour growth in the lung and the respiratory tract but also in the liver, the nasal cavity and the oesophagus of laboratory animals (Hecht et al 1986a, Hecht and Hoffmann 1988, Hecht 1998, Hecht 2003, Peterson 2010, Upadhyaya et al 2006).
Because of the evidence of NNK in urine indication of the exposure and metabolism of humans to that carcinogen were done (Hecht et al 1993, Lackmann et al 1999, Parsons et al

1998, Peterson 2010). NNN and its glucuronide are detectable in the urine of both smokers and second hand smokers and in toenails of smokers (Kavvadias et al 2009a, Kavvadias et al 2009b, Peterson 2010, Stepanov and Hecht 2005, Stepanov et al 2009).

9.4 Metabolism of NNN and NNK

For the establishment of the toxicological outcome, NNN and NNK have to be converted to a defined metabolism (Hecht 1998, Peterson 2010, Upadhyaya et al 2006). Metabolising of these substances leads to DNA adduct formation ending in miscoding and in the development of permanent mutations (Upadhyaya et al 2006).

The pyridyloxobutylating pathway results in the DNA adducts 7-[4-(3-pyridyl)-4-oxobut-1-yl]-2´-deoxyguanosine (7-pobdG) [42], *O*2-[4-(3-pyridyl)-4-oxobut-1-yl]-2´-deoxycytosine (*O*2-pobdC) [43], *O*2-[4-(3-pyridyl)-4-oxobut-1-yl]-2´-deoxythymidine (*O*2-pobdT) [43], and *O*6-[4-(3-pyridyl)-4-oxobut-1-yl]-2´-deoxyguanosine (*O*6-pobdG) (Hecht et al 2004, Peterson 2010, Wang et al 1997, Wang et al 2003). The schemes of these pathways are illustrated in figure 6.

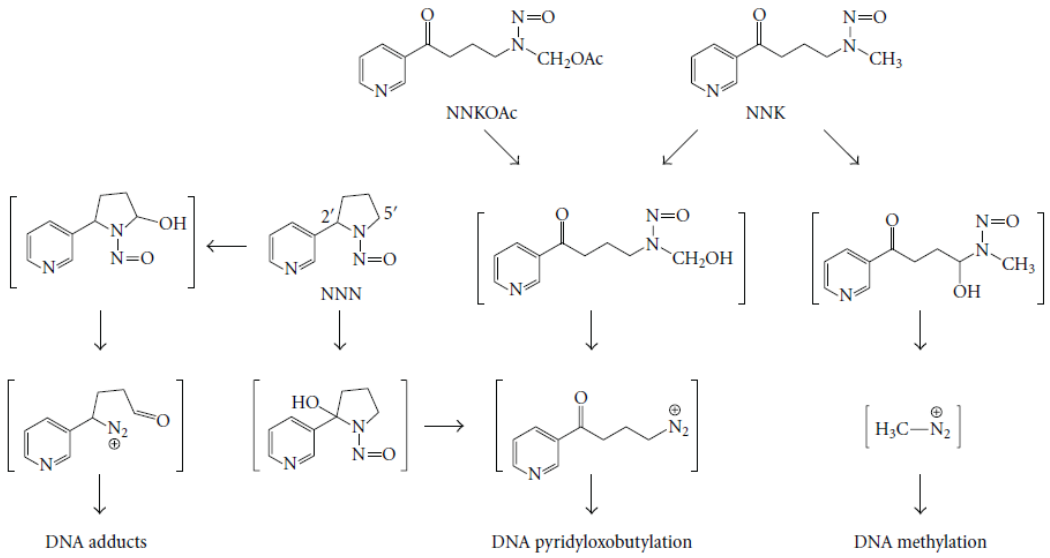


Figure 6. Biosynthetic pathways of NNK, NNN and pyridyloxobutylating agents. Figure adapted from reference (Peterson 2010).

Two pathways are known which lead to DNA adduct formation of 2´- and 5´-hydroxylation from NNN (Hecht 1998, Peterson 2010). These pathways are 2´hydroxylation of (S)-NNN and both 2´- and 5´hydroxylation of (R) NNN (McIntee and Hecht 2000, Peterson 2010). Methyl-hydroxylation of NNK and 2´hydroxylation of both (S) and (R) NNN build the same pyridyloxobutylating agent as shown in figure 2. The result of 5´hydroxylation is a metabolite which alkylates DNA (Figure 6) (Peterson 2010, Upadhyaya et al 2006).

In addition, other substances like cholesterol, steroids and some other lipids were identified to be involved in the development of potent carcinogens (Oyama et al 2008, Weng et al 2007). The CYP enzyme cytochrome P450 has major impact in the synthesis of these substances and plays an important role in the metabolism of therapeutic drugs and carcinogens for the inactivation of derivates (Oyama et al 2008, Singh et al 2011, Weng et al 2007). Cytochrome P450 is involved in NNK-induced carcinogenesis (Peterson 2010, Weng et al 2007). Metabolites of NNK are methylating or pyridyloxobutylating agents (Peterson 2010). The methyl DNA adducts are: 7-methylguanine (7-mG), *O*6-methylguanine (*O*6-mG), and *O*4-methylthymidine (*O*4-mT) (Belinsky et al 1986, Belinsky et al 1990, Hecht et al 1986b, Murphy et al 1990, Peterson 2010).

9.5 Non-small cell lung cancer

The two main types of lung cancer are small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (Brambilla et al 2001, Molina et al 2008). The main types of NSCLC are adenocarcinomas with approximately 50%, squamous cell carcinomas with about 30% and large cell carcinomas with of about 20% (Wang et al 2012). The appearance of adenocarcinomas rose by 10% in the last 20 years (Brambilla et al 2001, Ramalingam et al 1998).

9.6 Epigenetics

Conrad Hal Waddington (1905-1975) was a famous scientist in the areas of embryology and genetics. He created the concepts of the “epigenetic landscape” and “genetic assimilation” (Haig 2004). Waddington´s definition of epigenetics was: “The study of causal mechanisms by which the genes of the genotype bring about phenotypic effects” (Haig 2004). Developmental events occur from the oocyte fertilization to the mature organism and could be associated with a relationship between genes and development (Haig 2004, Slack 2002). Modern definitions for epigenetics were done by Gottschling: “A change in phenotype that is heritable but does not involve DNA mutation” and Riggs: “The study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence” (Haig 2004, Riggs 1996).

Epigenetics extends over a broad field of molecular biology including several organisms with correlating discovered effects. Epigenetic studies have been done in different model organisms like Saccharomyces cerevisiae, Schizosaccharomyces pombe, Drosophila, several plants and ciliates. The classic epigenetic mechanisms are “Dosage Compensation”, “Position effect of variegation”, “Mating type switching in yeast”, “Heterochromatin formation”, “Imprinting” and the influence of “Polycomb” and “Trithorax” group proteins on differentiation and cell identity. Because of the influence of “DNA Methylation”, which is the main aspect of this diploma thesis, on “Dosage Compensation” and “Genomic Imprinting, these two aspects are briefly mentioned below.

9.6.1 X-chromosomal inactivation/dosage compensation

First suggestions for “X-chromosomal inactivation” or “Dosage Compensation” in female mammals were done by Mary Lyon´s followed by several studies for the support of this hypothesis done by Beutler, Gartler and Linder and Nance (Beutler 1964, Gartler and Linder 1964, Lyon 1961, Nance 1964, Nance and Uchida 1964). Responsible for the inactivation of female X chromosomes resulting in one residual X chromosome is the X-inactivation centre (Xic). This centre harbours three essential genes. The noncoding RNA Xist is for in *cis* binding

and accumulation on the total chromosome (Brockdorff et al 1992, Brown et al 1991, Brown 1991) leading to the inactivation of the covered X chromosome (Lee et al 1996, Penny et al 1996, Wutz and Jaenisch 2000). The expression of Xist is regulated by Tsix (Lee et al 1999). It has been reported that DNA methylation plays a major role to stabilize the inactive status (Sado et al 2000, Sado et al 2004).

9.6.2 Genomic imprinting

Mammalians consists of a set of 2 matched chromosomes, each inherited from the parental genomes. Progenies so have 2 copies of every gene with basically equal capability for gene expression. Genomic imprinting influences expression of certain genes by DNA methylation in a manner of parental inheritance. If one imprinted parental copy of a gene is expressed, the other copy is silenced leading to parental-specific gene expression.

In the year 1985 more knowledge about chromosomal imprinting was gained by first nuclear transfers in mice (Cattanach and Kirk 1985, Solter et al 1985). It was concluded that not only DNA information but also information depending on the parental origin and the correlating gene expression might be important for further development. A mouse model experiment with uniparental disomy chromosomes (UPD) showed different phenotypes using either two maternal or two paternal copies of distinct genes leading to the implication of distinct gene expression depending on the origin (Cattanach and Kirk 1985). Further studies confirmed the influence of DNA methylation on genomic imprinting using transgenic mouse models (Reik et al 1990). Improvements in vertebrate epigenetics were done in the aspect of correlation of DNA methylation with chromosomal imprinting and X-inactivation with deeper interest in the Igf2-H19 locus (Ariel et al 1993, Li et al 1993, Tilghman et al 1993, Willard et al 1993).

So far, approximately 80 imprinted genes arranged in clusters have already been identified (Verona et al 2003). A regulatory DNA element, responsible for the regulation of imprinting and so responsible for the regulation of gene expression has been established and is called imprinting control element (ICE). DNA methylation in genomic imprinting could act as a de

novo imprinting mark by the chromosomes of one gamete or as silencer of parental alleles in terms of repression of gene expression.

9.6.3 Epigenetic gene silencing

Epigenetic mechanisms largely contribute to the regulation of transcriptional gene activities. Among these mechanisms, methylation and various chemical modifications of histone proteins (acetylation, methylation, phosphorylation, sumoylation, ubiquitination, ADP-ribosylation) are key regulators which affect the binding of transcription factors to DNA and which change the chromatin structure resulting either in gene activation or gene silencing (Rauch et al 2008, Zhong et al 2007).

9.6.4 DNA methylation

DNA methylation describes the covalent addition of a methyl group to the 5´carbon of cytosine bases within a CpG dinucleotide (Figure 13) (Cooper 1983). CpG dinucleotides are underrepresented in the mammalian genomes but are accumulated in so called CpG islands (CGIs) in a higher density. These CGIs are associated with ~70% of human genes and are harboured in approximately 60% of human gene promoter regions (Figure 7) (Ioshikhes and Zhang 2000, Larsen et al 1992, Wang and Leung 2004). CGIs are defined by the following criteria: 1) “a minimum DNA sequence length of 500 base pairs”, 2) “a G + C content of about 55%” and “ 3) “a CpG observed/expected ratio of 0.65” (Takai and Jones 2002, Wang and Leung 2004). In general, CGIs are unmethylated. CGI methylation occurs in healthy, non-malignant cells during developmental processes, genomic imprinting, X chromosomal inactivation and tissue-specific gene expression respectively (Ariel et al 1993, Bird 1986, Gardiner-Garden and Frommer 1987, Li et al 1993, Razin and Cedar 1994, Reik et al 1990, Sado et al 2000, Sado et al 2004, Tilghman et al 1993, Willard et al 1993). In addition, it has been shown that methylation of CGI associated CpG dinucleotides in the promoter region of cancer-associated genes occur frequently in malignant cells (Baylin et al 2001, Costello et al 2000, Esteller 2008, Herman and Baylin 2003, Jones and Baylin 2007). Moreover, it has been suggested, that up to 4.500 CpG islands (mean ~600) may be methylated in a tumour

(Costello et al 2000). As described in detail in section 9.8, CGI methylation is involved in regulation of transcriptional gene activity. While a gene which is associated with an un-methylated CGI is actively transcribed, a gene which is associated with a methylated CGI is transcriptionally silenced.

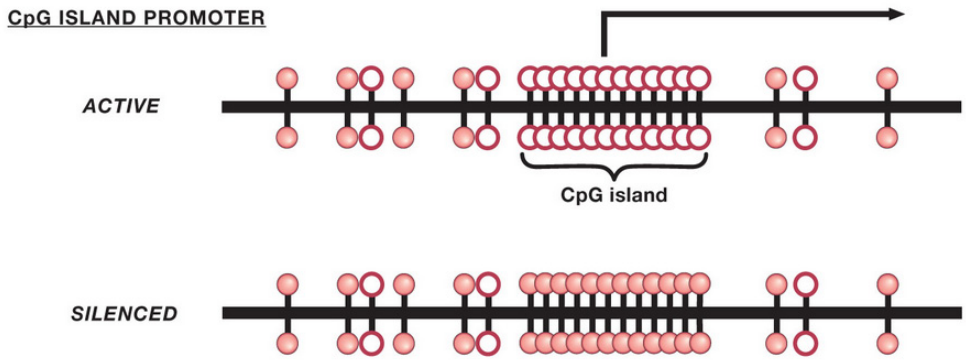


Figure 7. Genomic region with a CGI in a gene promoter which harbours a high density of CpG dinucleotides. The self-complementary CpG pairs are illustrated as vertical strokes. The active promoter is assigned with unmethylated cytosine in CpG dinucleotides (white spots). The silenced promoter is assigned with methylated cytosine (filled spots) (C. David Allis 2007). With permission from Cold Spring Harbor Laboratory Press, Copyright holder is Cold Spring Harbor Laboratory Press

It has been reported that certain transcription factors are able to recognize GC-rich motifs but a couple of them are not able to interact with methylated cytosine bases of CpG promoter sequences (Watt and Molloy 1988). This repression of interaction of transcription factors at transcription factor binding sites of gene promoters may lead to repression of gene expression. This has been proven by several studies which were engaged in the role of the CTC binding factor (CTCF) in imprinting at the H19/Ifg2 locus (Bell et al 1999, Wallace and Felsenfeld 2007).

9.6.4.1 Interaction between DNA methylation and histone protein changes

It has been shown that a crosstalk between methylated DNA and specifically modified histone proteins exists (Eden et al 1998, Espada et al 2004, Heller et al 2008, Ikegami et al 2009, Jaenisch and Bird 2003, Zhong et al 2007, Zochbauer-Muller et al 2005). Eukaryotic DNA is organized in a defined arrangement consisting of chromatin, chromatin-associated proteins and an octamer of histones. This histone octamer contains two copies of the histones H2A, H2B, H3 and H4 (Luger et al 1997, Peters et al 2003, Shogren-Knaak et al 2006). The N-terminal regions are called histone-tails and can variably be modified. These characterized modifications are phosphorylation, ubiquitylation, sumoylation, ADP-ribosylation, biotinylation, proline isomerization, methylation and acetylation (Heller et al 2010, Vaquero et al 2003). Transcriptional activity is positively or negatively influenced by altered chromatin structure depending on these modifications (Heller et al 2010, Shogren-Knaak et al 2006, Vettese-Dadey et al 1996). Gene silencing may also be a result of a crosstalk between DNA methylation and histone acetylation (Eden et al 1998, Espada et al 2004, Heller et al 2008, Ikegami et al 2009, Jaenisch and Bird 2003, Zhong et al 2007, Zochbauer-Muller et al 2005). A methylated CGI is recognized and bound by a methyl-CpG binding protein (MBD). Several MBDs are currently known: MeCP2, MBD1, MBD2, MBD3, MBD4 and additional the unrelated protein Kaiso which is also involved in repression of gene expression (Figure 8) (Bird and Wolffe 1999, Fujita et al 1999, Jin et al 2005, Kass et al 1997, Majumder et al 2006, Meehan et al 1989, Nan et al 1998a, Ng et al 1999, Ng et al 2000, Prokhortchouk et al 2001, Yoon et al 2003, Yu et al 2001).

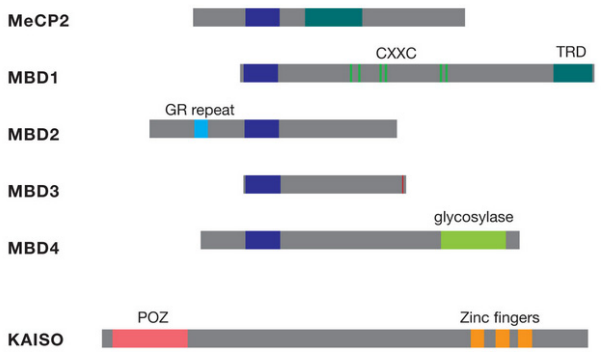


Figure 8. Methyl CpG binding proteins. Members of the MBD family with their significant domains, Transcriptional Repression Domains (TRD), CXXC, zinc fingers of KAISO is for bind-

ing of methylated DNA is shown (C. David Allis 2007). With permission from Cold Spring Harbor Laboratory Press, Copyright holder is Cold Spring Harbor Laboratory Press

The MBD-DNA complex is then recognized and bound by a protein complex which contains transcriptional repressors and co-repressors (e.g. mSins3A, SETDB1, NuRD) (Jin et al 2005, Jones et al 1998, Nan et al 1998b). Beside others, this protein complex also includes histone deacetylases. By binding of the protein complex to DNA, histone deacetylases deacetylate histone proteins H3 and H4. Because acetyl groups are negatively charged, loss of acetyl groups results in positive overall charge of histone proteins and higher affinity to negatively charged DNA. As a result, chromatin is tightly packed and transcription is blocked (Eden et al 1998, Espada et al 2004, Ikegami et al 2009).

9.6.4.2 Reversibility of epigenetic changes

Epigenetic alterations are reversible processes (Altucci and Minucci 2009, Das and Singal 2004, Egger et al 2004, Glaser 2007, Graham et al 2009, Heller et al 2012, Lyko and Brown 2005, Villar-Garea et al 2003, Villar-Garea and Esteller 2004). The reversibility of DNA methylation or the inhibition of histone deacetylation is performed pharmacologically resulting in re-expression of prior silenced genes (Cameron et al 1999, Egger et al 2004, Glaser 2007, Graham et al 2009, Heller et al 2010, Heller et al 2012, Lyko and Brown 2005, Villar-Garea et al 2003, Villar-Garea and Esteller 2004). Epigenetically active drugs are DNA demethylating agents and histone deacetylase (HDAC) inhibitors.
DNA demethylating agents may act as suicide substrates after incorporation into DNA, inhibition by masking the DNA methyltransferase (DNMT) target sequence or block active site of DNMT enzymes (Flynn et al 2003, Graham et al 2009, Lyko and Brown 2005, Santi et al 1984). Such drugs for instance are the nucleoside analogues 5-azacytidine (Vidaza), 5-aza-2´-deoxycytidine (Decitabine), 1-β-D-ribofuranosyl-2(1H)-pyrimidinone (Zebularine) and the non-nucleoside inhibitors procaine, epigallocatechin-3-gallate (EGCG) and RG108 (Figure 9) (Graham et al 2009, Heller et al 2012, Lyko and Brown 2005, Nakagawa et al 2004, Santi et al 1984). The incorporation mechanism of nucleoside analogues is shown in figure 10.

HDAC inhibitors may induce arrest of cell growth, differentiation and apoptosis by different mechanisms (Bi and Jiang 2006). Inhibitory effects are performed either by polar ends of HDAC inhibitors by binding zinc ion of the catalytic pocket structure or by hydroxamic acid groups acting on catalytic sites of HDACs (Bi and Jiang 2006, Finnin et al 1999). Another mechanism is the inhibition of active site of HDAC (Bi and Jiang 2006). Such HDAC inhibitors for instance are vorinostat (SAHA), romidepsin (depsipeptide, FK228), belinostat (PXD101), LAQ824/LBH589, MS-275 and trichostatin A (Bi and Jiang 2006, Glaser 2007, Heller et al 2012, Villar-Garea and Esteller 2004, Yoshida et al 1990).

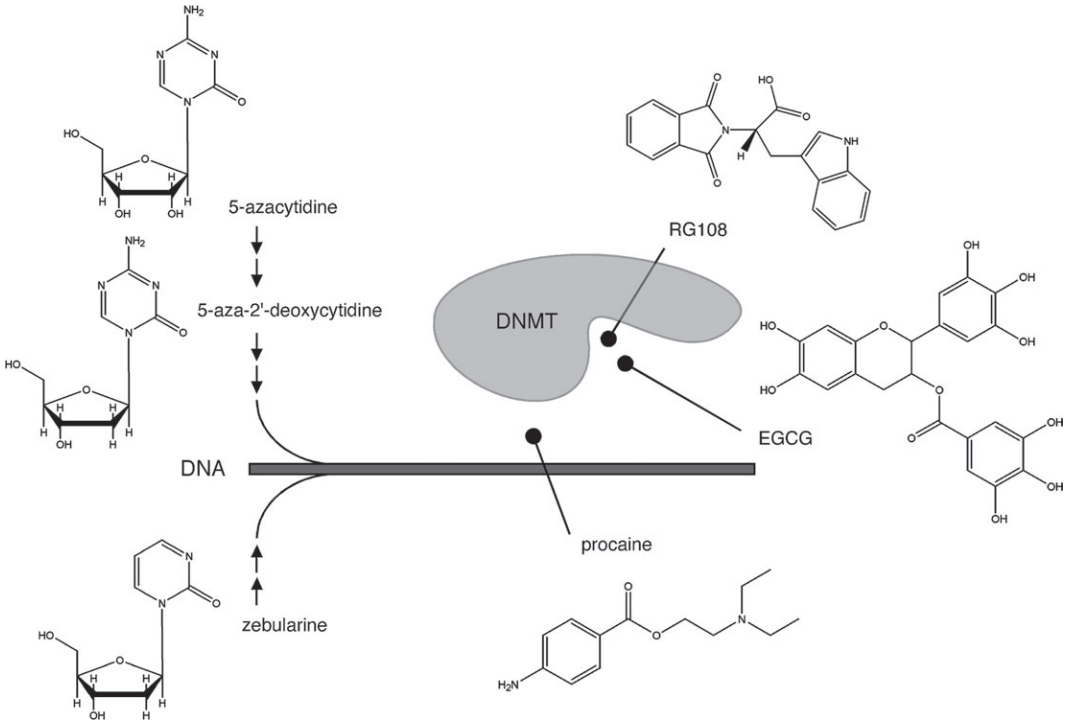


Figure 9. DNMT inhibitors and their mechanisms. 5-azazytidine, 5-aza-2´-deoxycytidine and zebularin get incorporated into DNA (black arrows); RG108 and EGCG block active sites of DNMTS (black dots); procaine masks DNMT target sequences (black dot) (Lyko and Brown 2005).

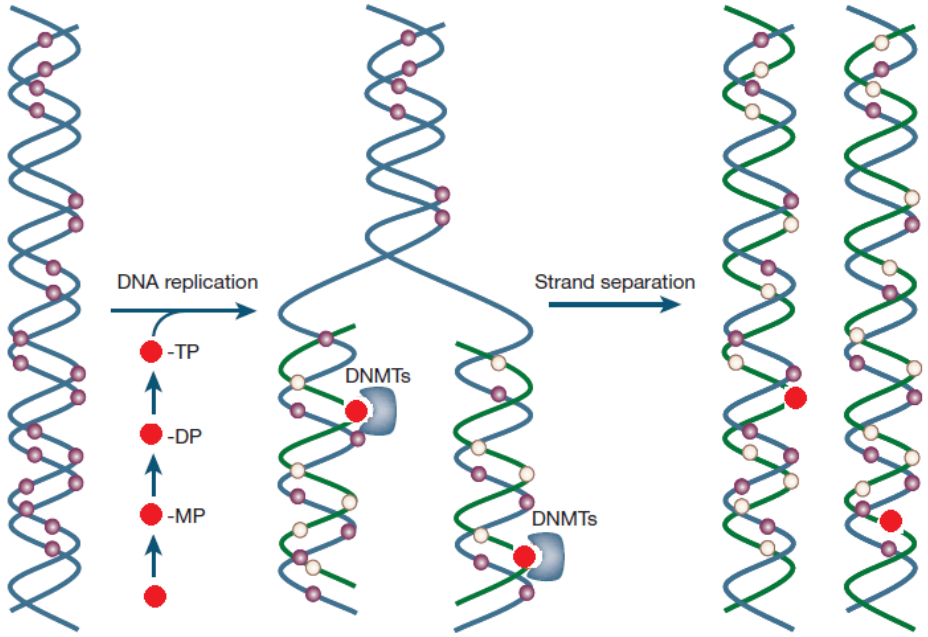


Figure 10. Incorporation and action mechanism of cytosine analogues. 5-azacytidine and zebularine are converted to nucleotide diphosphates (not shown), 5-aza-2´-deoxycytidine is converted to a nucleotide triphosphate and gets then incorporated into replicating DNA instead of cytosine. After incorporation, covalent duplexes of cytosine analogues and DNMTs get built leading to function-inhibition of active enzymes and demethylation of DNA. Pink circles, methylated CpG; white circle, unmethylated CpG; red circle, cytosine analogues; MP, monophosphate; DP, diphosphate; TP, triphosphate. Figure adapted from reference (Egger et al 2004).

9.6.4.3 De novo methylation of CpG dinucleotides

De novo methylation describes methylation of prior, fully unmethylated cytosine in CpG dinucleotides and mainly occurs during gametogenesis and developmental processes (Heller et al 2010, Okano et al 1998, Okano et al 1999, Sado et al 2004).

Experiments with retroviral proviruses and transgenic mice led to the identification of de novo methylation (Jahner et al 1982). Deletion experiments of the DNMT1 gene had no effect on the de novo methylation event leading to the presumption that other methyltransferases trigger de novo DNA methylation (Lei et al 1996). Two de novo methyltransferases, DNMT3a and DNMT3b are identified (Gowher and Jeltsch 2001, Okano et al 1998). Disruption experiments in mice resulted in developmental deficits (Hata et al 2002, Kaneda et al

2004), postnatal (DNMT3a) and embryonic (DNMT3b) lethality respectively, highlighting the importance of DNA methylation and DNA methylation catalysing enzymes (Hata et al 2002, Kaneda et al 2004, Okano et al 1999).

DNMT2 is also a member of the DNMT family but a change in the CpG methylation pattern could not be observed.

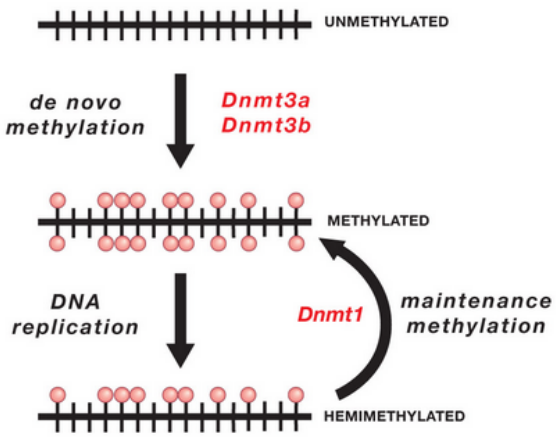


Figure 11. Illustration of De novo - and maintenance methylation. The vertical strokes indicate self-complementary CpG pairs. De novo methylation of prior unmethylated genomic regions is catalysed by DNMT3a and DNMT3b. Maintenance methylation is catalysed by DNMT1 leading to the original methylation status after DNA replication. Only prior methylated cytosine gets modified after replication. Prior unmethylated cytosine stays untreated (C. David Allis 2007). With permission from Cold Spring Harbor Laboratory Press, Copyright holder is Cold Spring Harbor Laboratory Press

DNMTs have highly conserved catalytic domains (Figure 12) opposite to regulatory domains located at amino terminal regions which show little conservation (Fatemi et al 2001, Kumar et al 1994). Both DNMT3a and DNMT3b have 2 conserved domains, the “proline-tryptophan-tryptophan-proline” (PWWP) domain which is involved in heterochromatin formation and a cysteine-rich region which harbours a C2-C2 zinc finger, the ATRX region.

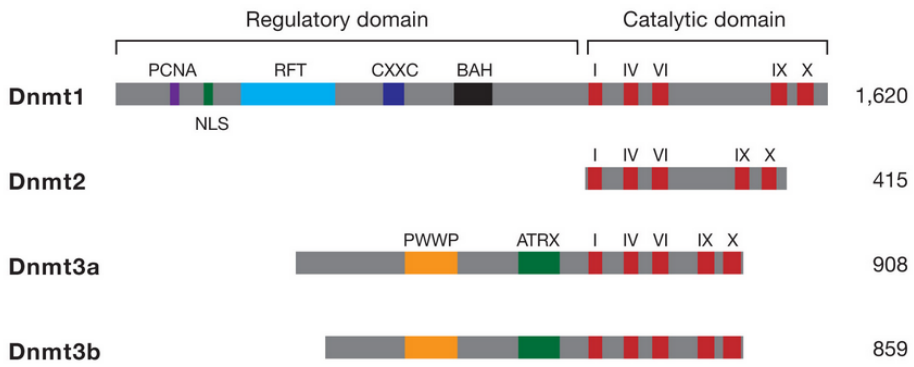


Figure 12. Mammalian DNA methyltransferases with their regulatory – and catalytic domains. Regions in the catalytic domains of the 4 DNMTs (I, IV, VI, IX and X) are largely conserved. The regulatory domains show great difference. DNMT1 contains the PCNA-interacting domain (PCNA), the nuclear localization signal (NLS), the replication foci-targeting domain (RFT) a cysteine-rich domain (CXXC) and the bromo-adjacent homology domain (BAH). Both DNMT3a and 3b have a proline-tryptophan-tryptophan-proline domain (PWWP) and a ATRX-related cysteine rich region with a C2-C2 zinc finger (ATRX) (C. David Allis 2007). With permission from Cold Spring Harbor Laboratory Press, Copyright holder is Cold Spring Harbor Laboratory Press

9.6.4.4 Maintenance methylation of CpG dinucleotide

It was postulated that a maintenance DNMT catalyses the methylation reaction of newly replicated cytosine bases according to the paternal methylation pattern. Unmethylated sites of the paternal strand are not influenced by the methylation event and keep their original pattern in the newly synthesised DNA strand. These postulations were confirmed a few years later by either DNA methylation sensitive restriction endonuclease experiments in *Xenopus laevis (Bird and Southern 1978)* or transfection experiments in cultured cells (Wigler 1981). DNMT1 was identified as the protein which is responsible for the maintenance methyltransferase reaction (Bestor and Ingram 1983, Li et al 1992).

DNMT1 harbours 5 motifs in its regulatory domain: the PCNA interacting domain (PCNA) (Chuang et al 1997, Fatemi et al 2001), the nuclear localization signal (NLS) (Bestor and Verdine 1994, Fatemi et al 2001), the replication foci-targeting domain (RFT) (Fatemi et al 2001, Leonhardt et al 1992), a cysteine rich domain (CXXC) and a bromo-adjacent homology

domain (BAH). The CXXC domain is involved in binding to CpG rich DNA sequences. The BAH domain is involved in protein-protein interactions (Kumar et al 1994). Regulatory and catalytic regions of DNMT1 are schematically illustrated in figure 12.

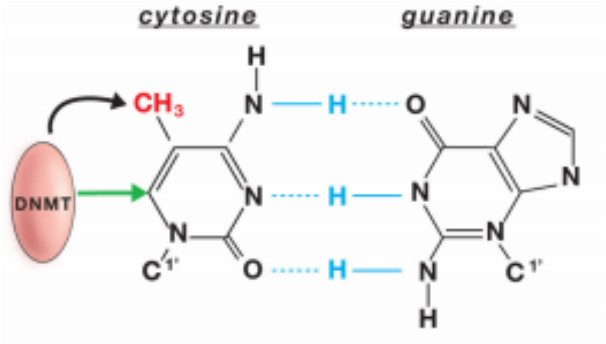


Figure 13. Illustration of the methyltransferase reaction. The addition of a methyl-group (CH_3 red) at the 5´carbon of cytosine bases in CpG dinucleotides is catalysed by DNA methyltransferases (DNMT). The green arrow indicates the covalently interaction of the DNMT with the 6´carbon of the cytosine during the methylation event (C. David Allis 2007). With permission from Cold Spring Harbor Laboratory Press, Copyright holder is Cold Spring Harbor Laboratory Press

9.7 Biosynthesis and function of microRNAs (miRNA)

MiRNAs are about 21 nucleotide-long non-coding RNAs and have major influence in the regulation of eukaryotic gene expression (Bushati and Cohen 2007, Filipowicz et al 2008, Kloosterman and Plasterk 2006, Rana 2007). The influence in gene expression happens post-transcriptionally in the cytoplasm by interaction with messenger RNAs (mRNA) (Filipowicz et al 2008, Jackson and Standart 2007, Nilsen 2007, Pillai et al 2007, Standart and Jackson 2007, Valencia-Sanchez et al 2006). The biosynthesis of miRNAs is a complex, multistep process which involves several enzymes and modification steps. Firstly miRNA encoding genes are transcribed by RNA polymerase II resulting in a pri-miRNA. The pri-miRNA is then modified in the nucleus by two enzymes, Drosha and Pasha, resulting in a ~ 70 nucleotide miRNA suboptimal stem loop structure (Basyuk et al 2003, Denli et al 2004, Esquela-Kerscher and Slack 2006, Gregory et al 2004, Lee et al 2002, Lee et al 2003, Zeng and Cullen 2003). The so formed pre-miRNA is then translocated into the cytoplasm by Exportin 5 (Bohnsack et al 2004, Esquela-Kerscher and Slack 2006, Lund et al 2004, Yi et al 2003). The RNAse III enzyme

Dicer cuts the pre-miRNA resulting in an 18 to 22 nucleotide long miRNA:miRNA duplex (Bagga et al 2005, Esquela-Kerscher and Slack 2006, Grishok et al 2001, Hutvagner et al 2001, Ketting et al 2001). This duplex has two 3´overhangs, the one with the 5´overhang at the less-stable end of the duplex becomes the mature miRNA the other strand is degraded (Du and Zamore 2005, Filipowicz et al 2005, Filipowicz et al 2008, Kim and Nam 2006, Rana 2007, Sontheimer 2005). The processed, mature miRNA associates with the RNA induced silencing complex (RISC) forming a miRISC complex to fulfil the function as negative regulators of target genes (Esquela-Kerscher and Slack 2006, Filipowicz et al 2008). Further, fundamental components of the RISC complex are Argonaute proteins (AGO 1 – 4) (Esquela-Kerscher and Slack 2006, Filipowicz et al 2008, Nilsen 2007, Rana 2007). An overview of miRNA biogenesis is shown in figure 14. After the miRISC complex is built, miRNAs bind to mRNA target sequences and inhibit the protein biosynthesis in 2 ways depending on the base complementary. While little sequence similarity and imperfect binding between miRNA and its mRNA target leads to translational repression high sequence similarity and so nearly perfect interaction of complementary sequences of miRNA and its mRNA target leads to target degradation (Figure 14) (Bushati and Cohen 2007, Chang and Mendell 2007, Esquela-Kerscher and Slack 2006, Filipowicz et al 2008, Kloosterman and Plasterk 2006, Krutzfeldt and Stoffel 2006).

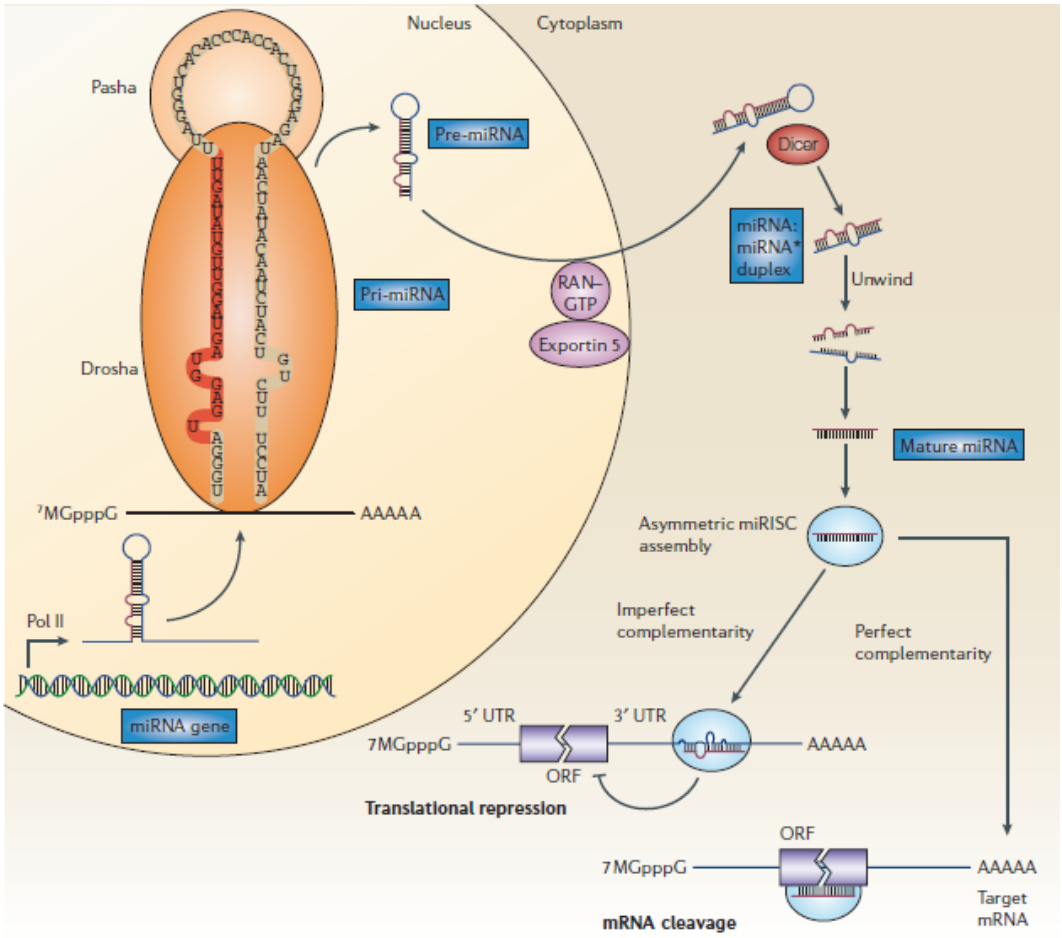


Figure 14. Biosynthesis of non-coding miRNAs. The miRNA biosynthesis starts with transcription and the procession to pri-miRNA and pre-miRNA in the nucleus. After translocation of pre-miRNA to the cytoplasm arranged by Exportin 5 further processions occur by several enzymes leading to a mature miRNA combined with the RISC complex. Micro RISC complexes are then able to fulfil their function as translational repressors. Figure adapted from reference (Esquela-Kerscher and Slack 2006).

It is estimated that miRNAs represent 1% to 4% of the expressed human genome suggesting that these non-coding RNAs are part of the largest gene regulators (Bentwich et al 2005, Berezikov et al 2005, Esquela-Kerscher and Slack 2006, Lim et al 2003a). Bioinformatics studies revealed that miRNA complementary sequences are in the 3´untranslated region (UTR) of mRNA target genes (Esquela-Kerscher and Slack 2006). These 2 to 8 nucleotide complementary region of one miRNA could be found in up to 200 mRNAs leading to the conclusion that miRNAs are multifunctional inhibitors of, e.g. transcription factors, secreted factors, receptors and transporters (Enright et al 2003, Esquela-Kerscher and Slack 2006, Grosshans et al

2005, John et al 2004, Kiriakidou et al 2004, Krek et al 2005, Lewis et al 2003, Lim et al 2003b, Lim et al 2005, Rajewsky and Socci 2004, Rehmsmeier et al 2004).

Several miRNAs and parts of the miRNA biosynthesis machinery have been identified to play a major part in tumour-genesis. Karube et al. (Karube et al 2005) reported about different expression levels of DICER and DROSHA in NSCLC patients with significant clinicopathologic characteristics (Esquela-Kerscher and Slack 2006, Karube et al 2005). Patients with a lower DICER expression level had poorly differentiated tumours and the hazard ratio in the aspect of earlier death was 17.6 (Karube et al 2005). Deletions in AGO1, 3 and 4 are involved in the formation of Wilms tumours and neuroectodermal tumours (Carmell et al 2002, Esquela-Kerscher and Slack 2006). Several miRNAs were identified as regulators of cancer-related processes like cell growth, differentiation and apoptosis (He and Hannon 2004, Heller et al 2012). For example the human homolog genes *lin-4* and *let-7* are involved in cell proliferation and are involved in the development of lung cancer (Esquela-Kerscher and Slack 2006, Farazi et al 2011, Iorio et al 2005, Johnson et al 2005, Takamizawa et al 2004, Yanaihara et al 2006) and breast cancer (Calin et al 2004, Esquela-Kerscher and Slack 2006). Thus, alterations and downregulation of miRNA gene expression may contribute to the development of a malignant phenotype (Du and Pertsemlidis 2010, Heller et al 2012). Downregulated miRNAs were already identified in several cancer types including lung cancer (Farazi et al 2011, Heller et al 2012, Yanaihara et al 2006). A significant miRNA downregulation in TU samples compared with NL samples were observed (Bandi et al 2009, Fabbri et al 2007, Heller et al 2012, Wang et al 2011b, Yanaihara et al 2006).

9.8 DNA methylation in NSCLC

In general, cancer arises from genetic lesions, both genetic alterations (point mutations, deletions, inversions and chromosome-aberrations) and epigenetics changes. Several publications have shown that epigenetic alterations especially DNA methylation influences gene expression and occurs in almost all cancer types (Hanahan and Weinberg 2000, Herman and Baylin 2003, Jones and Laird 1999, Jones and Baylin 2002).

Using approaches to detect single gene methylation, numerous tumour suppressor genes (TSG) have already been identified which are frequently methylated and thus, transcriptionally silenced in NSCLCs. Examples of these genes are *DLEC1* and *p16* (involved in cell cycle regulation), *CDH1*, *CDH13*, *TSLC1* and *DAL1* (involved in cell adhesion), *RASSF1A* and *RARβ2* (involved in cell growth), *DAPK*, *FHIT* and *RUNX3* (involved in apoptosis) or *MGMT* (involved in DNA repair) (Brabender et al 2001, Burbee et al 2001, Dammann et al 2000, Esteller et al 2001, Heller et al 2006, Heller et al 2010, Kashiwabara et al 1998, Kikuchi et al 2005, Kikuchi et al 2006, Kim et al 2003, Kuramochi et al 2001, Maruyama et al 2004, Nakata et al 2006, Seng et al 2008, Toyooka et al 2001a, Toyooka et al 2003b, Virmani et al 2001, Yanagawa et al 2007, Zochbauer-Muller et al 2001a, Zochbauer-Muller et al 2001b). It was found that TU samples are methylated up to 96% compared to NL samples, where no methylation or only a low percentage of methylation is detectable (Burbee et al 2001, Heller et al 2006, Toyooka et al 2001a, Zochbauer-Muller et al 2001b). In addition, it was shown that methylation of certain TSGs is tumour type-specific (Figure 15) (Burbee et al 2001, Heller et al 2006, Zochbauer-Muller et al 2001a, Zochbauer-Muller et al 2001b, Zochbauer-Muller et al 2005). Thus, DNA methylation of promoter regions of presumed TSGs may be one reason for the loss of function and may lead to the establishment of tumour growth (Fraga et al 2005, Herman and Baylin 2003, Jones and Laird 1999, Jones and Baylin 2002).

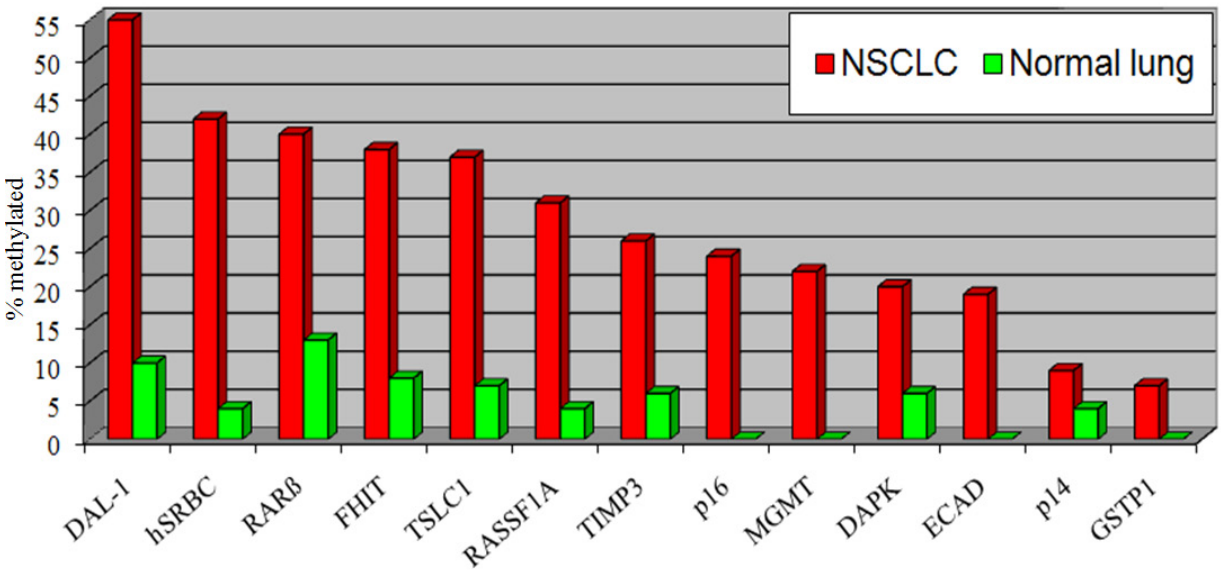


Figure 15. Tumour-specific methylation of certain tumour suppressor genes in NSCLCs. The percentage of methylation in TU and NL samples is shown. Data summarized from references (Burbee et al 2001, Heller et al 2006, Zochbauer-Muller et al 2001a, Zochbauer-Muller et al 2001b).

Moreover, certain associations between methylation of certain genes and clinical characteristics (e.g. histology, stage of disease, smoking history) were observed (Heller et al 2006, Heller et al 2010). For instance Toyooka et al. (Toyooka et al 2003b) reported statistically significant differences in methylation of the genes *APC*, *RARβ* and *CDH13* in primary adenocarcinomas and primary SCC (Figure 16). These genes were found to be more frequently methylated in adenocarcinomas than in squamous cell carcinomas.
Interestingly no methylation or only a small percentage of methylation was observed in NL samples. These results showed a clear difference in the methylation pattern of TU and NL samples of NSCLC patients suggesting that DNA methylation is tumour-specific and tumour type-specific respectively (Burbee et al 2001, Esteller et al 2001, Toyooka et al 2001b, Toyooka et al 2003a).

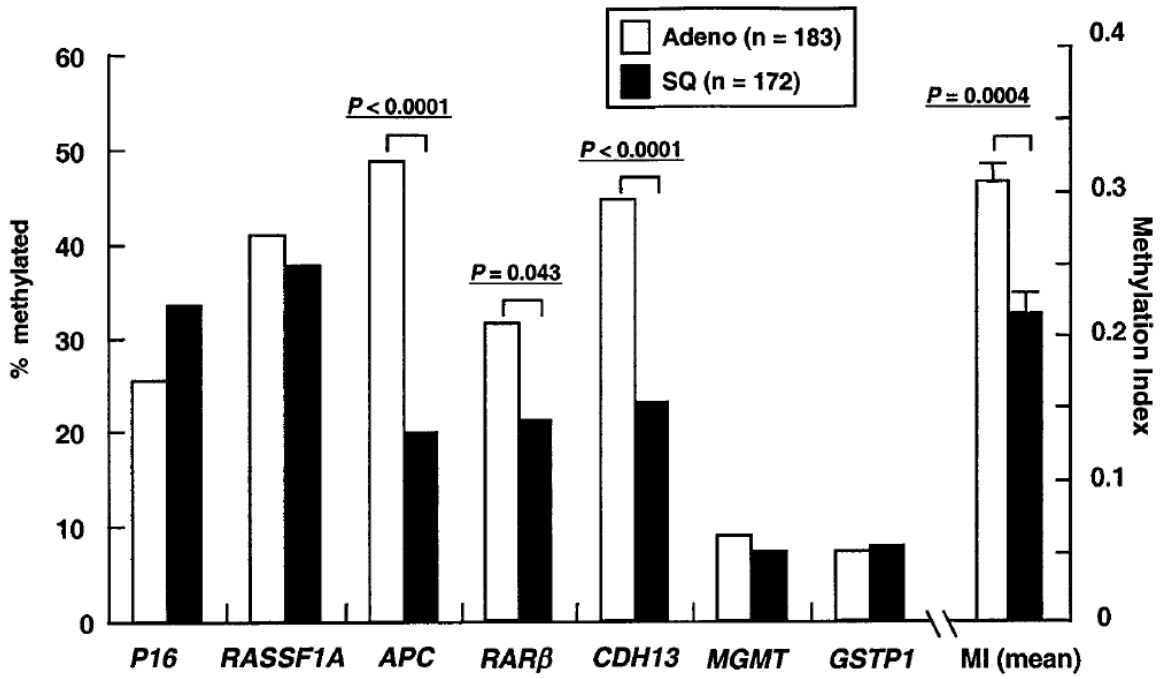


Figure 16. Methylation frequencies of 7 genes in primary adenocarcinomas and primary squamous cell carcinomas.

Moreover Toyooka et al. (Toyooka et al 2003b) compared the methylation status of the genes *p16*, *RASSF1A*, *APC*, *RARβ*, *CDH13*, *MGMT* and *GSTP1* in primary tumours of never smokers and ever smokers and found a statistically significant higher methylation frequency of *p16* and *APC* in primary tumours of ever smokers (Figure 17). Of note, methylation of all of these genes except *GSTP1* was also found in non-malignant lung tissue samples of ever smokers but not or in a lower frequency in never smokers (Figure 17).

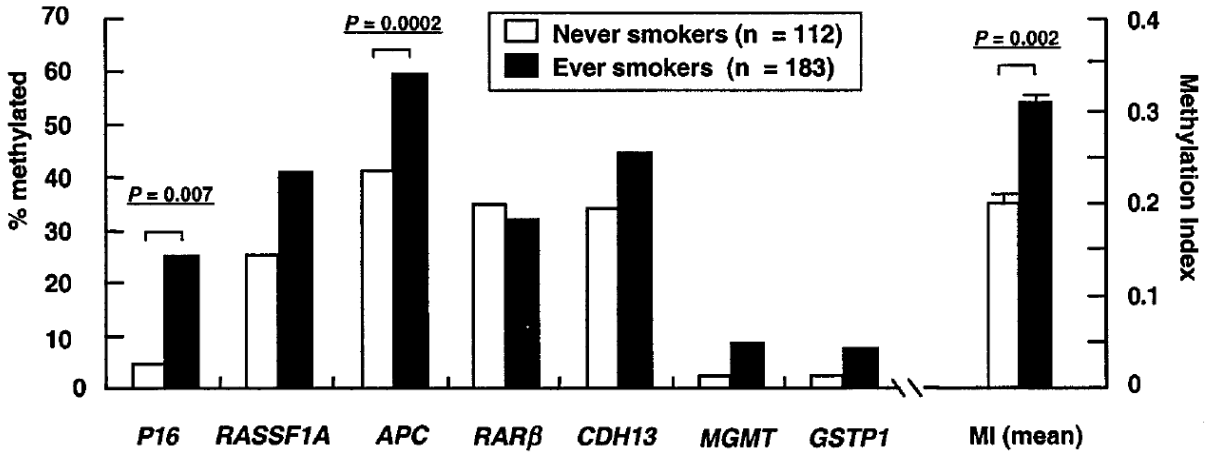


Figure 17. Methylation frequency of 7 genes in non-malignant lung tissue samples of never and ever smokers.

In addition, it was reported that methylation of some of them (e.g. *p16*, *RASSF1A*, *APC*) may be associated with a poor prognosis of NSCLC patients (Brock et al 2008).

DNA methylation analyses also have been done in blood, sputum, bronchial brushings and bronchioloalveolar lavage (BAL) samples of lung cancers. The methylation status of the genes *MGMT*, *p16*, *DAPK*, *APC*, *CDH13*, *FHIT*, *RARβ-2* and *RASSF1A* obtained from plasma or serum samples could be confirmed in the majority of samples, however, a lower percentage of methylation was found in these sample types compared to TU samples (Anglim et al 2008, Belinsky et al 2005, Esteller et al 1999, Fujiwara et al 2005, Hsu et al 2007, Usadel et al 2002, Wang et al 2007). In serum and plasma samples of control individuals no methylation of these genes was obtained (Esteller et al 1999, Wang et al 2007).

Recently, it was observed that also miRNA coding genes may be targets for methylation in NSCLCs (Ceppi et al 2010, Kitano et al 2011, Stanzer et al 2010, Yang et al 2010). However, researchers are still at the beginning of understanding the impact of methylation on miRNA gene silencing in cancers, particularly in NSCLCs.
Expression of several miRNAs was found to be tumour-specifically downregulated in NSCLC by genome-wide approaches to detect miRNA expression (Wang et al 2009, Yanaihara et al 2006). Tumour-specifically downregulated miRNAs in NSCLC included *let-7* family members (involved in regulation of *Ras* signalling), *miR-15a* and *miR-16-1* (involved in regulation of cell

cycle), *miR-451* (regulator of *Ras*-related protein 14, RAB14) (Bandi et al 2009, Johnson et al 2005, Takamizawa et al 2004, Wang et al 2011b).

Chromosomal loss, alterations of the miRNA processing machinery and methylation were identified as a mechanisms causing downregulated miRNA expression in cancer cells (Farazi et al 2011, Lujambio and Esteller 2007). Examples for miRNA encoding genes in NSCLC are members of the *miR-34* family, *miR-124a*, *miR-126*, *miR-415* and *miR-200c* (Ceppi et al 2010, Gallardo et al 2009, Lujambio et al 2007, Wang et al 2011c, Watanabe et al 2012). Interestingly, some of these miRNA genes are parts of molecular pathways whose deregulation may contribute to the development of a malignant phenotype. While *miR-34a* is part of the *p53* network, *miR-124a* regulates levels of the cell cycle progression factor CDK6 (Corney et al 2007, Lujambio et al 2007, Pierson et al 2008).

Recently, Wang et al. (Wang et al 2011c) observed that methylation of *miR-34b/c* may be associated with a poor overall and poor disease-free survival in stage I NSCLC patients. *MiR-451* is involved in *Ras* signalling pathway (Bandi et al 2009). Ceppi et al. (Ceppi et al 2010) reported, that *miR-200c* is a putative metastasis suppressor gene which is methylated in 63% of NSCLC cell lines analysed.

Moreover, Fabbri et al. (Fabbri et al 2007) demonstrated that certain miRNA genes are not only targets but also regulators of methylation. They reported that DNMT3A and DNMT3B are regulated by *miR-29* and that increased gene expression of *miR-29* in NSCLC cell lines results in restoration of methylation and gene expression patterns of the silenced TSGs *FHIT* and *WWOX* (Fabbri et al 2007, Volinia et al 2006, Yanaihara et al 2006).

10 Aims of this diploma thesis

This diploma thesis is based on 2 genome-wide approaches to detect DNA methylation of protein and miRNA encoding genes in NSCLC as described in detail in section “Basis of this diploma thesis”. A large number of protein and miRNA encoding genes were identified as targets for methylation in NSCLC cell lines and NSCLC patients. The aims of this diploma thesis were:

- To develop gene-specific approaches to analyse DNA methylation of the genes *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* and *miRNA-193a*.
- To confirm data obtained by genome-wide methylation analyses using gene-specific approaches
- To determine methylation of the 5 genes in primary tumours and corresponding non-malignant lung tissue samples of 97 NSCLC patients
- To compare methylation results with clinico-pathological characteristics of the NSCLC patients

11 Methods and material

11.1 Cell culture

NSCLC cell lines A549, NCI-H1993 and NCI-H2073 were purchased from the American Type Culture Collection (ATCC). Cells were stored in cryo tubes in liquid nitrogen. For thawing the cells cryo tubes were incubated for 1 minute at 37°C in a water bath. Then, cells were washed in 15 ml RPMI 1640 media + 10% FCS twice, the media was discarded, 5 ml fresh media were added and the cell pellet was dissolved by vortexing. Afterwards, cells were transferred into a T25 cell culture flask. 24 hours later we controlled adherent cell colonization microscopically. Cell culture medium was changed when necessary.

11.1.1 Cell passaging

For cell passaging, cell culture medium was discarded and 3 ml pre-warmed Trypsin-EDTA (stored at -20°C) were added directly to the cells. After cells were detached from the surface, 15 ml cell culture medium was added and cells were transferred to a 50 ml tube and centrifuged at 1520 rpm for 10 minutes. Fluids were discarded, 15 ml fresh cell culture medium was added and the cell pellet was dissolved by vortexing. This working step was repeated twice. Then the dissolved cells were transferred in equal parts in 2 new sterile T75 cell culture flasks.

11.1.2 Material

Components	Catalogue number	Company
RPMI + GLUTAMAX	758483	INVITROGEN
REFOBACIN	112214	MERCK
TRYPSIN-EDTA	197812	INVITROGEN
FCS	179822	INVITROGEN

11.1.3 Tissue samples

Overall, frozen TU and NL samples of 106 stage I-III NSCLC patients who underwent surgical resection of their tumour in a curative intent have been collected the years 2000 – 2004. 97 samples of NSCLC patients were analysed for methylation of protein encoding genes and of miRNA encoding genes. Clinical samples for MS-HRM were stored at -80°C until use. None of these NSCLC patients of the tumour stages I, II or III received adjuvant chemotherapy (Heller et al 2006, Heller et al 2012).

11.2 Isolation of genomic DNA

Genomic DNA was isolated from tumour cell lines and frozen tissue samples of 97 NSCLC patients using proteinase K digestion and phenyl-chloroform-isoamyl alcohol extraction. 500 µl PK buffer, containing 1 molar TRIS (pH 8.0), 0.5 molar EDTA (pH 8.0), sodium dodecyl sulphate (SDS) (1 g/ 5 ml), proteinase K (10 mg/ml) and water, were added either to cell pellets or to liquid-nitrogen disrupted tissue samples of NSCLC patients and incubated the mixture for 1 hour at 50°C. After incubation 500 µl phenyl-chloroform-isoamyl alcohol were added and followed by centrifugation at 14000 rpm for 10 minutes resulting in a clear phase separation. The upper phase containing genomic DNA was collected and transferred to a new tube. The phenyl-chloroform-isoamyl alcohol step was repeated followed by ethanol (EtOH) precipitation of genomic DNA using EtOH absolute. After incubation at -80°C for 30 minutes and subsequent centrifugation at maximum speed, the resulting DNA pellet was washed with 70% EtOH. Then, the DNA pellet was air dried and dissolved in TE-buffer.

11.2.1 Material

Components	Catalogue number	Company
Isoamyl alcohol 98%	I-3643	SIGMA-ALDRICH
Phenol S/P buffer saturated	K-168-400	AMRESCO
Chloroform	C-5312	SIGMA-ALDRICH
Ethanol absolute 96%	1.0097	MERCK
TRIS	50005	BIOMOL
EDTA	43178-8	SIGMA-ALDRICH
SDS	L-4390	SIGMA-ALDRICH
Proteinase K	745723	ROCHE APPLIED SCIENCE

11.3 Sodium bisulfite treatment of genomic DNA

Prior detection of methylated cytosines in CpG dinucleotides in the promoter regions of the genes of interest, genomic DNA was treated with sodium bisulfite. Sodium bisulfite treatment of DNA leads to deamination and conversion of unmethylated cytosine to uracil. Methylated cytosines remain unchanged (Clark et al 1994, Clark et al 2006, Frommer et al 1992, Zilberman and Henikoff 2007). In the following amplification step uracil is replaced by thymine (Figure 18).

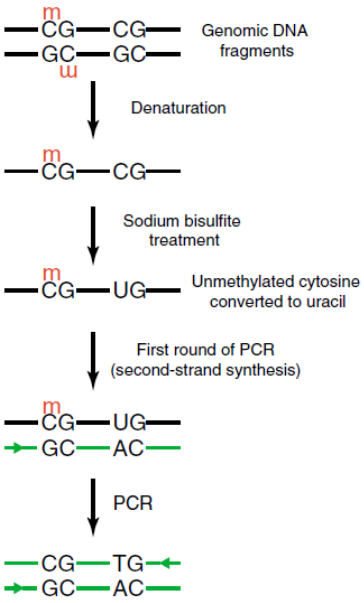


Figure 18. Sodium bisulfite treatment of genomic DNA. Methylated and unmethylated genomic regions are shown. After denaturation and treatment with sodium bisulfite, unmethylated cytosine is converted to uracil while methylated cytosine remains 5-methylcytosine. After PCR amplification the incorporated uracil is replaced by thymine. This change of DNA sequence allows distinguishing methylated and unmethylated DNA sequences in subsequent PCR based approaches for detection of DNA methylation. Figure adapted from reference (Zilberman and Henikoff 2007).

For sodium bisulfite treatment the EpiTect Bisulfite Kit form Qiagen was used according to the instructions of the manufacturer “Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA”. 1 µg genomic DNA was used (C. David Allis 2007).

11.3.1 Material

Components	Catalogue number	Company
EpiTect 96 Bisulfite Kit	59110	Qiagen

11.4 Primerdesign for methylation-sensitive high resolution melt analyses (MS-HRM) and bisulfite genomic sequencing (BGS)

Primerdesign for methylation-sensitive high resolution melt analysis (MS-HRM) and bisulfite genomic sequencing (BGS) was performed using the "Methyl Primer Express v 1.0" software. These primers were designed to amplify the regions of interest regardless of the methylation status. Genomic sequences were obtained from Ensembl database. Settings for MS-HRM primer design were: a) primer length of 18 to 25 nucleotides, b) amplicon between 100 and 160 nucleotides c) G-C content in the primer sequence of more than 50%. Settings for BGS primer design were: a) primer length of 18 to 25 nucleotides, b) amplicon between 250 and 350 nucleotides and c) G-C content in the primer sequence of more than 50%. Primers were synthesized by VBC Biotech (Vienna, Austria). Lyophilised primers were dissolved in ddH_2O according to the instructions of the manufacturer to obtain a concentration of 50 nm per µl. For a ready to use solution 20 µl of forward and reverse primer were mixed with 160 µl ddH_2O in a sterilized Eppendorf tube. Primer sequences are shown in table 1. Sequencing primers were designed to harbour the MS-HRM primer binding sites.

Table 1. Oligonucleotide primer sequences for MS-HRM analyses and BGS of 5 genes

Gene	Forward sequence	Reverse sequence	Amplicon length
HOXA2	TTAGATTGAGGTGTTTAAATGATTTG	ATAACTACCCTCTACCTCCCCC	98
HOXA2 BGS	AATAGAATTTATGTGGTTGGG	ACCTCCTAATCTACAAAAATCTATC	284
SHOX2	GGTGGGAGTTTTGAGATTTTAG	AACAAATTTCCCCAACAACTAT	118
SHOX2 BGS	GGTGGGAGTTCTGAGACCTCAG	GGAGAAGGTGCAGGCAACCCTGTC	309
TAL1	GATTGTGTTGGATTGTGTGTT	CACCTAACACTACCCCAAAAA	110
TAL1 BGS	TGGTGTTCTCAGCAGGGATCTG	GTGTGCCTGTGTCCTTTAGAGGT	299
miRNA-9-3	GTGYGTGTGTTTGTTTATTTT	ACCTCCCTTAACCAATACC	141
miRNA-9-3 BGS	GTGYGTGTGTTTGTTTATTTT	AACACTACAAATATCCCCAAAAA	393
miRNA-193	TTTGAGGGATATTTAGAGTTTYGG	CAACTCCCATCCTCRAAATT	115

miRNA-193a BGS	TTTTGATGTGTTATTAGTAYGTGGAG	ACCCAACTCCRCTCTACC	385

MS-HRM, methylation sensitive high resolution melt analyses; BGS, bisulfite genomic sequencing; Y in primers, random integration of C or T; R in primers, random integration of G or A.

11.5 Methylation-sensitive high resolution melt analyses

After bisulfite conversion of genomic DNA the methylation status of the promoter regions of the genes *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* and *miRNA-193a* were quantified by the use of EpiTect HRM PCR kit in a RotorGene®Q cycler (Qiagen, Hilden, Germany) according to the manufacturer´s instructions. This technique is based on 3 main facts, the intercalation of a fluorescent dye in double stranded DNA (dsDNA), the base pair constitution of the DNA fragments analysed and thus the melting behaviour depending on methylation of the region of interest (Wojdacz and Dobrovic 2007, Wojdacz et al 2010). The DNA intercalating dye of the EpiTect HRM PCR kit is EvaGreen which enables high fluorescence during intercalation in dsDNA without inhibiting the PCR reaction (Wittwer et al 2003, Wojdacz and Dobrovic 2007, Wojdacz et al 2010). Because of temperature gradients in HRM analysis from low to high temperature, dsDNA denature into ssDNA resulting in disability of intercalation of the fluorescent EvaGreen and furthermore a decrease in light emission.

The next aspect of DNA constitution refers to the hydrogen bridge bonds (H-bonds) between complementary nucleotides. It is known that G-C base pairs are connected by 3 H-bonds, A-T base pairs are connected by 2 H-bonds. As explained before, prior bisulfite conversion leads to a sequence change depending on the methylation status. Unmethylated cytosine gets converted to uracil and after PCR to thymine further resulting in the loss of one H-bond. Methylated cytosine stays untreated and keeps the 3 H-bonds between CpG dinucleotides. The more H-bonds are present in a DNA sequence, the more energy is needed to break these bonds and denature dsDNA to ssDNA.

For construction of a regression line and subsequent calculation of the % of methylation, differentially methylated DNA standards were included in each MS-HRM run. DNA standards

were prepared from dilutions of commercially available 100 % and 0 % methylated standard control DNA (Qiagen, Hilden, Germany) at 100 %, 75 %, 50 %, 25 %, 10 % and 0 % ratios (Stanzer et al 2010). PCR amplification was performed using the following conditions: 5 minutes hold at 95°C for enzyme activation followed by 40 times amplification cycle rotating with denaturation for 10 seconds at 95°C, annealing for 30 seconds at 55°C and an extension step for 10 seconds at 72°C. After amplification the RotorGene®Q software directly starts with HRM starting from 60°C and rising up to 90°C in 0.1°C steps.

11.5.1 Material

Components	Catalogue number	Company
EpiTect Control DNA Set (100)	59695	Qiagen
EpiTect HRM PCR Kit (100)	59445	Qiagen

11.6 Bisulfite genomic sequencing

11.6.1 Polymerase chain reaction (PCR)

DNA samples (stored at -20°C), and reaction components were thawed at room temperature (RT). For homogenization DNTPs, PCR buffer and primer mixes (forward and reverse) were vortexed. The total volume for one PCR reaction was 21 µl, consisting of 13.1 µl H_2O, 3.2 µl DNTPs, 2.5 µl PCR buffer, 1 µl primer mix, 0.2 µl TAQ polymerase and 1 µl of bisulfite treated, genomic DNA. After the combination of all reaction components the thermo cycler was programmed according to the beneath program. To obtain a higher amount of amplicons for subsequent cloning reactions all samples were amplified in triplicates.

Step	Temperature in °C	Time
DENATURATION	94	12 minutes
DENATURATION	95	30 seconds
ANNEALING	56°C *HOXA2* 60°C *SHOX2* 56°C *TAL1* 55°C *miRNA-9-3* 64°C *miRNA-193a*	40 seconds
EXTENSION/ELONGATION	72	30 seconds
FINAL ELONGATION	72	7 minutes
HOLD	4	endless

11.6.1.1 Material

Components	Catalogue number	Company
DNTP SET	120281	AMERSHAM
HOT-STAR TAQ DNA POLY-MERASE	203205	QIAGEN
BUFFER	203205	QIAGEN
PCR SINGLE CAP SOFT-STRIPS	710980	BIOZYM

11.6.2 Agarose gel electrophoresis

For visualization of PCR products, agarose gel electrophoresis using a 2 % agarose gel was performed. Using this technique, the DNA was separated depending on their mass-to-weight ratio. 180 mL 1X TAE buffer were mixed with 3.6 g agarose and heated in a microwave. After cooling down 15 µl GelRED™ for autoradiographical detection were added. DNA samples were prepared by adding 2 µl loading buffer (bromphenol-blue) and transferred in separate slots. For band length classification a 100 base pair DNA ladder was used. Gel electrophoreses was performed at 170 V for 25 minutes. Band detection was performed using Chemi-Doc™ XRS (Biorad).

11.6.2.1 Material

Components	Catalogue number	Company
DNA LADDER 100 base pairs	15628-019	INVITROGEN
GelRED™	41003	BIOTREND
TAE BUFFER	161-0743	BIO-RAD

11.6.3 Agarose gel purification

Before DNA purification bands were excised from the agarose gel under the influence of UV detection. For each specific DNA sample a new scalpel was used to avoid contamination and result falsification. PCR products were then purified from agarose gel using the QIAquick Gel Extraction Kit (Qiagen) according to the instructions of the manufacturer.

11.6.3.1 Material

Components	Catalogue number	Company
QIAquick GEL EXTRACTION KIT	28704	QIAGEN

11.6.4 Cloning of amplicons

For bisulfite genomic sequencing of the promoter regions of the genes *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3 and miRNA-193a* the gel purified PCR products were cloned using the TOPO® TA Cloning® Kit for Sequencing (Invitrogen) according to the instructions of the manufacturer. In the previous PCR step, regions of interest were amplified with TAQ polymerase. Because of the activity as a terminal transferase, the TAQ polymerase adds a single deoxyadenosine (A) to 3´ends of PCR products. Before inserting a fragment into the pCR™4-TOPO® vector, the vector has linear constitution with a single 3´overhanging deoxythymidine (T). These overhangs, one at the PCR product and one at the vector and the enzymatic activity of a covalently bound topoisomerase I (TOPO I) from the *Vaccinia* virus allow integration and ligation

(Shuman 1991). Integration occurs in the open reading frame of the fusion construct of the *LacZα−ccdB* gene. Expression of this gene leads to the death of *E. coli* but integration of a fragment leads to disruption of the lethal gene and furthermore to growth of transformed *E. coli* cells (Bernard and Couturier 1992, Bernard et al 1993, Bernard et al 1994). The pCR™4-TOPO® harbours 2 antibiotic resistant cassettes, one for Ampicillin and one for Kanamycin for further growth selection. In addition, 2 necessary sequences for later amplification and sequencing procedures are M13 forward and reverse primer binding sites (Figure 19).

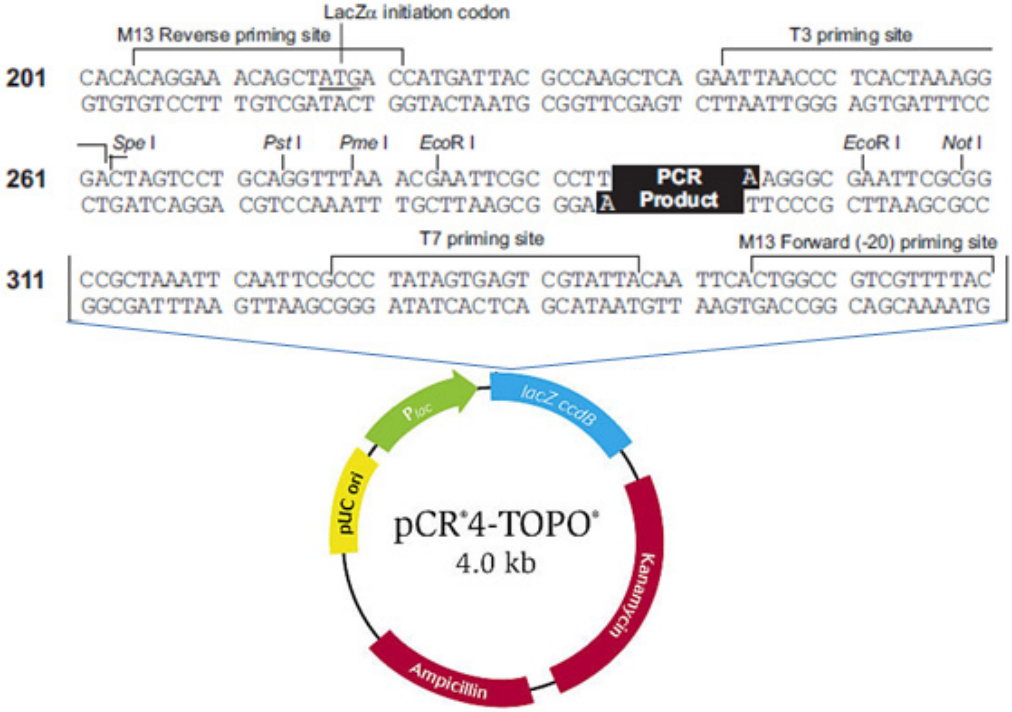


Figure 19. Schematic representation of the 3956 base pair vector which are essential for the integration of the PCR product at the TOPO® cloning site at position 294-295, for screening (Kanamycin resistant gene: bases 1159 – 1953, Ampicillin resistant gene: bases 2203 – 3063) and for amplification (M13 forward priming site: bases 355 – 370 and M13 reverse priming site: bases 205 – 221) Figure adapted from Invitrogen™ life technologies.

11.6.5 Production of LB culture medium

Before the cloning procedure was started, the growth medium was prepared for chemically competent *E. coli* cells as follows:

5 g Bacto Tryptone,

2.5 g Yeast-extract,

5 g NaCl

Total volume: 500 ml, pH: 7.2

11.6.6 Production of LB plates

10 g agar were added to the prior prepared LB medium. For dissolution and sterilization the MultiControl (CertoClav) autoclave was used. After sterilization and cooling down to 50°C, the antibiotic Kanamycin was added (50 µg/ml). Then the medium was poured into cell culture plates for later cell cultivation and in 1.5 ml Eppendorf tubes for sequencing. Cell culture plates were stored on 4°C until use.

11.6.6.1 Material

Components	Catalogue number	Company
BACTO TRYPTON	211705	BD
BACTO YEAST EXTRACT	212750	BD
BACTO AGAR	214014	BD
SODIUMCHLORID	S3014	SIGMA-ALDRICH
KANAMYCIN	K1876	SIGMA-ALDRICH

11.6.7 TOPO® cloning

Before seeding chemically competent *E. coli* cells, cell culture plates were pre-warmed in the BBD 6220 Heraeus® incubator (Thermo Scientific) at 37°C for 30 minutes to reach the optimum growing temperature. The first cloning step, the incorporation of our PCR amplified fragments into the vector, was done at RT. After thawing all components, 4 µl PCR amplified genomic DNA, 1 µl salt solution and 1 µl TOPO® vector were mixed in a 1.5 ml Eppendorf tube and incubated for 15 minutes at RT. During that time, the PCR amplified genomic DNA incorporated in the linear vector leading to circularization. The salt solution was then stored at 4°C, PCR products and TOPO® vector were stored at -20°C.
The chemically competent *E. coli* cells were stored at -80°C in separate vials. Cells were thaw on ice. Transformation was performed in a 4°C cooling chamber. For each transformation 2 µl of the cloning reaction were added into a separate *E. coli* vial and mixed gently by agitation. After 30 minutes incubation at 4°C a heat shock reaction was performed at the pre-warmed 42°C water bath for exactly 30 seconds. Afterwards the vials were immediately put on ice, 250 µl of pre-warmed S.O.C growth media were added and the vials were incubated for 1 hour at 37°C shaker at 200 rpm. After 1 hour of incubation cells were (50 µl and 20 µl) plated on pre-warmed LB cell culture plates with sterile drigalsky scoop, and incubated at 37°C overnight.

Afterwards *E. coli* culture growth was analysed optically. As mentioned before, the used *E. coli* strain has only growing ability, when 1) cloning of our PCR products and 2) transformation of the vector into the cells were successful. The transformation of the vector harbouring Kanamycin resistance leads to growth ability of the *E. coli* strain.

In a next step, single colonies were picked from the LB plate and transferred to new pre-warmed LB cell culture plates (37°C) using sterilized toothpicks. A raster was signed at the bottom of each cell culture plate and each square was for exact single colony identification. Then the patterned LB cell culture plates with transferred single *E. coli* colonies were incubated at 37°C for further 4 hours.

For the confirmation of successful cloning and transformation steps PCR and agarose gel electrophoreses were performed. 20 µl master mix including 13.µl ddH_2O, 3.2 µl DNTPs, 2.5 µl PCR buffer, 1 µl of individual sequencing Primer mix (*HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* and *miRNA-193a*) and 0.2 µl TAQ polymerase was prepared. Cells form the re-colonized single *E. coli* colonies were picked using sterile toothpicks and transferring into the prepared master mix. PCR reactions were performed in a vapo.protect thermocycler (Eppendorf). The specific PCR program is mentioned in section 11.6.1. In the meantime patterned LB cell culture plates were put on 4°C. After positive identification of colonies with the inserts of interest by agarose gel electrophoreses further incubation of selected *E. coli* colonies for 4 hours was performed. Furthermore pre-warmed LB containing 1.5 ml Eppendorf tubes with open cap were incubated at 37°C for 30 minutes. After 4 hour incubation specific *E. coli* cells with sterile toothpicks were transferred to the pre-warmed LB containing Eppendorf tubes and *E. coli* containing tubes with half open caps were incubated for further 5 hours. Afterwards transformed *E. coli* cells, harbouring the vector with specific promoter inserts, were sent for sequencing to LGC genomic Berlin.

11.7 Statistical analyses

Wilcoxon signed rank test were used to calculate differences between TU samples and NL samples obtained by MS-HRM analyses. Receiver operating characteristic (ROC) curve analyses was done using GraphPad Prism 5 software.
MS-HRM data of the genes *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* and *miRNA-193a* were compared with clinico-pathological parameters (gender, age, histology, tumour stage, lymph node stage, stage of disease recurrence, disease free survival (DFS) and overall survival (OS) of NSCLC patients. Chi^2 tests / Fisher´s exact tests were used to calculate differences between groups and t-tests were used to calculate differences between means. Survival analyses of NSCLC patients were performed using log rank testing. The Cox proportional model was used for multivariate analyses on DFS/OS. Factors included in the multivariate analyses were gender, age, tumour stage, lymph node stage and stage of disease. A p-value < 0.05 was considered as statistically significant. These analyses were performed using the statistic software PASW (version 18).

12. Results

12.1 Establishing MS-HRM assays for 5 genes in NSCLC cell lines

The major aim of this diploma thesis was to confirm results of genome-wide DNA methylation analyses as described in "Basis for this diploma thesis". To reach this goal, we developed MS-HRM assays for the genes *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* and *miRNA-193a*. In a first step, these assays were tested for quality and efficacy on the NSCLC cell lines A549, NCI-H1993 and NCI-H2073 and on various DNA methylation standards (0%, 10%, 25%, 50%, 75% and 100% methylated). As shown in figures 20 - 24, all differentially methylated DNA standards were amplified at comparable Ct values in all 5 assays tested. After normalization of melt curves a clear curve separation depending on the % of methylation was observed (Figure 20 - 24). The % of methylation was then plotted against the normalized fluorescence and regression lines were calculated. The resulting R^2 values of the assays range from 0.971 to 0.9972 indicating high linearity of our MS-HRM assays.

Methylation of all genes was observed in all 3 cell lines analysed. The percentage of methylation of *HOXA2* was 90% in A549, 93% in NCI-H1993 and 84% in NCI-H2073. The percentage of methylation of *SHOX2* was 84% in A549, 96% in NCI-H1993 and 36% in NCI-H2073. The percentage of methylation of *TAL1* was 77% in A549, 88% in NCI-H1993 and 100% in NCI-H2073. The percentage of methylation of *miRNA-9-3* was 41% in A549, 98% in NCI-H1993 and 44% in NCI-H2073. The percentage of methylation of *miRNA-193a* was 41% in A549, 94% in NCI-H1993 and 83% in NCI-H2073. These results are summarized in table 2.

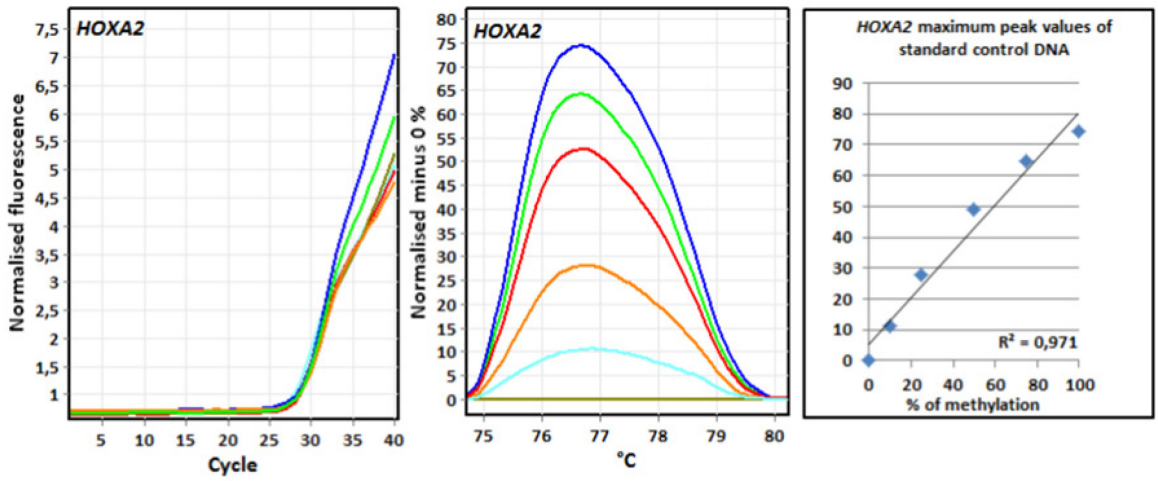


Figure 20. Test of linearity of MS-HRM assay for *HOXA2*. Left panel: Amplification plot showing amplification of differentially methylated DNA standards at comparable Ct values. Middle panel: Normalization of melting curves against 0% methylated DNA. A clear curve separation depending on the % of methylation is shown. Right panel: Maximum peak values were plotted against the % of methylation resulting in high linearity regression lines.

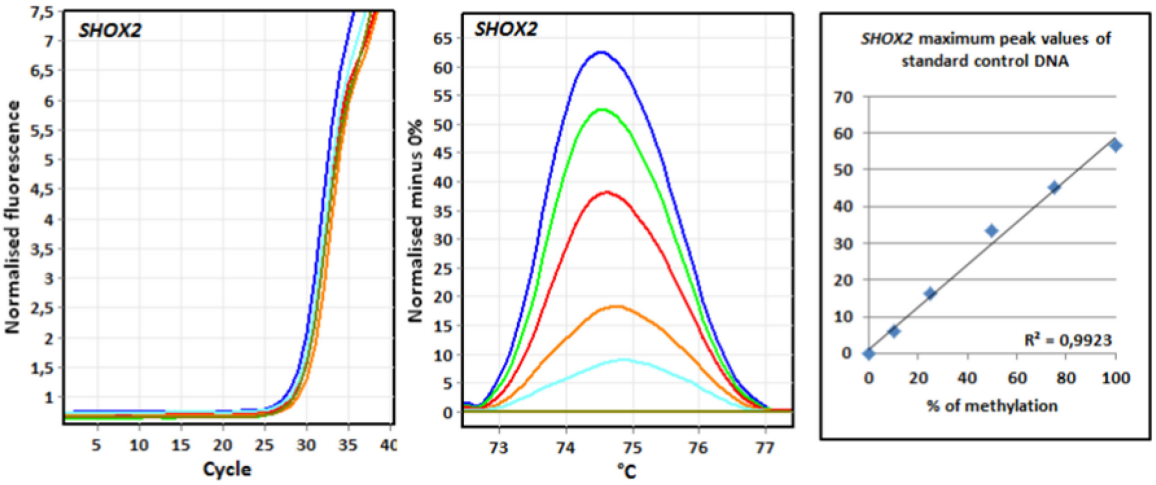


Figure 21. Test of linearity of MS-HRM assay *SHOX2*. Left panel: Amplification plot showing amplification of differentially methylated DNA standards at comparable Ct values. Middle panel: Normalization of melting curves against 0% methylated DNA. A clear curve separation depending on the % of methylation is shown. Right panel: Maximum peak values were plotted against the % of methylation resulting in high linearity regression lines.

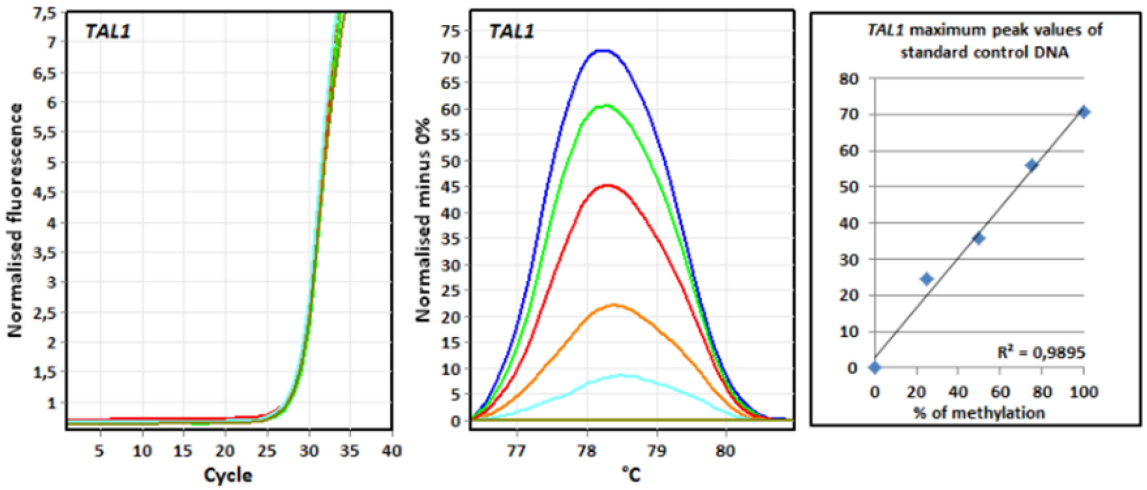


Figure 22. Test of linearity of MS-HRM assay *TAL1*. Left panel: Amplification plot showing amplification of differentially methylated DNA standards at comparable Ct values. Middle panel: Normalization of melting curves against 0% methylated DNA. A clear curve separation depending on the % of methylation is shown. Right panel: Maximum peak values were plotted against the % of methylation resulting in high linearity regression lines.

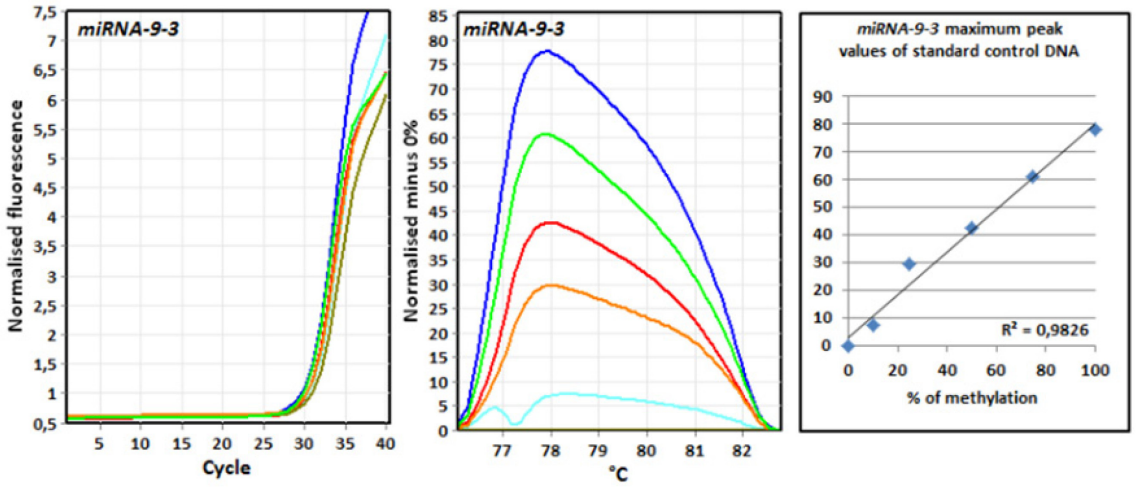


Figure 23. Test of linearity of MS-HRM assay *miRNA-9-3*. Left panel: Amplification plot showing amplification of differentially methylated DNA standards at comparable Ct values. Middle panel: Normalization of melting curves against 0% methylated DNA. A clear curve separation depending on the % of methylation is shown. Right panel: Maximum peak values were plotted against the % of methylation resulting in high linearity regression lines.

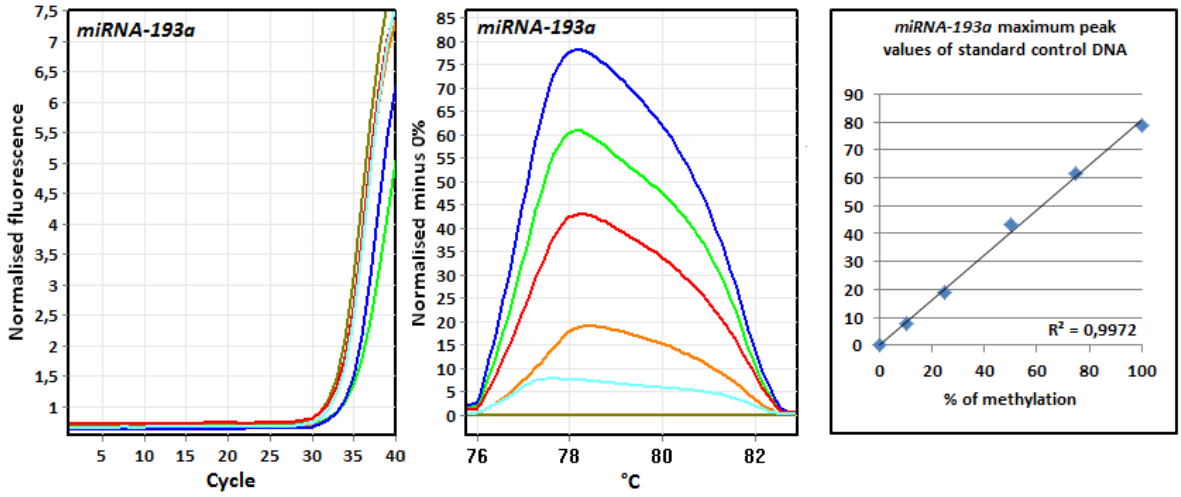


Figure 24. Test of linearity of MS-HRM assay *miRNA-193a*. Left panel: Amplification plot showing amplification of differentially methylated DNA standards at comparable Ct values. Middle panel: Normalization of melting curves against 0% methylated DNA. A clear curve separation depending on the % of methylation is shown. Right panel: Maximum peak values were plotted against the % of methylation resulting in high linearity regression lines.

Legend for HRM plots:

% of methylation

Table 2. Percentage of methylation of 5 genes in the NSCLC cell lines A549, NCI-H1993 and NCI-H2073

Cell line	**% of methylation**				
	HOXA2	*SHOX2*	*TAL1*	*miRNA-9-3*	*miRNA-193a*
A549	90	84	77	41	41
NCI-H1993	93	96	88	98	94
NCI-H2073	84	36	100	44	83

12.2 MS-HRM analyses of 5 genes in clinical samples of 97 NSCLC patients

Next, we performed MS-HRM analyses of the genes *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* and *miRNA-193a* in TU and NL samples of 97 stage I, II and III NSCLC patients. Consistent with our data derived from cell line experiments, we observed amplification of the 5 genes in clinical samples at comparable Ct values (Figures 25 - 29). Overall, clinical samples of 97 NSCLC patients were analysed in 8 MS-HRM runs. The mean R^2 values of these runs are 0.977 (*HOXA2*, range 0.961 -0.988, 0.982 (*SHOX2*, range 0.9592 - 0.992), 0.976 (*TAL1*, range 0.9439 – 0.997). 0.995 (*miRNA-9-3*, range, 0.984 – 0.999) and 0.994 (*miRNA-193a*, range, 0.986 – 0.998).

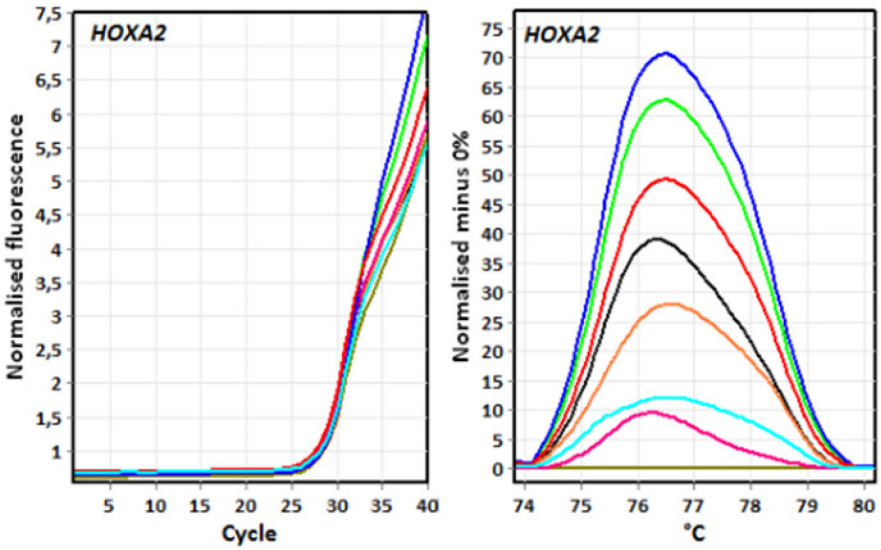


Figure 25. Example of MS-HRM analyses of the gene *HOXA2* in differentially methylated DNA standards and in clinical samples of NSCLC patients. A clear difference in methylation of this gene is shown for a TU and a NL.

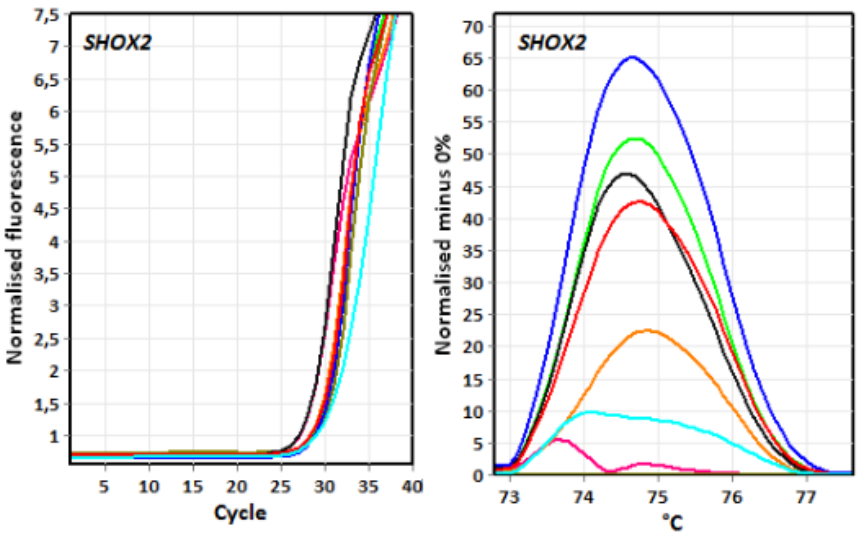


Figure 26. Example of MS-HRM analyses of the gene *SHOX2* in differentially methylated DNA standards and in clinical samples of NSCLC patients. A clear difference in methylation of this gene is shown for a TU and a NL.

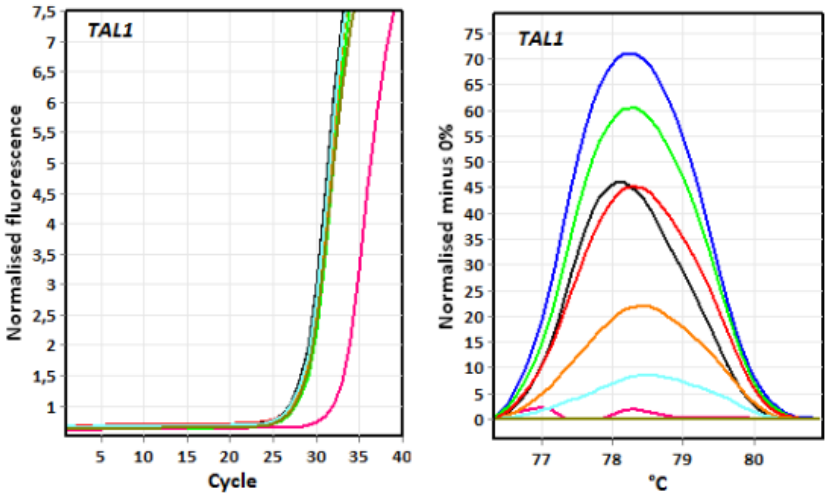


Figure 27. Example of MS-HRM analyses of the gene *TAL1* in differentially methylated DNA standards and in clinical samples of NSCLC patients. A clear difference in methylation of this gene is shown for a TU and a NL.

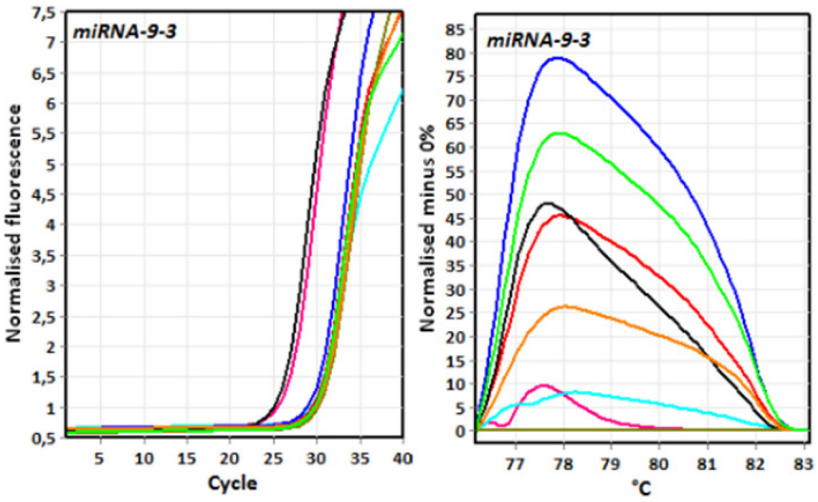


Figure 28. Example of MS-HRM analyses of the gene *miRNA-9-3* in differentially methylated DNA standards and in clinical samples of NSCLC patients. A clear difference in methylation of this gene is shown for a TU and a NL.

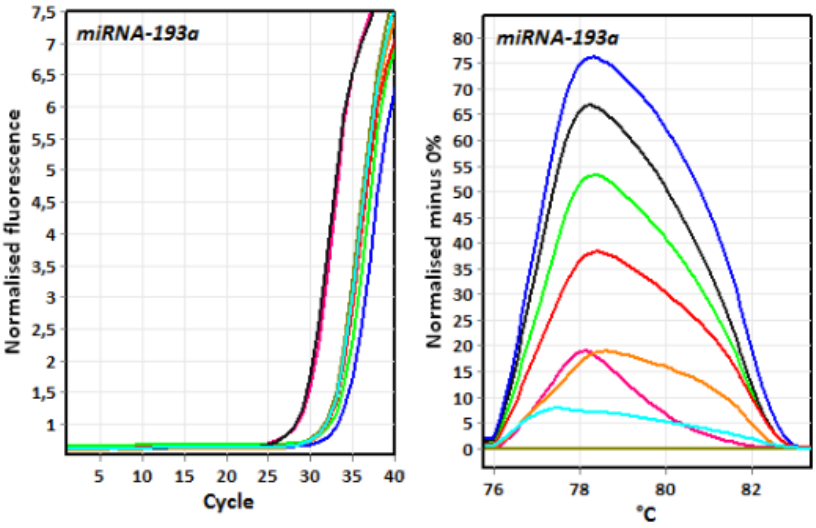
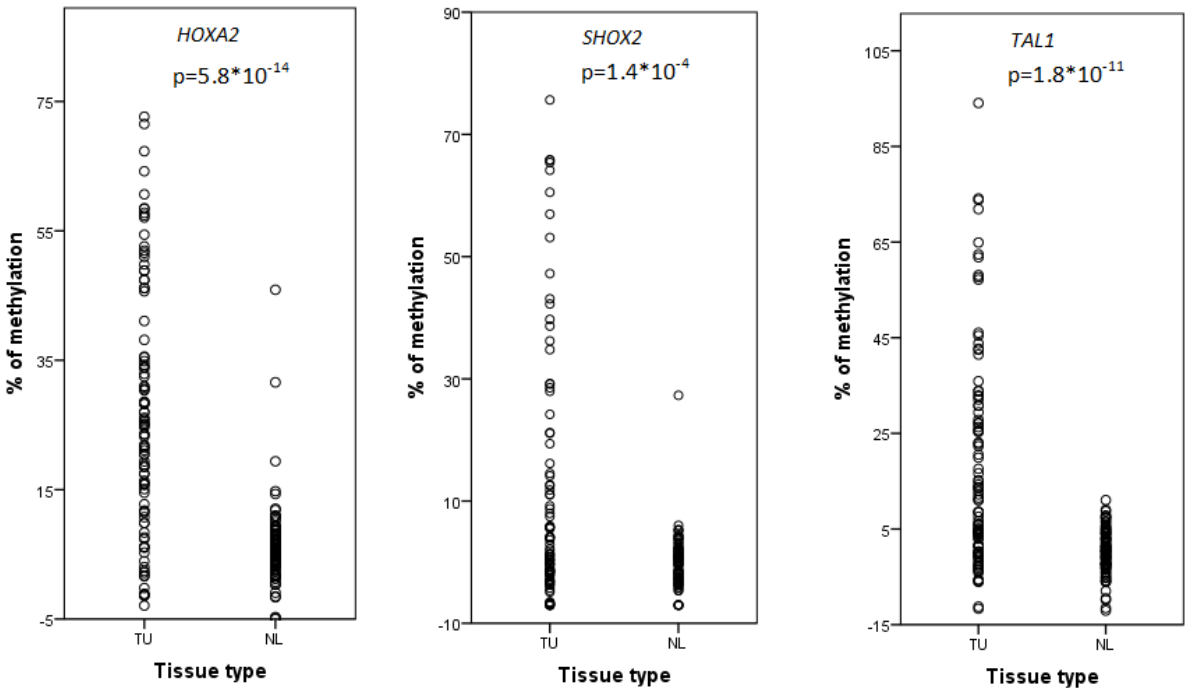


Figure 29. Example of MS-HRM analyses of the gene *miRNA-193a* in differentially methylated DNA standards and in clinical samples of NSCLC patients. A clear difference in methylation of this gene is shown for a TU and a NL.

Legend for HRM plots:

% of methylation

In addition, we observed statistically significant differences in methylation between TU and NL samples for all genes analysed. Consistent with our results of genome-wide DNA methylation analyses, we found that *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* and *miRNA-193a* are tumour-specifically methylated MS-HRM analyses (Figure 30). The mean % of *HOXA2* methylation in TU samples and NL samples were 76% (range 57% - 86%) and 0%, respectively. The mean % of *SHOX2* methylation in TU samples and NL samples were 71% (range 41% - 94%) and 0%, respectively. The mean % of *TAL1* methylation in TU samples and NL samples were 87% (range 61% - 100%) and 0%, respectively. The mean % of *miRNA-9-3* methylation in TU samples and NL samples were 89% (range 82% - 95%) and 6.65% (range 0% - 15.4%), respectively. The mean % of *miRNA-193a* methylation in TU samples and NL samples were 77% (range 75% - 81%) and 0.7% (range 0% - 0.7%), respectively.



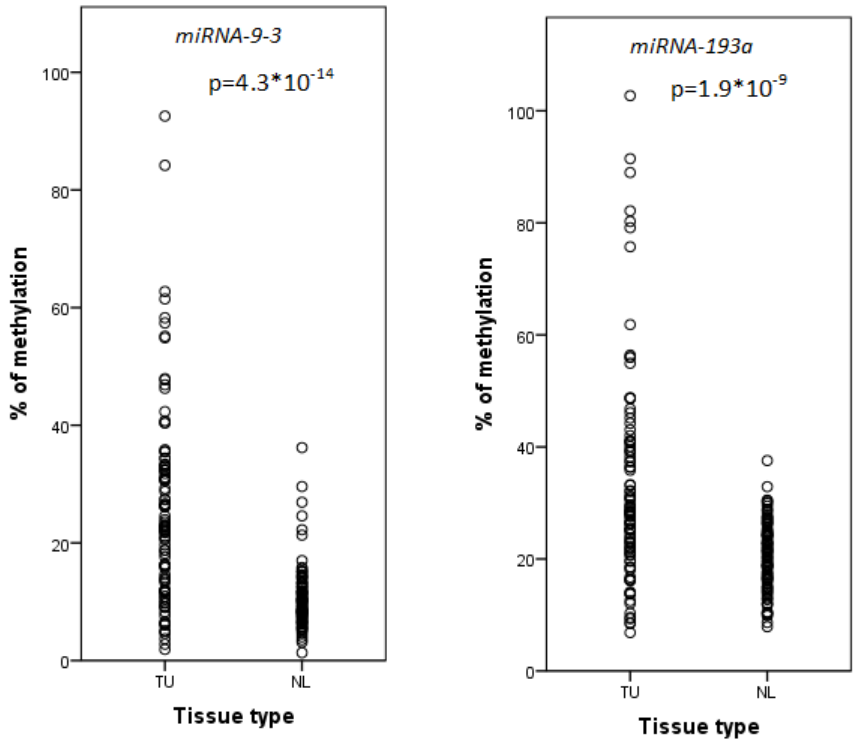


Figure 30. Summary of MS-HRM data of the genes *HOXA2*, *SHOX2*, *TAL1, miRNA-9-3 and miRNA-193a* in TU and NL samples of 97 NSCLC patients. Each circle represents a single tissue sample.

Moreover, we performed ROC curve analyses and found that methylation of all genes analysed statistically significant distinguishes TU samples from NL samples (Figure 31).

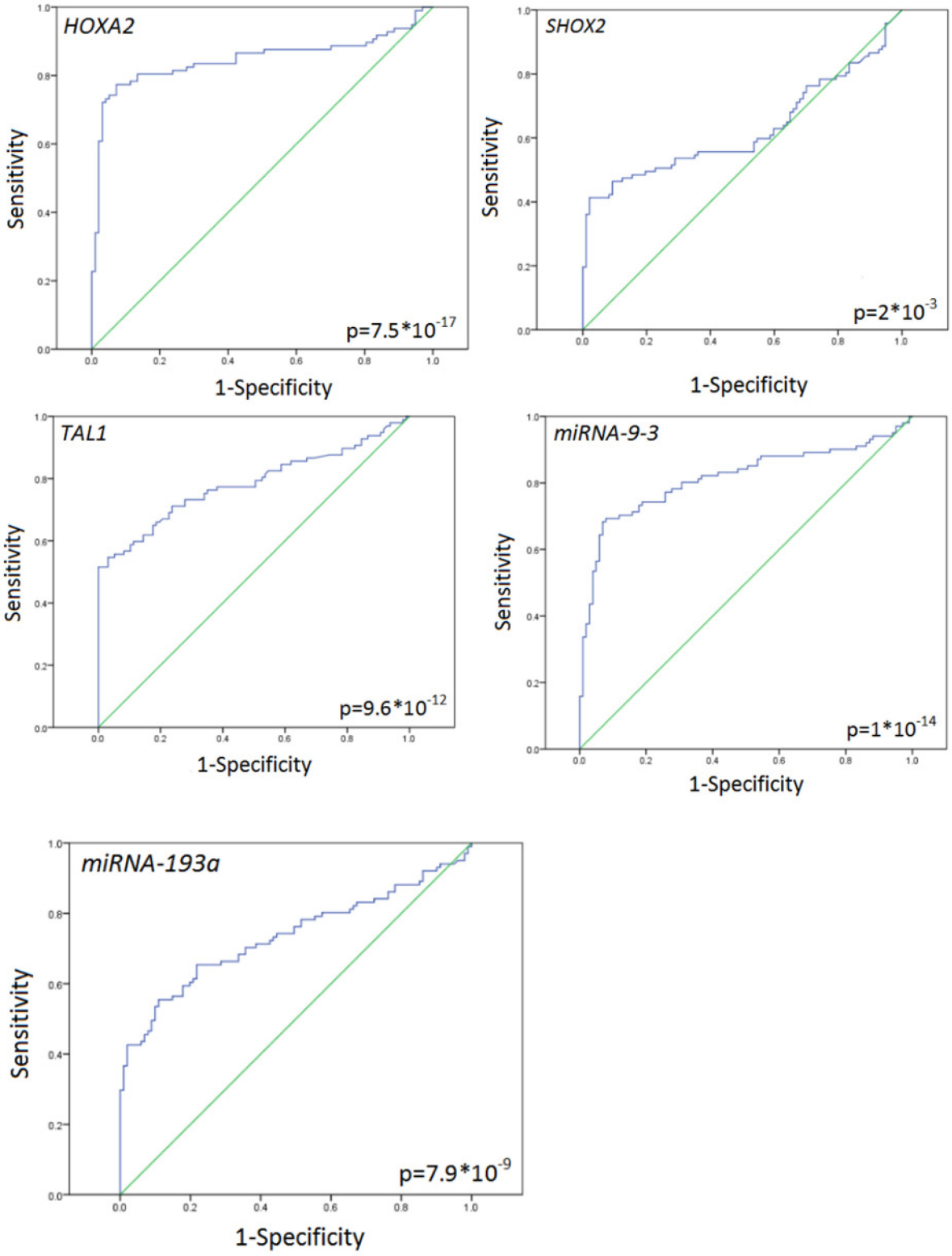


Figure 31. ROC curve analyses of *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* and *miRNA-193a* methylation determined by MS-HRM analyses of TU and NL samples of 97 NSCLC patients. Extend of methylation of these genes allows to distinguish between TU and NL samples. Green line, reference line; blue line, methylation of particular gene.

Next, for each patient T/N methylation ratios of the 5 genes were calculated and patients with a T/N ratio ≥ 1.5 were considered as methylated. The most frequently methylated genes was *HOXA2* (78%) followed by *miRNA-9-3* (68%), *TAL1* (61%), *miRNA-193a* (42%) and *SHOX2* (39%).

Finally, a heat map which summarizes methylation of the genes *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* and *miRNA-193a* in all NSCLC patients analysed was generated (Figure 32). Methylation of one gene was detected in 17/106 (16%) NSCLC patients, methylation of two genes in 19/106 (17.9%) NSCLC patients, methylation of three genes in 23/106 (21.7%) NSCLC patients, methylation of four genes in 25/106 (23.6%) NSCLC patients, and methylation of five genes in 12/106 (11.3%) NSCLC patients. Only 9.5% of NSCLC patients were not methylated for one of the genes analysed.

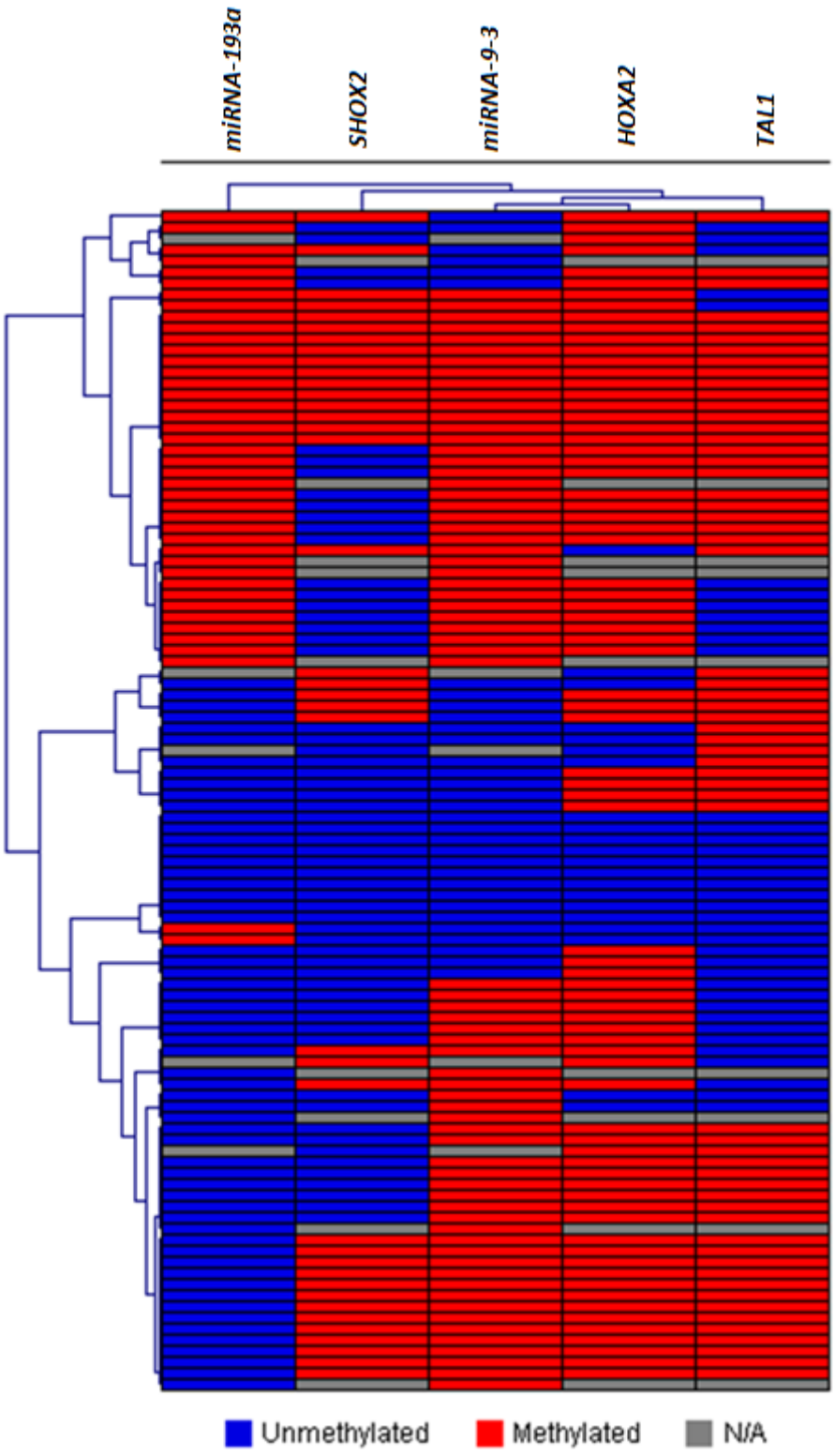


Figure 32. Heat map summarizing the methylation status of the genes *miRNA193a*, *SHOX2, miRNA-9-3, HOXA2 and TAL1* in 106 NSCLC samples. Blue, unmethylated, red, methylated, grey, not analysed.

12.3 Bisulfite genomic sequencing

To prove that genomic regions analysed by MS-HRM indeed are methylated, we performed BGS of a part of the 5´ regions of *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* and *miRNA-193a* in TU and NL samples (Figures 33 - 37). While in the TU specimens 76% of CpG sites analysed were found to be *HOXA2* methylated, in the NL samples 0% of CpG sites analysed were observed to be *HOXA2* methylated. In TU specimens 71% (*SHOX2*), 87% (*TAL1*), 89% (*miRNA-9-3*) and 77% (*miRNA-193a*) of CpG sites analysed were found to be methylated. In NL specimens 0% (*SHOX2*), 0% (*TAL1*), 21% (*miRNA-9-3*) and 3% (*miRNA-193a*) of CpG sites analysed were found to be methylated.

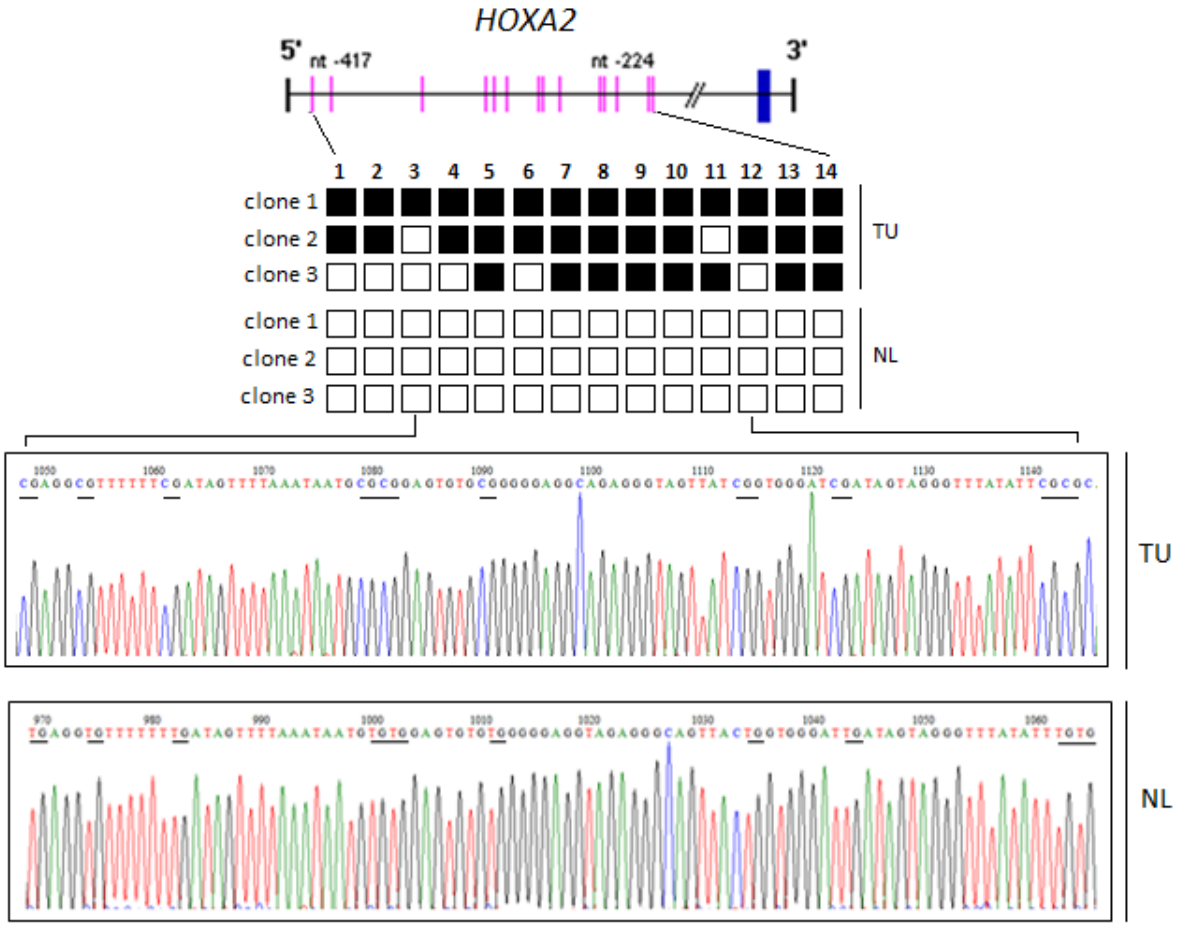


Figure 33. BGS results of parts of *HOXA2* 5´regions in a TU and NL sample. Fourteen CpG sites were analysed for methylation, respectively. CpG sites (pink bars) analysed by BGS relative to transcription start sites (blue bars) of the gene are shown. Three clones of a TU and NL sample were sequenced. Black squares indicate methylated cytosines at CpG sites, white

squares indicate unmethylated cytosines at CpG sites. In addition, representative chromatograms from BGS of parts of the gene in a TU and the NL sample are shown. CpG sites are underlined.

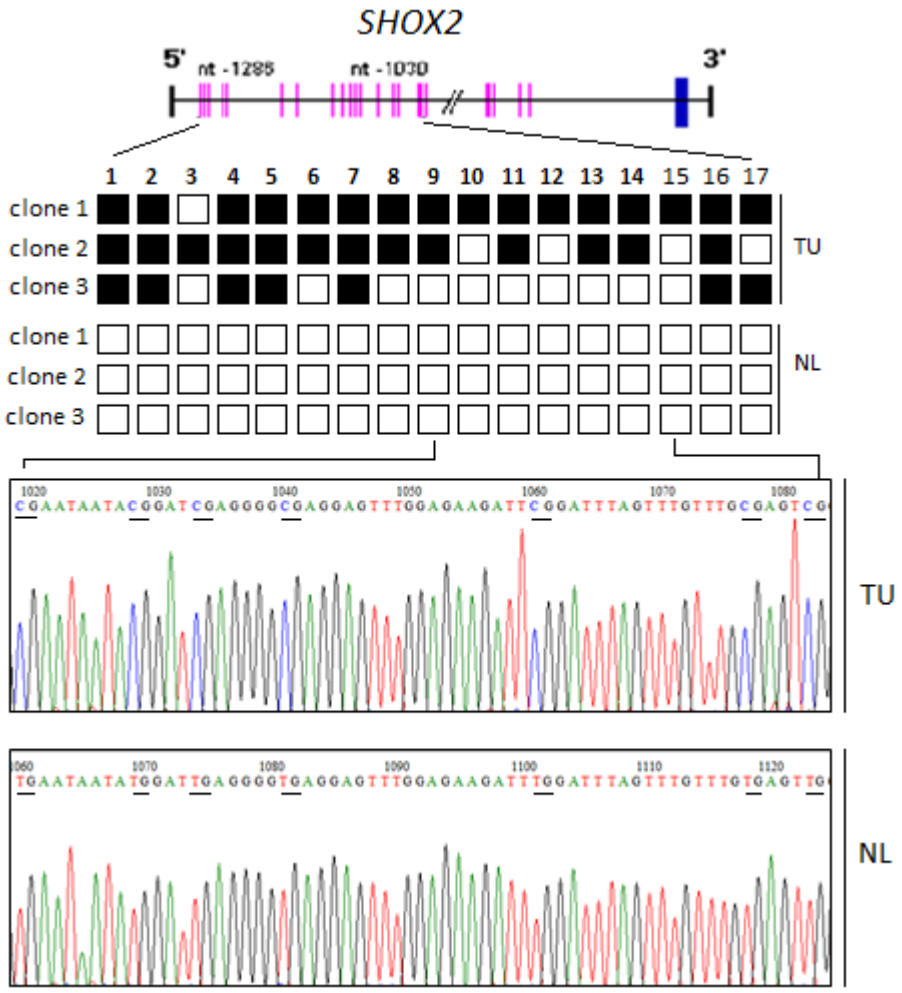


Figure 34. BGS results of parts of *SHOX2* 5´regions in a TU and NL sample. Seventeen CpG sites were analysed for methylation, respectively. CpG sites (pink bars) analysed by BGS relative to transcription start sites (blue bars) of the gene are shown. Three clones of a TU and NL sample were sequenced. Black squares indicate methylated cytosines at CpG sites, white squares indicate unmethylated cytosines at CpG sites. In addition, representative chromatograms from BGS of parts of the gene in a TU and the NL sample are shown. CpG sites are underlined.

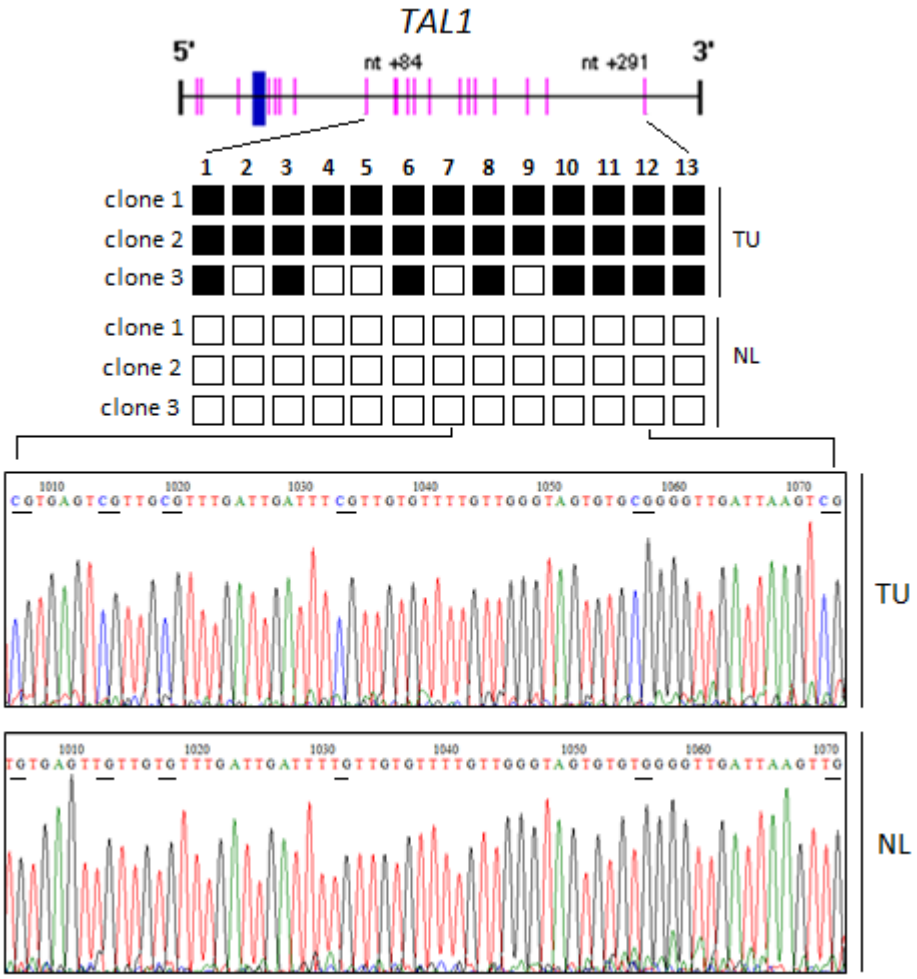


Figure 35. BGS results of parts *TAL1* 5´regions in a TU and NL sample. Thirteen CpG sites were analysed for methylation, respectively. CpG sites (pink bars) analysed by BGS relative to transcription start sites (blue bars) of the gene are shown. Three clones of a TU and NL sample were sequenced. Black squares indicate methylated cytosines at CpG sites, white squares indicate unmethylated cytosines at CpG sites. In addition, representative chromatograms from BGS of parts of the gene in a TU and the NL sample are shown. CpG sites are underlined.

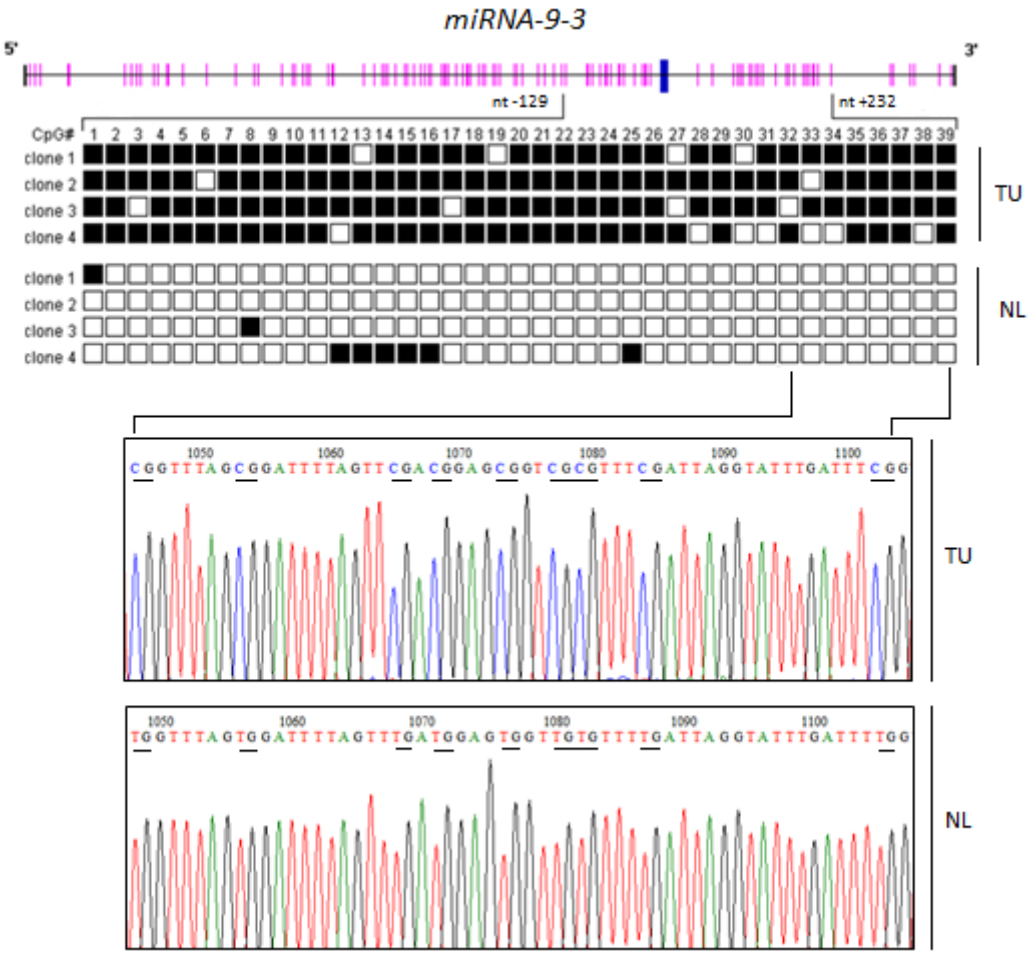


Figure 36. BGS results of parts of *miRNA-9-3* 5´regions in a TU and NL sample. Thirty-nine CpG sites per gene were analysed for methylation. CpG sites (pink bars) analysed by BGS relative to transcription start sites (blue bars) of the gene are shown. Four clones of a TU and NL sample were sequenced. Black squares indicate methylated cytosines at CpG sites, white squares indicate unmethylated cytosines at CpG sites. In addition, representative chroma-tograms from BGS of parts of the gene in a TU and the NL sample are shown. CpG sites are underlined.

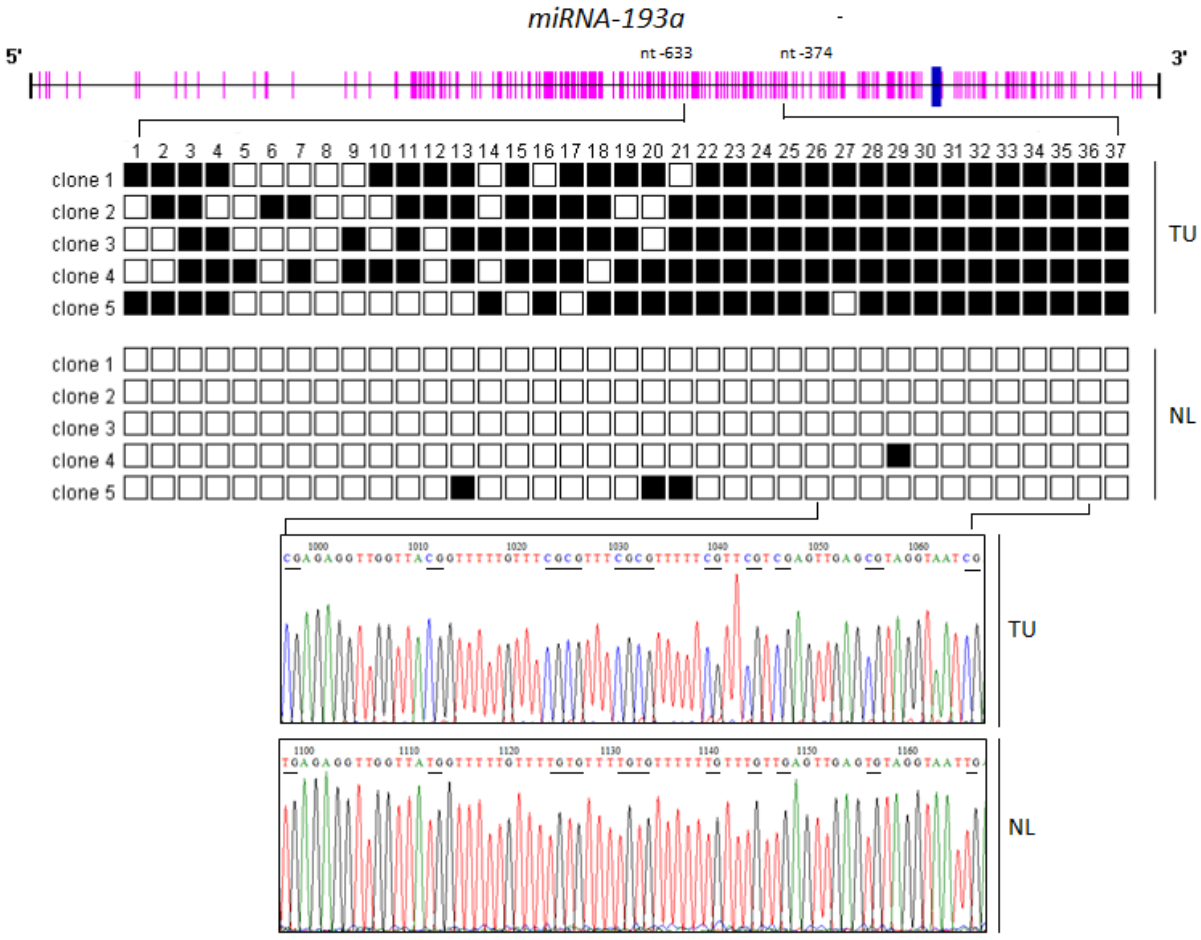


Figure 37. BGS results of parts of *miRNA-193a* 5´regions in a TU and NL sample. Thirty-seven CpG sites per gene were analysed for methylation. CpG sites (pink bars) analysed by BGS relative to transcription start sites (blue bars) of the gene are shown. Five clones of a TU and NL sample were sequenced. Black squares indicate methylated cytosines at CpG sites, white squares indicate unmethylated cytosines at CpG sites. In addition, representative chromatograms from BGS of parts of the gene in a TU and the NL sample are shown. CpG sites are underlined.

12.4 Comparison of MS-HRM results with clinico-pathological characteristics of NSCLC patients

Moreover, we compared the results with clinico-pathological characteristics of NSCLC patients. Therefore we used T/N rations of methylation obtained by calculation after MS-HRM analyses. T/N ratios of patients greater than 1.5 were determined to be methylated (Heller et al 2012).

T/N ratios of methylation of the 5 genes analysed by MS-HRM were used for comparison of clinico-pathological characteristics of the patients. No associations between methylation and DFS or OS of the whole study population were observed. However, *HOXA2* methylated squamous cell carcinoma (SCC) patients had a statistically significant shorter DFS than *HOXA2* not methylated SCC patients (median survival: 45 month vs. not reached, p = 0.034) in univariate analyses (Figure 38A). Similar findings were observed for OS with a statistically significant shorter OS of *HOXA2* methylated SCC patients compared to *HOXA2* not methylated patients (median survival: 39 month vs. not reached, p = 0.043, Figure 38B).

Also *miRNA-9-3* methylated SCC patients had a statistically significant shorter DFS than *miRNA-9-3* not methylated SCC patients (median survival: 35 month vs. not reached, p = 0.046) and a statistically significant shorter OS than *miRNA-9-3* not methylated SCC patients (median survival: 35 month vs. not reached, p = 0.046, Figure 38C and 38D).

In addition, multivariate analyses identified both *HOXA2* and *miRNA-9-3* methylation as independent prognostic factor for shorter DFS of SCC patients (*HOXA2* methylation: hazard ratio (HR) = 5.8, 95% confidence interval (CI) = 1.2 to 28.2, p = 0.031; *miRNA-9-3* methylation: HR = 4.6, 95% CI = 1.3 to 16.1, p = 0.018) (Heller et al 2012).

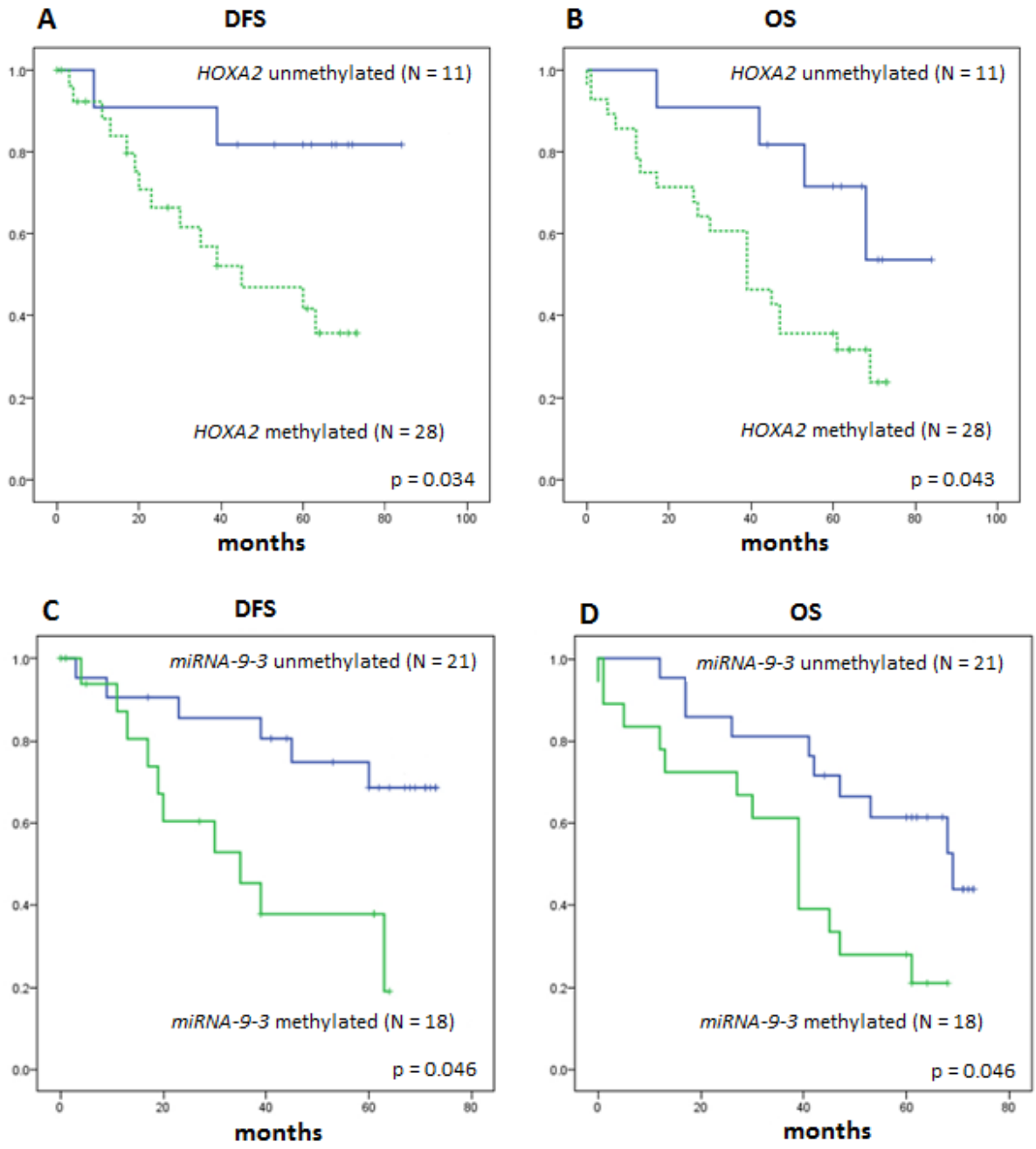


Figure 38. Results for *HOXA2* and *miRNA-9-3* regarding DFS and OS. Results are shown by Kaplan-Meyer plots for 39 patients (N = 39). Nine of them were treated with platin-based chemotherapy and two of them with radiotherapy after disease recurrence. A: DFS for *HOXA2* methylated (N = 28) and not methylated (N = 11) SCC patients; B: OS for *HOXA2* methylated (N = 28) and not methylated (N = 11) *HOXA2* SCC patients; C: DFS for *miRNA-9-3* methylated (N = 18) and not methylated SCC (N = 21) patients; D: OS for *miRNA-9-3* methylated (N = 18) and not methylated (N = 21) SCC patients (Heller et al 2012).

13 Discussion

About 1.200.000 people are diagnosed with lung cancer per year worldwide. In 2009, 4.239 people were diagnosed with lung cancer in Austria. Despite advances in developing new treatment strategies and new anticancer drugs in recent years, the prognosis of lung cancer patients is still poor with 5-year OS rates of about 14%. Thus, defining molecular markers of prognostic relevance would be the basis for a more personalized therapy promising improved outcome.

Beside genetic changes (e.g. mutations, deletions, chromosomal loss) epigenetic abnormalities, especially DNA methylation, are involved in the pathogenesis of NSCLC. So far, several genes have been identified which are frequently methylated in NSCLCs (Heller et al 2010). Recently, members of the research lab of Ao. Univ. Prof. Dr. Sabine Zöchbauer-Müller performed genome-wide screens to identify protein encoding and miRNA encoding genes which are targets for methylation in NSCLCs. Overall, 298 tumour-specifically methylated protein encoding genes were identified using MeDIP-chip analyses of 101 NSCLC patients. In addition, 33 miRNAs were found to be targets for methylation in NSCLC cell lines. Three protein encoding genes (*HOXA2*, *SHOX2* and *TAL1*) and 2 miRNA encoding genes (*miRNA-9-3* and *miRNA-193a*) were selected for further methylation analyses in NSCLC cell lines and in TU and NL samples of 97 NSCLC patients during this diploma thesis.

DNA methylation of these genes was analysed using the recently developed technique MS-HRM. This method is based on methylation-insensitive amplification of sodium bisulfite-modified DNA followed by high resolution melt analysis. The major advantages of this method are that MS-HRM analysis is an in-tube, cost-effective, very sensitive and quantitative alternative to other PCR based techniques (e.g. methylation-specific PCR) for detection of DNA methylation (Wojdacz et al 2008). The quality of our MS-HRM assays was strengthened by plotting fluorescence values of predefined methylation standards (0% methylated – 100% methylated) against the percentage of methylation. A strong linear relationship (R^2-values >0.95) between fluorescence and percentage of methylation was observed for all MS-HRM assays used for subsequent analyses of clinical samples.

The genes *HOXA2* and *SHOX2* are members of the homeobox gene family. Homeobox genes are transcription factors which are involved in regulation of various developmental proc-

esses including morphogenesis and cell differentiation (Abate-Shen 2002, Gehring and Hiromi 1986, Kim et al 2009, Stein et al 1996). To date more than 200 human homeobox genes are known. Thirty-nine of them are located in 4 HOX gene clusters (HOXA on chromosome 7, HOXB on chromosome 17, HOXC on chromosome 12 and HOXD on chromosome 2) (Abate-Shen 2002, Kim et al 2009). Besides their involvement in developmental processes some HOX genes are supposed to have tumour suppressor gene function (Shah and Sukumar 2010). For example, *HOXA5* and *HOXA10* are involved in G1 cell cycle checkpoint regulation by regulating expression of *p53* and *CDKN1A* (Bromleigh and Freedman 2000, Raman et al 2000). *HOXB13* represses the β-catenin-TCF pathway and acts as a tumour suppressor in colorectal cancer (Jung et al 2005). Moreover, *HOXB13* suppresses the transactivation of the androgen receptor, thus, functioning as a tumour suppressor in prostate cancer cells (Jung et al 2004). Interestingly, Rauch et al. (Rauch et al 2007) observed that all 4 HOX gene clusters are targets for methylation in the lung adenocarcinoma cell line A549. Very similar data were observed by the research group of Ao. Univ. Prof. Sabine Zöchbauer-Müller. *HOXA2* is one member of the HOX gene family and *HOXA2* methylation was reported in up to 94% of extrahepatic cholangiocarcinomas analysed (Shu et al 2011). In addition, it was reported that *HOXA2* expression is downregulated in gastric carcinomas compared with non-malignant gastric tissue samples (Rossi Degl'Innocenti et al 2007). Using MS-HRM analyses, we found highly significant tumour-specific methylation of *HOXA2* in NSCLC patients. BGS was performed to validate MS-HRM data in some clinical samples. Those samples which were found to be *HOXA2* methylated by MS-HRM analyses were also found to be methylated by BGS. Overall, 90% (A549), 93% (NCI-H1993) and 84% (NCI-H2073) of NSCLC cell lines and 78% of NSCLC patients analysed were found to be *HOXA2* methylated suggesting that methylation of this gene is an important event in the pathogenesis of NSCLCs.

SHOX2 (short stature homeobox 2) is located on chromosome 3 and is involved in regulation of gene transcription. Methylation of *SHOX2* in NSCLCs was reported recently (Kneip et al 2011, Schmidt et al 2010, Schneider et al 2011). These data are in concordance with our results showing that 77% (A549), 96% (NCI-H1993) and 36% (NCI-H2073) of NSCLC cell lines and 71% of NSCLC patients analysed are *SHOX2* methylated. Again, MS-HRM results of some patients were confirmed by BGS.

The *TAL1* (T-cell acute lymphocytic leukemia 1) gene is located on chromosome 1. *TAL1* is known to be a serine phosphoprotein, basic helix-loop-helix transcription factor and is asso-

ciated with embryonic haematopoiesis and T-cell acute lymphoblastic leukemia (Bash et al 1995, Bernard et al 1995, Chetty et al 1997, Hsu et al 1991, Huang and Brandt 2000, Kelliher et al 1996, Pulford et al 1995). Differences in the methylation status of *TAL1* were reported in pancreatic cancers and non-malignant control samples (Pedersen et al 2011). No other studies about *TAL1* methylation and the role of *TAL1* in the pathogenesis of NSCLCs have been reported so far. We found *TAL1* frequently methylated in both, NSCLC cell lines 77% (A549), 88% (NCI-H1993), 100% (NCI-H2073) and in primary NSCLCs (87%). Besides its oncogenic function in hematologic malignancies *TAL1* was found to upregulate expression of the prostate cancer tumour suppressor gene *NKX3.1* (Armstrong and Look 2005, Kusy et al 2010). Our data suggest that *TAL1* is involved in the pathogenesis of NSCLC, however, its role in the development of this disease needs to be investigated in future studies.

Deregulated expression of miRNA genes is a relatively new abnormality which was observed in many cancer types (Calin and Croce 2006). Recent data suggest that downregulated expression of miRNA genes is an important event in the pathogenesis of lung cancer, however, knowledge about mechanisms leading to silencing of many of these miRNA genes is still limited (Peltier and Latham 2008, Volinia et al 2006, Wang et al 2011a, Yanaihara et al 2006). During this diploma thesis, we analysed methylation of the genes *miRNA-9-3* and *miRNA-193a,* whose expression was found to be upregulated in Aza-dC or Aza-dC/TSA treated NSCLC cell lines recently, in NSCLC cell lines and in clinical samples of NSCLC patients (Heller et al 2012). Interestingly, both genes were found to be methylated at various extents in NSCLC cell lines. Moreover, statistically significant tumour-specific methylation of *miRNA-9-3* and *miRNA-193a* was observed in NSCLC patients. Again, *miRNA-9-3* and *miRNA-193a* MS-HRM results of some clinical samples were confirmed by BGS. While *miRNA-9-3* was found to be methylated in 89% of NSCLC patients *miRNA-193a* methylation was observed in 77% of NSCLC patients. The frequency of *miRNA-9-3* methylation in primary NSCLCs is very similar compared to data reported recently by Lujambio et al (Lujambio et al 2008) who found 53% of primary lung tumours *miRNA-9-3* methylated. Besides in lung cancers, *miRNA-9-3* methylation was also observed in acute lymphoblastic leukemia (Rodriguez-Otero et al 2011). Furthermore, deregulated *miRNA-9-3* expression was reported in ovarian cancer, breast cancer and gastric carcinomas (Laios et al 2008, Lehmann et al 2008, Luo et al 2009) suggesting that *miRNA-9-3* is involved in the pathogenesis of various cancer types. *MiRNA-193a* was found to regulate expression of certain oncogenic factors and thus, is suggested to be a tumour

suppressor miRNA (Gao et al 2011, Kozaki et al 2008). Recently, it was reported that *miRNA-193a* is silenced by methylation in myeloid leukemia and in oral squamous cell carcinoma (Gao et al 2011, Kozaki et al 2008).

Next, we compared methylation results of the 5 genes with clinico-pathological characteristics of the NSCLC patients. No statistically significant associations between *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* or *miRNA-193a* methylation and age, sex, tumour stage, lymph node stage and stage of disease were found. While no associations between methylation of the 5 genes and DFS or OS of the whole study population were observed, *HOXA2* and *miRNA-9-3* methylated SCC patients had a statistically shorter DFS and OS compared to *HOXA2* and *miRNA-9-3* not methylated SCC patients. Compatible to these findings, we detected *HOXA2* and *miRNA-9-3* methylation more frequently in SCC patients with disease recurrence compared to SCC patients with no disease recurrence, however, this association did not reach statistical significance. Although the numbers of patients in our survival analyses are low, we believe that *HOXA2* and *miRNA-9-3* methylation may be of prognostic impact for patients with SCC. However, prospective studies are necessary to confirm these results. Overall, our findings might be potentially helpful for a more personalized treatment and follow-up care of patients with SCC after surgery.

In summary, our methylation results suggest that MS-HRM is a very useful technique for detection of methylation. Using this method, we were able to confirm results of previous genome-wide approaches for detection of methylation of protein encoding genes and miRNA encoding genes. Statistically significant differences of *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* and *miRNA-193a* methylation in TU and NL samples of a large number of NSCLC patients were observed. In addition, we suggest that *HOXA2* and *miRNA-9-3* methylation might be useful prognostic markers for SCC patients, however, these findings need to be confirmed in additional studies. Overall, our results stress the importance of methylation of both protein encoding genes and miRNA encoding genes for the pathogenesis of NSCLCs.

In conclusion, using gene specific methylation approaches in NSCLC cell lines and TU samples, an accurate quantification of methylation could be performed. The BGS approach confirms and strengthens genome-wide as well as gene-specific results in NSCLC. Major differences of promoter methylation of *HOXA2*, *SHOX2*, *TAL1*, *miRNA-9-3* and *miRNA-193a* in TU and NL NSCLC samples could be obtained. DNA methylation of *HOXA2* and *miRNA-9-3* in SCC

patients is associated with shorter DFS and OS suggesting that these genes could be potential prognostic factors. Overall these results implicate that DNA methylation is an important mechanism in the pathogenesis of NSCLC.

14 Literature

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Curriculum Vitae

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10.2011	Internship at the Medical University Vienna, Clinical Institute of Pathology
09.2010 – 02.2011	Diploma Thesis at the Medical University Vienna, University Clinic for Inner Medicine I, Clinical Oncology
06.2010 – 08.2010	Internship, Medical University Vienna, University Clinic for Inner Medicine I, Clinical Oncology
03. 2008	Start of the second part of the diploma studies Genetics and Microbiology
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10. 2005	Additional major exam for Biology at the Grammar-School Baden Biondekgasse
09. 2005	University-entrance diploma at the Vienna Business School
06. 2005 – 09. 2005	Preliminary courses for the University-entrance exam and the additional mayor exam Biology
10. 2004	Qualification exam for the officialised business “Forwarding- and Transport-Agents

09. 2004 – 06. 2005	Preliminary courses for the University-entrance exam at WIFI Mödling and WIFI St. Pölten (Institute of the Austrian Economic Centre of Trade)
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Work Experience:

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Additional Skills:

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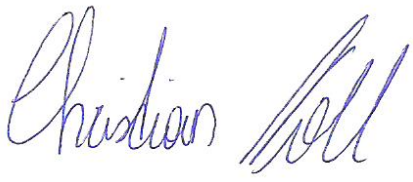
Kottingbrunn, October 7th 2012



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Posters

Gerwin Heller, Valerie Babinsky, Barbara Ziegler, Marlene Weinzierl, **Christian Noll**, György Lang, Adelheid End-Pfützenreuter, Irene Womastek, Sonja Zehetmayer, Balasz Döme, Britt-Madeleine Arns, Kwun M. Fong, Casey M. Wright, Ian A. Yang, Rayleen V. Bowman, Walter Klepetko, Martin Posch, Christoph C. Zielinski, Sabine Zöchbauer-Müller (2011)
Genome-wide DNA methylation analysis identifies tumor-specifically methylated genes in non-small cell lung cancer patients.

Papers

Heller G, Weinzierl M, **Noll C**, Babinsky V, Ziegler B, Altenberger C, Minichsdorfer C, Lang G, Dome B, End-Pfutzenreuter A, Arns BM, Grin Y, Klepetko W, Zielinski CC, Zochbauer-Muller S. (2012)
Genome-wide microRNA expression profiling identifies miR-9-3 and miR-193a as targets for DNA methylation in non-small cell lung cancers (Clin Cancer Res. 2012 Mar 15;18(6):1619-29. Epub 2012 Jan 26).

References

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