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Role of carcinogens factors in epigenetic silencing of Rassf1a, DAPK, p16, MT1A genes in human lung

Dissertation
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Umwelttoxikologie

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Referat

In der vorliegenden Arbeit haben wir zunächst die Häufigkeit von epigenetischen Inaktivierungen durch Methylierung bei den tumorassoziierten Genen *Ras Association Domain-Containing Protein* (Rassf1a), *Death-Associated Protein Kinase* (DAPK), *Cyclin Dependent Kinase Inhibitor 2A* (p16) und dem Detoxifikationsprotein *Metallothionein 1A* (MT1A) untersucht. Von 26 Lungenkrebspatienten wurden Proben genommen und mittels methylisierungsspezifischer Polymerase-Kettenreaktion (PCR) untersucht. Die Häufigkeit der DNA-Methylierung lag bei: 62% für Rassf1a, 46% für DAPK, 54% für p16 und 39% für MT1A. Die Prüfung auf statistisch signifikante Korrelation zwischen Methylierungsstatus, Genen und Gewebeursprung einerseits sowie klinischer Anamnese und Alter, Geschlecht, Rauchgewohnheiten und Schadstoff-Exposition des Patienten am Arbeitslatz andererseits wurden untersucht. MT1A (p = 0,007), DAPK (p < 0,001) und p16 (p = 0,005) zeigten signifikante Assoziationen. Metallexposition am Arbeitsplatz war ebenfalls signifikant assoziiert mit einer Methylierung des p16-Gens (p = 0,03) im gesunden Gewebe. Die allgemeinen Unterschiede im Methylierungsstatus der Gene der Probanden im Tumorgewebe und gesundem Gewebe wurden bei allen Proben auf patientenspezifischem Level geprüft. Die Ergebnisse zeigen signifikante Assoziationen für DAPK (p = < 0.001), p16 (p = 0,005) und MT1A (p = 0,007). Bei rauchenden Patienten gab es signifikante Assoziationen Im Methylierungsstatus zwischen Tumor und gesundem Gewebe für DAPK (p = 0,00004), p16 (p = 0,01522) und MT1A (p = 0,0345). Daneben haben wir an Primärkulturen von normalen humanen Lungenzellen von 12 Patienten die Rolle einer Langzeitexposition [14 Wochen für NHBEC und 11 Wochen für PLC] gegenüber den karzinogenen Faktoren Tabakrauchkondensat (eng. cigarette smoke condensate (CSC)) und Cadmium(II) bei der Inaktivierung der Gene Rassf1a, DAPK, p16 und MT1A untersucht. Im MTT-Assay wurden die Substanzen zuvor auf zelluläre Toxizität untersucht. Wir konnten zeigen, dass Cadmium (II) signifikant die aberrante Methylierung von DAPK in PLC (p = 0,019) und in NHBEC (p = 0,097) induzierte. CSC führte zu einer signifikanten Induktion von p16-Methylierung in PLC (p = 0,047), CSC führte auch zu einer Senkung der p16-Gentranskription bei den methylierten NHBC-Proben [(p = 0,022); 70%], und bei methylierten PLC-Proben [(p = 0,002) 85%]. Die vorliegenden Ergebnisse zeigen, dass Zellkulturen als In-vitro-Modelle einen Beitrag zur Langzeituntersuchung der Wirkung verschiedener Umweltschadstoffe leisten können und ein besseres Wissen über die Epigenetik von Tumorsuppressorgenen in Bezug auf die Ätiologie von Lungenkrebs ermöglichen können.

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Contents

List of Abbreviations

µg	Microgramm
AECG	Airway Epithelial Cell Growth Medium
AMD	a central auto modification domain
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BER	Base excision repair
BSA	Bovine Serum Albumin
Cacl2	Calcium chloride
Cal/EPA	California Environmental Protection Agency
CDC	Centers for Disease Control and Prevention
cDNA	Complementray DNA
CHD	Chronic hypertensive disease
COPD	Chronic obstructive pulmonary disease
CPT	Camptothecin
CSC	Cigarette Smoke Condensate
CSE	Cigarette Smoke Extract
CuSO4	Copper sulphate
DAPK	Death associated protein kinase
DBD	DNA-binding domain
DEPC	Diethyldicarbonat
DMEM	Dulbecco´s modified Eagle´s medium
DNA	Deoxyribonucleic acid
dNTPs	Nucleoside triphosphate
DTT	Dithiothreitol

EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic Acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal Bovine Serum
FITC	Fuoresceine isothiocyanate
GAPDH	Glyceraldehde 3-phosphate dehydrogenase
HCL	Hydrochloric acid
Hg Cl2	Mercuric chloride
IARC	International Agency for Research on Cancer
KD	Kilo Dalton
LEC	Liver endothelial cells
MgCl2	Magnesium chloride
MgSo4	Magnesiumsulfat
MMLV RT	Moloney murine leukemia virus reverse transcriptase
MMR	Mismatch repair
MT1A	Metallothionein1A
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaOH	Sodiumhydoxide
NHBE	Normal human bronchial cells
NLS	Nuclear localization signal
NSCLC	Nonsmall lung cell lung carcinoma
P16	Cyclin dependent kinase inhibitor 2A
PAD	peripheral artery disease

PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PLC	Peripheral lung cells
PTCA	Percutaneous transluminal coronary angioplasty
Rassf1a	Ras association domain-containing protein
ROS	Reactive oxygen species
RT	Room temperature
SDS	Sodium dodecyl sulfate
SG	SYBR green
SIDS	Sudden infant death syndrome
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TE	buffer Tris and EDTA buffer
TPP	Techno plastic products
Tris	Tris (hydroxymethyl) aminomethane
USDHHS	U.S. Department of Health and Human Services
WHO	World Health Organisation

1 Introduction

1.1 Lung cancer

Lung cancer is the most prevalent cancer related death with 1.37 million deaths per year (Ferlay et al., 2010). The majority of primary lung cancers are lung carcinomas and can be divided into two groups; Small cell lung carcinoma (SCLC) and Non-Small Cell Lung Carcinoma (NSCLC). SCLC is an aggressive neuroendocrine tumor consisting of small tumor cells deriving from epithelial and neuroendocrine cells. This type of lung cancer is strongly associated with smoking and with a poor prognosis. People with small-cell lung cancer in the advanced stage cannot be cured. They usually survive less than one year. NSCLC accounts for approximately 80% of all lung cancers and includes three histological subtypes; adenocarcinoma (AdC), squamous cell carcinoma (SCC), and large cell carcinoma (LCC) (Travis et al., 2002). In recent years, AdC of the lung has replaced SCC as the most frequent histologic subtype for both men and women (Devesa et al., 2005), (AdC) arises from cells with glandular or secretary properties in the periphery of the lung (Sun et al., 2007).

Staging and TNM classification of NSCLS

Staging is one of the most important components in the clinical management of lung cancer. Accurate staging is important because it allows the clinician to predict prognosis and assign appropriate therapy and also provides a system that allows clinicians and researchers to stratify patients into reasonably homogenous groups so that treatment outcomes can be appropriately compared. The pathologic stage refers to the best prediction of the stage following pathologic analysis of the patient's tumor, lymph nodes, and/or metastases tumor/node/metastasis (TNM) and is usually applied following surgical resection or exploration. The last updated staging system is the 7th edition of the American Joint Commission on cancer (AJCC) and the International Union Against Cancer (UICC) Staging Manual (Goldstraw, 2010).

1.2 Risk factors of lung cancer

1.2.1 Cigarette smoking

Cigarette smoking is the most important risk factor for lung cancer, and it is considered to be responsible for 85% of lung cancer in men and 47% of lung cancer in women. The risk of lung cancer in cigarette smokers depends on duration of smoking, number of cigarettes

smoked, type of cigarette smoked and inhaling pattern. The relative risk for lung cancer between smokers with respect to no smokers is 15 and 30 times. Although the risk of lung cancer in former smokers remains higher than in individuals who have never smoked, the risk for former smokers decreased over time. The benefit of cessation becomes apparent approximately 5 years after quitting. Passive smoking carries a risk of developing lung cancer. It has been estimated a relative risk between 1.14 to 5.20 in people who had never smoked but who lived with a smoker (Zhong et al., 2000). Cigarette smoke contains more than 6,000 components, many of which can lead to DNA damage (Lofroth, 1989). Consistent with this, cigarette smoke exposure has been reported to induce DNA damage in a variety of cell types (La Maestra et al., 2011). Clinically, one of the major cell types affected by cigarette smoke is the airway epithelium, where damage can lead to cancer and may contribute to the development of chronic obstructive pulmonary disease (Agusti et al, 2002; Tuder et al, 2003; Hodge et al., 2003). Cigarette smoke condensate (CSC) refers to the sticky particles comprised of thousands of chemicals created by burning tobacco. It is the particulate component of tobacco smoke without nicotine and water. Cigarette smoke extract (CSE) is prepared by combustion of cigarette without filter and bubbling of the smoke through distilled water. This solution is considered to be 100% CSE. CSC is a chemical mixture containing different compounds, of which 100 or more are known carcinogens, co-carcinogens, mutagens and tumor promoters (Hoffmann et al., 2001), oxidants and aldehydes, all of which have the potential to cause inflammation and damage cells. Oxidants are thought to play a major role in cell injury induced by tobacco smoke since each puff of tobacco smoke contains approximately 1017 oxidant molecules (Church et al., 1985). Exposure to cigarette smoke activates an inflammatory cascade in the airway epithelium resulting in the production of a number of potent cytokines and chemokines, with accompanying damage to the lung epithelium, increased permeability, and recruitment of macrophages and neutrophils to the airway (Adler et al., 1994). Cigarette smoke has been associated with damage to the alveolar epithelium. In particular it can induce suppression of cell proliferation, increase detachment of cells, DNA strand breaks, and reduced surfactant production (Hoshino et al, 2001; Yokohori et al., 2004). When damage occurs to the DNA of a cell, several responses are possible. Often apoptosis or programmed cell death occurs, a response thought to protect the integrity of the genome (Liu et al., 2006).

1.2.2 Cadmium

Cadmium as an environmental pollutant is toxic to a number of tissues. Acute exposure to cadmium produces hepatic, pulmonary, and testicular injuries, whereas chronic exposure can result in renal and bone injury and subsequently cancer. The human exposure to heavy metals such as cadmium arises from widespread sources including cigarette smoke, air pollution, leaching of landfills, industrial waste, from fossil fuels, fertilizers, and corrosion of pluming. The exposure of lung to cadmium is associated with the development of pulmonary damage such as emphysema and lung cancer (Chubatsu et al., 1992). The cadmium absorption, distribution, and elimination are not yet well understood, but it is known that cadmium is poorly absorbed after oral ingestion. However, metallothionein decreases cadmium elimination through the bile and is a major factor for tissue retention of cadmium. Several studies also suggest both acute and chronic Cd exposure is associated with elevated lipid peroxidation in the lung, brain, kidney, liver, erythrocytes, and testis (Patra et al., 2011). Epidemiological cohort studies of workers found that exposure to various cadmium compounds increased the risk of death by lung cancer. Cadmium is an established carcinogen that has very low mutagenicity. Cadmium arises from widespread sources including cigarette smoke, air pollution, the leaching of landfills, industrial waste, fossil fuels, fertilizers, and the corrosion of pluming. The exposure of lung to cadmium is associated with the development of pulmonary damage such as emphysema and lung cancer (Askarinejad et al., 2009). Many possible mechanisms of cadmium carcinogenesis have been suggested and, among them, the induction of ROS and an alteration of DNA methylation seem to play a predominate biological role. Takiguchi et al., 2003 showed that cadmium reduces genome methylation, inhibiting DNA methyltransferases in a non-competitive manner. This finding is suggestive of an interference in enzyme-DNA interaction possibly through an interaction of cadmium with the methyltransferase DNA binding domain.

1.3 Epigenetic and lung cancer

Epigenetic changes have found in an early stage of diseases or even before the disease can be detected by any other diagnostic studies. Therefore, understanding the epigenetic changes induced by lung cancer risk factors like exposure to cigarette smoke (CS), and particulate matter (PM) is important. These factors triggered inflammation are considered to play a central role in various pathologies by a mechanism that stimulates the release of pro-inflammatory cytokines.

During this process, epigenetic alterations are known to play important roles in the specificity and duration of gene transcription (Zong et al, 2019; Li et al., 2015). Aberrant epigenetic patterns associated with the development and progression of cancer have a potential clinical application. In mammalian cells, DNA methylation is the most widely studied epigenetic modification, it involves the covalent binding of a methyl group to the 5 position of cytosine residues at cytosine preceding guanosine in (CpG) islands, which most typically suppresses transcription by impeding the binding of transcription factors to cis-DNA–binding elements. Most CpG rich genomic regions are sites of transcription initiation and are ≈200 to 300 base pairs in length, with a GC (guanine-cytosine) content of ≈50%, predominantly nonmethylated. DNA methylation is catalysed by 3 different DNMTs (DNA methyltransferases); DNMT1 is the maintenance enzyme, and its inhibition leads to passive demethylation, whereas DNMT3a and DNMT3b are associated with de novo methylation. Methylated CpG islands can be oxidized by the TET (ten-eleven translocation) family of enzymes through the base excision repair pathway, leading to active demethylation and gene reactivation. It is also important to point out that RNA can be methylated/demethylated, thereby regulating gene expression, which represents a less well studied epigenetic regulatory mechanism (Zarzour et al., 2019). Since the aberrant DNA methylation events occurring in cancer cells have been extensively mapped, DNA methylation of tumor suppressor genes can be used as the biomarkers for cancer detection and prediction of therapy responses (Campbell et al., 2014). In contrast with the static alterations in DNA, epigenetic changes can potentially be reversed, making the epigenetic machineries involved in such processes promising therapeutic targets (Jones et al., 2016). Aberrant methylation of CpG-rich areas, also known as CpG islands, located in or near the promoter region of many genes, has been associated with the initiation and progression of NSCLC, transcriptional inactivation of important tumor suppressor, DNA repair, and metastasis inhibitor genes, among others, has been reported (Herbst et al, 2008; Hu et al., 2002). Therefore, the detection of aberrant promoter methylation of cancer-related genes may be essential for the diagnosis, prognosis and/or detection of metastatic potential of tumors, including lung cancer.

Tumor suppressor genes

Tumor suppressor genes are important regulatory genes which encode proteins regulating transitions in and out of the cell cycle and which also have a role in the gateway to terminal differentiation (Tripathy et al., 1992). Defects in tumor suppressor genes in uncontrolled cell division leads to cancer (Tripathy et al., 1992). Tumor suppressor genes encode proteins that

suppress cell growth and most frequently result in exit from the cell cycle. Loss-of-function mutations in tumor suppressor genes result in tumor malignancy and can account for hereditary cancers. Every gene has two alleles present in the genome (with a few exceptions in the hemizygous regions of the sex chromosomes). For tumor suppressor genes to be inactivated either deletion of one allele and somatic mutation of the other allele is required resulting in a loss of heterozygosity (Swellam et al., 2004), or somatic deletion of both of the alleles is required resulting in a complete loss of homozygosity (Quelle et al., 1997). Tumor suppressor genes can also be inactivated by hypermethylation of the gene resulting in promoter suppression so that genes cannot be transcribed further (Ng et al., 2015). DNA methylation of tumor suppressor genes was found to be frequently abnormal in lung carcinomas, which may influence treatment strategies for lung cancer patients. On the other hand, the detection of methylation in the bronchial epithelium of heavy smokers could be used in a population with a greater risk for lung cancer to assess the individual risk (Muller et al., 2002). Ras association domain-containing protein 1 is a protein that in humans is encoded by the Rassf1 gene. This gene encodes a protein similar to the RAS effector proteins. Rassf1a inactivation is implicated in the development of many human cancers. The loss of a Rassf1a allele is a frequent phenomenon in primary human cancer (Burbee et al, 2001; Pfeifer et al., 2005). Similar findings were detected in non-small-cell lung cancer (NSCLC) (Tomizawa et al., 2002) and bladder transitional carcinoma (Chan et al., 2003). The inactivation of this gene was found to correlate with the hypermethylation of its CpG island promoter region. This protein was shown to inhibit the accumulation of cyclin D1, and thus induce cell cycle arrest. An overexpression of RASSF1A promotes apoptosis and cell cycle arrest and reduces the tumorigenicity of cancer cell lines. On the other hand, a downregulation of RASSF1a enhances genetic instability and the loss of cell cycle control (Agathangelou et al., 2005). DAPK is a 160-kDa calcium/calmodulin-dependent serine threonine kinase protein that has been shown to play a role in the apoptosis pathways. DAPK was initially identified as a mediator of cell death in the apoptosis pathway induced by IFN-γ and mapped to chromosome 9q34. It has a death domain at its COOH-terminal end that is critical to its function. The hypermethylation of DAPK has been shown to be an important epigenetic alteration in lung, oesophageal, head and neck, prostate, bladder, and gastric malignancies (Reddy et al., 2003). p16 is a 16 kD cell cycle inhibitor protein, which normally blocks abnormal cell growth and proliferation by binding to complexes of the cyclin-dependent kinases (CDK) 4 and 6, and cyclin D, which cause the inhibition of G1 phase progression.

A loss of function of p16 results in higher cyclin D-dependent protein kinase activity and thus leads to aberrant phosphorylation of the retinoblastoma protein, which accelerates cell growth. Inactivation of p16 by homozygous deletion, point mutation or hypermethylation is one of the most commonly observed aberrations in tumors (Hu et al., 2009). Herman et al., (1995) showed that silencing p16 by promoter hypermethylation has been detected in the cell lines of breast, colon and prostate cancers as well as primary tumors.

Metallothioneins (MTs)

Metallothioneins (MTs) are a class of low molecular weight proteins with metal-binding and antioxidant properties. Human metallothioneins are encoded by a family of genes located at chromosome 16q13 and some of the isoforms seem to be expressed in an organ-dependent manner. Metallothionein expression is regulated by several factors, including zinc, glucocorticoids, cytokines, and reactive oxygen species. In several human cancers, a metallothionein expression was found to correlate with cell proliferation, tumor progression, and drug resistance. MT expression could be a potential prognostic biomarker for breast cancer (Bay et al., 2008). An absence of MT-3 mRNA demonstrates the hypermethylation of the MT-3 intron1 in gastric cancer cell lines, so far as 5-azacytidine results in a new expression of MT-3 mRNA in these cells (Deng et al., 2003). However, a major problem in lung cancer is the lack of clinically efficient non-invasive methods to facilitate an early detection and therapeutic monitoring of lung cancer. Molecular biology techniques might effectively estimate the expression of particular appointed genes not only in tumor cells, but also in other materials like sputum, bronchoalveolar lavage (BAL), and serum/plasma (Wynimko et al., 2007). Molecular changes in tumor suppressor genes have been detected in all stages of lung tumorigenesis (Li et at., 2003). Genetic abnormalities of proto-oncogenes and tumor suppressor genes are well-known changes that are frequently involved in lung cancer pathogenesis.

2 Aim of the work

The understanding of the epigenetic silencing process is of fundamental importance for the etiology of lung cancer. Nevertheless, the mechanism of aberrant promoter methylation of tumor suppressor genes is still widely unknown and has attracted considerable interest in the cancer research community. However, having in mind that cancer of the lung develops over a long period of time; any experimental system should be adapted to repeated and long-term exposure. The following aspects should be analysed:

• In search of biomarkers for the early detection of lung cancer it was then our task to find out whether the frequency of inactivation by aberrant CpG island methylation of several tumor-associated genes such as Rassf1a, DAPK, p16 and the detoxification protein metallothionein IA could be helpful as indicators.

• The second aim of this study is to clarify whether and how epigenetic silencing occurs in vitro upon contact to carcinogenic substances. This will offer deeper insight into the cascade of events leading to tumor formation as a final outcome.

• It is very likely that gene silencing can be induced by cellular stress from exposure to cigarette smoke and other environmental carcinogens, Therefore, the experimental design aims to clarify whether cigarette smoke condensate and lung carcinogenic metals, like Cadmium, trigger aberrant promoter methylation and silencing of cancer related genes in normal lung cell cultures, initially showing no promoter hypermethylation.

3 Materials and Methods

3.1 Materials

Cell culture reagents

AECG-Medium	Promo-Cell, Heidelberg
Fetal Bovine Serum	promo Cell, Heidelberg
Trypsin, PBS, L15-Leibovitz Buffer	Biochrome, Berlin
Penicillin / Streptomycin	Biochrom AG, Berlin
Fibronectine, Collagen R, DMSO	Sigma, Taufkirchen

MTT-Assay

PBS	Biochrome,Berlin
MTT-Reagent	Gibco-Lifesicience
Isopropanol	Roth, Karlsruhe
Formic Acid	Roth, Karlsruhe

RNA Isolation

TRItidy G	Applichem, Darmstadt
1-Brom-3-chlor-propan, Chloroform	VWR, Darmstadt
Isopropanol, Ethanol	Roth, Karlsruhe
DEPC Diethyl pyrocarbonate	Sigma, Taufkirchen

DNA-Isolation

Proteinase K	Fermentas, Thermo Fisher
RNase	Scientific, Schwerte
Phenol, Phenol/Chloroform	GmbH + Co, Karlsruhe
Na-Acetate,Isopropanol,	Merck KGaA, Darmstadt
Ethanol, EDTA,Tris	

Bisulfit Treatment

NaOH	Carl Roth GmbH + Co, Karlsruhe
Sodium Bisulfit	Sigma-Aldrich GmbH,Steinheim
Hydrochinon	Merck KGaA, Darmstadt
Ammoniumacetat	Carl Roth GmbH + Co, Karlsruhe
Glycogen	Roche GmbH, Mannheim
DNA Clean-up system	Promega,USA

Invito-methylation	
SAM, SSS1 Enzyme	New England BioLabs, Schwalbach
RT-PCR	
DNase, DNase-Buffer, DNase Reagent	Promega, USA
Random-Primer,MMLVReverse, Transcriptase	Fermants Canada
5xBuffer dNTP, RNasin,Master-Mix	Fermants Canada
MgCl2 (25mM)	Promega,Mannheim
Sybr Green	Biozym, Oldendorf
Primers	Invitrogen,USA
Lab-Materials	
24Well,96er-Well,Cellculture-pates,Cellcuture-dishes	Nunc, Wiesbaden
Centrifugetubes,Chamber,Slides, Coverslips	Nunc, Wiesbaden
Capillary Pipete (5ml, 10ml, 25ml)	Schütt-Labortechnik, Göttingen
Pipettips (1000µl, 200µl, 10µl)	Eppendorf, Hamburg
Tubes RNase, DNase free	Brand, Wertheim
Cuvettes	Hellma, Mühlheim
Devices	
Autoclave,Incubation	Schütt- Labortechnik, Göttingen
Centrifuge	Heraeus-Instruments; Hanau
Photometer: DU 7500	Beckman, USA
Microscope: Nikon Eclipse TS100	Nikon, USA
Mastercycler gradient	Eppendorf, Hamburg
Rotorgene 2000	Corpett Research, UK
Evaluation Softwares	
SPSS.15	SPSS Incorporation, Chicago, USA.
Tina 2.09	Raytest, Straubenhardt

Reagents

AECG (Airway Epithelial Cell Growth Medium promo cell)

The medium-formulation contains no growth factors or antibiotics. After adding the Supplement mix the concentrations of growth factors in the complete medium are as follows:

BPE 0.4 %

Epidermal Growth Factor 10 ng/ml

Insulin 5 µg/ml

Hydrocortisone 0.5 µg/ml

Epinephrine 0.5 µg/ml

Triiodothyronine 6.7 ng/ml

Transferrin 10 µg/ml

Retinoic Acid 0.1 ng/ml

The medium was stored between 4 °C and 8 °C in the dark, and the Supplement-Mix at 20°C. We added Penicillin/ Streptomycin combination (1% of 10.000 I.E./ml) and before use all media were warmed up to 37°C.

Chemicals for the treatment of cultured cells

Cigarette Smoke Condensate

Was a generous gift of Dr. Schramke and Dr.Haußmann from Philip Morris Research Laboratories

Cadmium II

Sterilization of Materials and solutions

Articles for the cell preparation, as well as cell culture, were sterile from factory. Heatproof glass and plastic materials as well as solutions were sterilized by 25 minutes by autoclave with 120°C. Non heatproof solutions were sterile-filtered. The assigned sterile filters (0.45µm or 0.2µm mesh size) depended on sterility degrees and consistency of the solutions.

Proteinase K Buffer

Tris/HCl pH 7,6	50 mM
EDTA x Na2	25 mM
NP40	0,5%
SDS	0,5%
added H2O	

TBE Buffer (10x)

Tris/HCl	1 M
Boric Acid	0,8 M
EDTA x Na2	10 mM
added H2O	

TBS Buffer (10x)

Tris/HCl	50 mM
NaCl	1,4 M
HCl	PH 7,6

TE Buffer (1x)

Tris/HCl pH 7,6	10 mM
EDTA x Na2	1 mM
added H2O	

Tab.1. Characteristics of the study group.

IDs	Gender	Age	Smok -ing	Clinical Diagnosis	Work	Primary tumor classification	Chemoth erapy	Chronic diseases
114*	M	63	Yes a	NSCLC IIa	Locksmith	T2, N0, M0	Yes	CHD, PTCA
178*	M	58	Yes	NSCLC IIIb	Driver	T2, N1, M0	Yes	COPD gold II, MT
180*	M	64	Yes a	NSCLC Ib	Nurse	T2, N0, M0, R0	No	No
182*	F	85	Yes a	NSCLC IIa	Saleswoman	T2, N0, M0, R0	Yes	No
192*	M	62	Yes a	NSCLC IIIa	Construction worker	T3, N2, M0, R0	No	DM, CHD, Pancreatitis
200*	M	67	No	NSCLCIIa	Unemployed	T1, N1, M0	No	DM, CHD
202*	M	54	Yes	NSCLC	Unemployed	T1, N1, Mx	Yes	No
207**	F	48	No	NSCLC IIb	Teacher	T2, N1, M0	Yes	No
208*	F	78	Yes a	NSCLS Ib	Saleswoman	T2, N0, M0	No	CHD, COPD gold II ,MT
213*	M	77	Yes	NSCLC IIa	Locksmith	T2, N0, M0	Yes	CHD
214*	M	50	No	NSCLC IIa	Mechanic	T1, N1, M0, R0	Yes	Aortic insufficiency
215**	M	64	Yes b	NSCLC IIb	Stonemason	T2, N1, M0	Yes	DM
216**	M	68	Yes b	NSCLC IIa	Truckdriver	T2, N0, M0	No	PAD II, DM
219**	M	68	Yes b	NSCLC IV	Unemployed	T2, N2, M1	No	No
220**	M	52	Yes	NSCLC IIa	Seller	T2, N0, M0	No	Alcohol Abuse
221**	M	52	Yes a	NSCLC IIb	Unemployed	T2, N1, M0	No	Chronic pain syndrome
222**	M	74	Yes a	NSCLC IIa	Welder	T2, N1, M0	No	COPD gold III
223**	M	50	No	SCLC Ib	Unemployed	T2 N0, M0	No	Alcohol Abuse
224**	F	73	Yes b	NSCLC IIIa	Unemployed	T3, N1, M0	No	DM
225**	M	77	Yes b	NSCLC Ib	Locksmith	T2, N0, M0	No	CHD, DM
226**	M	80	Yes	NSCLC IIb	Seller	T3, N0, M0	No	COPD gold III, CHD, Prostate cancer
228*	M	68	Yes	NSCLC IIIa	Unemployed	T2, N2, M0	No	CHD

229*	M	75	No	NSCLC, IIIa	Industry businessman	T3, N1,M0	No	diabetes, CHD,COPD I
230*	M	75	Yes a	NSCLC IIIa	Welder	T2, N2, M0	Yes	COPD gold III, DM, renal failure
232*	M	68	Yes a	NSCLC IIIa	Locksmith	T2, N2, M0	Yes	COPD gold III
234*	M	68	Yes a	NSCLC, IIIa	locksmith	T2,N2,M0	Yes	No
235*	M	73	Yes b	NSCLC IIb	Driver	T2, N1, M0	No	No
236*	M	74	Yes a	NSCLC IIb	Train driver	T3, N0, M0	No	Alcohol Abuse
238*	M	58	Yes b	NSCLC Ia	Plumper	T1, N0, M0	No	Neuroendocrine carcinoma
243*	M	62	Yes b	NSCLC Ia	Unemployed	T1, M0,N0	No	CHD
245*	M	54	Yes b	NSCLC IIIa	Stonemason	T2, N2, M0	No	COPD gold II
246*	M	71	No	NSCLC	Pensioner	T2, Nx, Mx	Yes	DM, CHD
268**	F	74	No	NSCLC Ia	Nurse	T0,N0,M0	No	No
276**	M	63	Yes a	NSCLC IIb	Umemployed	T2,N1,M0	No	No
278**	M	60	No	NSCLC IIIa	Unemployed	T2,N2,M0	Yes	COPD gold III, renal failure

* Patients from Martin Luther University Hospital / Halle (Saale),Kröllwitz, ** patients from Martha Maria hospital / Halle (Saale).. (a) 10-30 pack years of cigarettes, (b) > 40 pack years of cigarettes. All patients had primary lung cancer and underwent lob- or pneumectomy due to a clinical indication for an operation (better prognosis or curative). The smoking status was assessed by a self-report of current smokers, but most patients had ceased smoking 3–4 weeks before the operation. Non-smokers are those who had stopped smoking more than ten years before the operation. (CHD) chronic hypertensive disease, (COPD) chronic obstructive pulmonary disease, (DM) diabetes mellitus, (PTCA) percutaneous transluminal coronary Angioplasty, (PAD) peripheral arterial disease, (NSCLC) Non-small cell lung carcinoma, (MT) Metabolism Syndrome.

Tab. 2. PCR primer sequences of MSP and the Primer sequences of RT-PCR.

Gen	Primer's sequences of methyl-specific-PCR	FA %	Bp	Tan in C°
Rassf1a	**M**:GTGTTAACGCGTTGCGTATCAACCCCGCGAACTAAAAACGA	2	94	60°
	U:TTTGGTTGGAGTGTGTTAATGTGCAAACCCCACAAACTAAAAACAA	2	108	60°
P16	**M:**TTATTAGAGGGTGGGGCGGATCGCGACCCCGAACCGCGACCGTAA	1	150	60°
	U:TTATTAGAGGGTGGGGTGGATTGTCCACCTAAATCAACCTCCAACCA	1	234	65°
DAPK	**M**:GGATAGTCGGATCGAGTTAACGTCCCCTCCCAAACGCCGA	1	98	64°
	U:GGAGGATAGTTGGATTGAGTTAATGTTCAAATCCCTCCCAAACACCAA	1	106	64°
MT1A	**M:**TTAAGGTTGGGTTTTCGGAACGCAAATTTACGCCCGAAATACGAAACG	0.5	110	65°
	U:TGTTAAGGTTGGGTTTTTGGAATGTGTAATTTACACCCAAAATACAAAACATCCCC	0.5	111	65°
Gen	**Primer's sequences of Rt-PCR**		**Bp**	**Ann - Tem**
GAPDH	5- GAA GAT GGT GAT GGG ATT TC 5- GAA GGT GAA GGT CGG AGT		226	60°
Rassf1a	5-GGCTGGGAACCCGCGGTG 5-TCCTGCAAGGAGGGTGGCTTCT		239	60°
DAPK	5-CGGCTGGAGGCGAGTTTGGA 5-CCCAGAAGCCCCAGCATCCA		122	60°
P16	5-GCTGCCCAACGCACCGAATAGT 5-CTCCCGGGCAGCGTCGTG		157	60°
MT1A	5-TCCACGTGCGCCTTATAGCC 5-GGGGCAGCAGGAGCAGCA		178	60°

This table shows:

I)PCR primer sequences and PCR product sizes of MSP M: methylated-specific primers U: unmethylated specific primers. FA: formamide (%) in 25µl. Rassf1a, P16 M-PCR primers taken by (Schagdarsurengin et al., 2003). DAPK primers of M-PCR taken by (Ahmed et al., 2011) MT1A M-PCR primers designed by Prof.Dr Dammann/ Institut für Genetik Justus-Liebig-Universität Gießen, Germany.
II)The Primer sequences, product length, annealing temperature of RT-PCR.

3.2 Normal Lung Tissue specimens and corresponding tumors

Primary tumor samples and their corresponding nonmalignant lung tissues were obtained from (NSCLC) non-small cell lung carcinoma patients at all stages. Patients were diagnosed at the University hospital, Halle (Saale) from 2000 to 2010 and the diagnosis was confirmed by histo-pathologists. Tissue samples had underwent lob- or pneumectomy due to a clinical indication for an operation (better prognosis or curative). The smoking status was assessed by a self-report of current smokers, but most patients had ceased smoking 3–4 weeks before the operation. Nonsmokers are those who had stopped smoking more than ten years before the operation. Most of cases had a chronic disease such as chronic hypertensive disease (CHD), (COPD) chronic obstructive pulmonary disease, (DM) diabetes mellitus, (PTCA) percutaneous transluminal coronary Angioplasty. The samples were immediately frozen and stored at -80C° until being analysed. The selection of these patients was based on the availability of archived fresh tumor and corresponding normal lung tissues and normal bronchial tissues for the investigators. Clinical information and follow-up see (Tab.1). The patients ranged in age from 48 to 85 years.

3.3 Explant culture from human lung

Bronchial and peripheral lung tissues were obtained from lung resections of cancer patients. Normal tissue as used in our cell cultures was obtained in the proximity of the tumor. Before transport, the tissue material was transferred as soon as possible in cold, sterile Leibovitz L15-buffer. The donors were all well-documented patients from different hospitals that underwent surgery at the University Hospital of Halle because of a clinical indication. Ages, the use of medication or smoking were not exclusion criteria. All patients have given written consent that tissue may be used for cell culture purposes. The medical treatment of patients and diagnosis was not influenced by the study at any time. This study was approved by the ethics committee of the University of Halle-Wittenberg (Tab 1).

3.4 Isolation and cultivation of normal human bronchial epithelial cells and parenchymal lung cells

The method of cultivation which was established in our laboratory by Mr. Thomas Stock and modified by Mrs. Dr. Dorothee M. Runge. The modification of the cultivation of normal human bronchial cells (NHBECs) and peripheral lung cells (PLCs) was established according to the method of (Lechner et al., 1985). Bronchial tissue was obtained from lung resections of cancer patients. Morphologically normal tissue used in our cell cultures was obtained in

proximity of the tumor. The diagnosis was done by histopathological examination of the institute of pathology. Before transport the tissue material was transferred as soon as possible in cold, sterile Leibovitz L15-buffer. Bronchi were dissected and cut into pieces of approx. 0.2-0.5 cm^2. These pieces were transferred onto culture dishes that were pre-coated with 1 % fibronectin/ 2 % collagen/ 0,1% Bovine Serum Albumin in L-15 medium. After five minutes serum-free medium was added. The medium contained 0,4% Bovine hypophyseal extract; 0.5 ng/ml epidermal growth factor (EGF).; 5 µg/ml insulin; 0.5 µg/ml hydrocortisone; 0.5 µg/ml epinephrine; 6.7 ng/ml triiodothyronine; 10 µg/ml transferrin; 0.1 ng/ml retinoic acid. The tissue cultures were cultivated for 2-3 weeks, cells grew out of the bronchial pieces and adhered to the coated dish surface, until they were 80-90 % confluent (= first generation, 10-14 days). The pieces were then transferred onto new-coated dishes for new and further generations and culturing continued until 80-90 % confluence was reached again (= second generation). Medium was changed every 72 hours. Primary culture of normal peripheral lung cells (PLC) Peripheral lung tissue was obtained distal to the bronchial tree, which was microscopically free of bronchial epithelium. Immediately after resection the tissue was maintained in cold L15 Leibovitz buffer. The material was cut into pieces of approximately 1 mm thickness by hand. The pieces were placed onto uncoated 57 mm culture dish. The cultures were maintained in serum-free AECG medium containing 0.4% bovine hypophyseal extract; 0.5 ng/ml epidermal growth factor (EGF).; 5 µg/ml insulin; 0.5 µg/ml hydrocortisone; 0.5 µg/ml epinephrine; 6.7 ng/ml triiodothyronine; 10 µg/ml transferrin; 0.1 ng/ml retinoic acid. The first sub-confluent monolayer was obtained after 4- 5 weeks. Then the pieces could be transferred to new culture dish for new and many generations and the monolayer could be splitted and seeded into new culture dish (10x 103 cells/ cm2) for new and several passages.

3.5 Seeding of cells in passages

After obtaining the first subconfluently monolayer, cells could be splitted into several passages as fellow; Cells in plates were washed 2 times with HBSS and incubated with Trypsin for approx. 5 min by 37°C. The cells were resuspended in TNS (4ml/Plate) and centrifuged with 900/rpm for 5 min; finally, the cell pellet was loosened in suspended AECG medium. And the cell number was determined by means of counting slide. Cell pellet (40,000 cells/ml) solved in AECG medium and the cell suspension was used on coated plates for (NHBEC) and on non-coated plate for PLC. Cells need approx. 1 week to reach (80 % -90 %) confluence.

3.6 Staining of cultivated cells with Haematoxylin and Eosin

For this purpose, the cells were grown as monolayers on cover glasses placed in cell culture dishes. The medium was removed and the cover glasses were washed with distilled water and fixed by 10% formalin for 10 min and washed with distilled water to remove excessive formalin. Subsequently, cover glasses incubated with haematoxylin for 1 min to stain the nuclei with blue colour and washed with Tape water and subsequently incubated with eosin 5 min to stain the cytoplasm with pink colour. Subsequently, followed by successive 5 min washing in 70%, 90% and absolute ethanol to remove the remnant of water. Cover glasses were cleaned with xylol for 5 min and were mounted on micro slides with mounting medium.

3.7 Treatment with Test Substances

The primary lung, bronchial cells (NHBEC) and Human lung cells (PLC) were exposured for a long-term to (5µM) Cadmium Cd (II) and (10mg/l) cigarette smoke condensate (CSC) [for 14 weeks in NHBEC, and 11 weeks in PLC]. 12 culture dishes were prepared for each experiment, including two controls and two dishes for the two different concentrations.

3.8 Vitality test (MTT-Test)

Cell viability is a determination of the fraction of vital metabolically active cells. There are numerous tests and methods for measuring cell viability e.g. measurement of lactate dehydrogenase (LDH) and ATP which are used as indicators for membrane integrity. One parameter used as an indicator for the metabolic activity of viable cells is the colorimetric assays. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, first described by (Mosmann et al., 1983), which based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product. The colour can then be quantified using a simple colorimetric assay. All used substances were tested for toxic effects in the (MTT) assay on lung cells in order to check subtoxic concentration ranges. NHBECs, and PLC were cultivated on 1.9 cm2 growth area plastic gamma sterilized tissue culture test plates 24 (TPP-Switzerland-Europa). When adherent, cells were incubated with the substance to be tested at 37°C. Afterwards the old medium containing the test-substance was removed and the cells were washed with PBS.

Subsequently, the cells were incubated with the MTT-reagent (3-(4-5dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide) 0.7 mg/ml in PBS for 30 min (37°C) and then washed with PBS. Subsequently a mixture of formic acid (98%) and isopropanol (5: 95 v/v) was added to the MTT-reagent treated cells for 2 min. Formazan was determined by photometric measurement at 570nm. All assigned substances (Cd, CSC) were used in the following concentration 5µM, 10 mg/ml respectively which were proved by MTT test to be non-toxic, and more than 80 % of cells were alive after incubation with these substances up to 24h.

3.9 Genomic DNA Extraction

Genomic DNA was isolated by the standard phenol/chloroform extraction, cell pellets were harvested by 0.05% Trypsin EDTA and washed with PBS once, and centrifuged at 5000 rpm for 5 min. and stored at -80˚C until use. Proteinase K were added to cell pellets. Cell pellets were then incubated at 50°C for 3 hr on the thermo-shaker. 500µl phenol was added to the tubes and the upper layer was collected by centrifugation (9000 rpm, 4 °C). 500µl of Mixture phenol Chloroform was added to the tubes and then they were centrifuged (9000 rpm, 4 °C). the supernatants were then collected, as well as 500 µl 0f Chloroform and the upper layer was collected by centrifugation and DNA then precipitated with 3 M Sodium Acetate and 100% alcohol. Genomic DNA pellets were centrifuged at 13,000 rpm for 15 min. DNA pellets were washed once with 70% ethanol and leaved to be air-dried. Dried pellets were resuspended DNA resuspended in 100µl TE buffer. The DNA was quantified by spectrophotometric. The absorbance was checked at 260 and 280 nm. All DNA stock solutions were stored at -20°C.

3.10 Bisulphite treated

Bisulphite-conversion-based methods are the most widely used in recent years because they permit the rapid identification of methylated cytosine. In the bisulphate reaction, all unmethylated cytosines are deaminated and sulfonated, converting them to uracil's, while 5-methylcytosines remain unaltered. The method of bisulphite modification was performed according to previous methods (Dammann et al., 2000). DNA (2 µg) in a volume of 18µl TE buffer was denatured by 2µl NaOH (final concentration of 0.3 mol/l) at 37˚C for 15 min and then treated with 3,6 M sodium bisulphate 208 µl (PH 5.0) and 0,1M hydroquinone 12 µl at 55˚C for 16 h. All solutions were prepared freshly, DNA was purified using the Wizard DNA clean-up system following the manufacturer's recommendations. The modified DNA was re-suspended in 50µl TE (PH 8. and stored at -20˚C.

3.11 Methyl specific- PCR

MS-PSR of Rassf1a

1.Only for the methylated DNA appear a PCR product

25µl-Mixture:
4 µl Buffer (10x NH4)
2 µl dNTPs (2,5 mM)
0,5 µl Formamide (100%)
1 µl RAM 1 Primer (10 pmol/ µl)
1 µl RAM 2 Primer (10 pmol/ µl)
1,5 µl MgCl2 (25 mmol)
11,6 µl H2O
0,4 µl Taq-Polymerase (5 units/ µl)
3 µl Bisulphite-treated DNA

2.Only for the unmethylated DNA appear a PCR product

25µl- Mixture:
2,5 µl Buffer (10x NH4)
2 µl dNTPs (2,5 mM)
0,5 µl Formamide (100%)
1 µl RAU 1 Primer (10 pmol/ µl)
1 µl RAU 2 Primer (10 pmol/ µl)
1,5 µl MgCl2 (25 mmol)
13,1 µl H2O
0,4 µl Taq-Polymerase (5 units/ µl)
3 µl Bisulphite-treated DNA

PCR-Program:

1.) 95 C° 2 min
2.) 95 C° 30 sec
3.) 60 C° 30 sec
4.) 72 C° 30 sec
5.) back to step 2.) and repeat 34 cycles
6.) 60 C° 2 min
7.) 72 C° 4 min

Product size:
RAM1/RAM2: 93bp
RAU1/RAU2: 105 bp

MS-PSR of DAPK

1.Only for the methylated DNA appear a PCR product

25µl- Mixture:
2,5 µl Buffer 10x NH4)
2 µl dNTPs (2,5 mM)
1 µl DAPK m1 Primer (10 pmol/ µl)

2.Only for the unmethylated DNA appear a PCR product

25µl- Mixture:
2,5 µl Buffer (10x NH4)
2 µl dNTPs (2,5 mM)
1 µl DAPK u1 Primer (10 pmol/ µl)

1 µl DAPK m2 Primer (10 pmol/ µl)
1,5 µl MgCl2 (25 mmol)
3,5 µl H2O
3 µl Bisulphite-treated DNA

1 µl DAPK u2 Primer (10 pmol/ µl)
1,5 µl MgCl2 (25 mmol)
3,5 µl H2O
3 µl Bisulphite-treated DNA

Taq-Polymerase/H2O-Mix:
Firstly, heat the mixture at 68C°
0,5 µl Taq-Polymerase
10 µl H2O

PCR-Program:
1.) 95 C° 5 min
2.) 95 C° -> Add Taq polymerase
3.) 95 C° 45 sec
4.) 64 C° 45 sec
5.) 72 C° 45 sec
6.) back to step 3.) and repeat 40 cycles
7.) 64 C° 2 min
8.) 72 C° 4 min

Product size:
DAPK m1/m2: 98 bp
DAPK u1/u2: 106 bp

MS-PCR of p16

1.Only for the methylated DNA appear a PCR product

25µl- Mixture:
2,5 µl Buffer (10x NH4)
2 µl dNTPs (2,5 mM)
1 µl p16 m1 Primer (10 pmol/ µl)
1 µl p16 m2 Primer (10 pmol/ µl)
1,5 µl MgCl2 (25 mmol)
1 µl Formamide (100 %)
3 µl Bisulphite-treated DNA

Taq-Polymerase/H2O-mixture:

2. Only for the unmethylated DNA appear a PCR product

25µl- Mixture:
2,5 µl Buffer (10x NH4)
2 µl dNTPs (2,5 mM)
1 µl p16 u1 Primer (10 pmol/ µl)
1 µl p16 u2 Primer (10 pmol/ µl)
1,5 µl MgCl2 (25 mmol)
1 µl Formamide (100%)
3 µl Bisulphite-treated DNA

Firstly, heat the mixture alt 68C°
0,4 µl Taq-Polymerase
12,6 µl H2O

PCR-Program of p16
Methylated DNA:
1.) 68C° ->Add Taq-Polymerase
2.) 92C° 30 sec
3.) 65C° 30 sec
4.) 72C° 30 sec
5.) back to step 2.) and repeat 38 cycles
6.) 65C° 2 min
7.) 72C° 4 min
Product size:
P16 m1/m2: 150 bp

PCR-Program of p16
Unmethylated DNA:
1.) 68C° ->Add Taq-Polymerase
2.) 92C° 30 sec
3.) 60C° 30 sec
4.) 72C° 30 sec
5.) back to step 2.) and repeat 38 cycles
6.) 60C° 2 min
7.) 72C° 4 min
Product size:
P16 u1/u2: 234 bp

MS-PCR of MT1A

1. **Only for the methylated DNA appear a PCR product**

25µl- Mixture:
2,5 µl Buffer (10x NH4)
2 µl dNTPs (2,5 mM)
1 µl MT1A m1 Primer (10 pmol/ µl)
1 µl MT1A m2 Primer (10 pmol/ µl)
1,5 µl MgCl2 (25 mmol)
1 µl Formamide (100%)
3 µl Bisulphite treated DNA
Taq-Polymerase/H2O-Mixture:
0,4 µl Taq-Polymerase
12,6 µl H2O
PCR Program:
1.) 68C° ->Add Polymerase
2.) 92c° 30 sec
3.) 65C° 30 sec
4.) 72C°30 sec

2. **Only for the unmethylated DNA appear a PCR product**

25µl- Mixture:
2,5 µl Buffer (10x NH4)
2 µl dNTPs (2,5 mM)
1 µl MT1A u1 Primer (10 pmol/ µl)
1 µl MT1A u2 Primer (10 pmol/ µl)
1,5 µl MgCl2 (25 mmol)
1 µl Formamide (100%)
3 µl Bisulphite treated DNA
Taq-Polymerase/H2O-Mixture:
0,4 µl Taq-Polymerase
12,6 µl H2O

5.) back to step 2.) and repeat 38 cycles

6.) 65C° 2 min

7.) 72C° 4 min

Product size:

MT1A m1/m2: 110 bp

MT1A u1/u2: 111 bp

3.12 RNA Extraction

RNA was isolated by Guanidiniumthioisocyanat / phenol acid, cell pellets were harvested by TRItidy G reagent Method has the advantage, that both RNA, this proteins can isolated from the same sample. The process was described and developed by (Chomczynski et al., 1995) with some modifications. Cells were harvested from culture dishes with 6 cm diameter by 1 ml TRItidy G. Cell suspension could be stored at -80 ° C until use. 100µl of 1-chloro-3-bromo-propane was added. After a few seconds centrifugation at 11,000 rpm for 15 min at 4 ° C, the aqueous phase, which contains the RNA was collected into new tubes, RNA was precipitated by 1:1 (V/V) 200µl isopropanol, washing with ethanol (75%and 96%) and leaved to be air-dried. Dried RNA pellets were resuspended 20µl DEPC water. Thereafter RNA was measured in a quartz cuvette at 260 nm using a phosphate buffer. The quantity of total RNA was determined by the spectrophotometer. The ratio was between 1.7 to 2.0 to be used for further reactions. RNA stock solutions were stored at -80 °C.

3.13 cDNA synthesis

2 µg RNA were used. The RNA was treated with RQ1 Rnase in a Volume of 10 µl (40mM Tris-HCl PH 8.0 1 mM MgSO4, 1 mM CaCl2) for 30 min at 37 ° C. The reaction was stopped with 1µl stop Solution (20 mM EGTA pH 8.0) and heated at 65 ° C for 10 min. 500 ng random hexamers were added for the c-DNA synthesis and the samples were denatured at 70 ° C for 5min. Then 5x buffer (final concentration: 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM DTT), MMLV reverse transcriptase (200U) and dNTP Mix (final concentration 500 microns of each nucleotide) were added to the samples. The synthesis were incubated at 37 ° C for 1 h and terminated by heating at min 94 ° C for 5 minutes.

3.14 Real-time PCR

10 µl of each cDNA sample were taken to set up the standard pool, then the residual set of cDNAs were diluted 1:4 using DEPC-water. To prepare the standard calibration row, the standard pool was diluted according to the following scheme

Undiluted Standard	≅ 106 Copies
1.diluting (1:2)	≅ 5x105 Copies
2.diluting (1:10)	≅ 105 Copies
3 diluting (1:10 diluted from 1.)	≅ 5x104 Copies
4.diluting (1:10 diluted from 2.)	≅ 104 Copies

For the PCR reaction, 2,5µl of diluted, undiluted standard and samples (15µl cDNA+60µl DEPC water 1:4) were added to the following substances 12,5 µl PCR-Master mix , 6,5 µl DEPC water, 1,5 µl of primer mixture (concentration 0,6 µM; Primer see Tab.2.B), For the detection we used the sequence-nonspecific Dye Sybr Gr1 µl (1:50.000, diluted from stock solution) with excitation wavelength: 470nm, emission wavelength: 585 nm and 1 µl MgCl2 3 mM. The final volume was 25 ml. In addition, a blank sample without c-DNA should be conducted. The measurement was performed in duplicate. The PCR started with an initial step at 95 ° C for 2 min to denature the DNA. This was followed by 35-40 cycles with the following.

Steps:

1) Denaturation :95° C 30 s

2) Primer's annealing: 30 s (Temperature: see Table2)

3) Extension: 72°C 30 s

4) Fluorescence measurement: 15 s

Finally, specific PCR product was generated at a temperature of 72 ° C for 5 min followed by measured of the melting curve at (up to 95 ° C). The selting curve is used to assess the clean product. The temperature of the melting of double-stranded DNA are sequence and size-dependent. The threshold and the threshold cycles were evaluated, the samples concentrations are in relation to the standard calibration curve. The evaluation was semi-quantitatively by

compression with the (GAPDH). The expression changes were determined in comparison to an untreated control.

Gel electrophoresis

To visualise genomic DNA and PCR products, gel electrophoresis was performed in 2 % agarose gels prepared in 1X TBE. Gel electrophoresis was performed at 60 ~ 150 V using a Phero-STAP 550. PCR products and DNA were visualised by UV light. For the quantification of each band, a rectangular region of interest was drawn around each PCR band. The signal density was measured and normalised with local background measurement around a rectangular region of interest using Tina 2.09 software. Then, normalised values were exported into Microsoft Excel for analysis. Statistical analysis was performed using SPSS 15.0 software. Methylation frequencies of candidate genes were analysed by Fisher's exact test. Results with $p < 0.05$ were considered statistically significant (one-tailed test).

4 Results

4.1 The Role of DNA hypermethylation of Rassf1a, DAPK, p16, and Metallothionein 1A in normal and tumor tissues of lung cancer patients

We examined the role of methylation by studying Rassf1a, DAPK, p16 and MT1A promoters in 26 lung carcinoma patients. Fig. 1 shows representative pictures of agarose gel electrophoresis of the methyl specific PCR (MSP) products for the 4 gene loci. Two patients were presented as an example of the 4 candidate genes. Bisulfite-modified DNA samples from bronchus, lung, and tumor were amplified with a methylation-specific primer set and with a primer set specific for non-methylated CpG-Islands (Tab.2) and run on a gel, respectively as shown in (Fig.1).

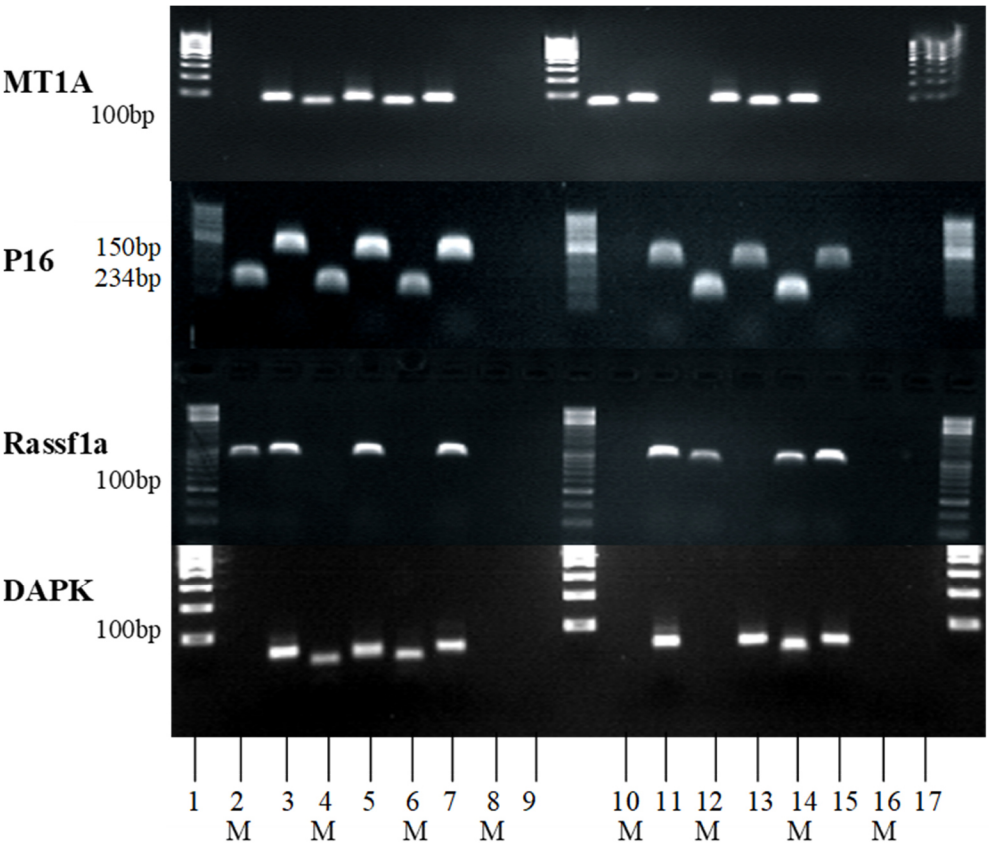

Fig. 1. Methylation analysis of MTIA, p16, Rassf1a and DAPK gene promoters by MS-PCR in lung cancer patients. Representative samples of tumor and corresponding adjacent normal lung and bronchial tissue. Lanes 2, 3, 10, 11 = bronchial tissues; lanes 4, 5, 12, 13 = lung tissues; lanes 5, 6, 14, 15 = tumor tissues. Lane1= size marker. Lanes 2, 4, 6, 10, 14 = bisulfite-modified DNA amplified with methylation-specific primer set; lanes 3, 5, 7, 11, 13, 15 = bisulfite-modified DNA amplified with a non-methylated-specific primer set. Lanes 8, 16 = H2O negative control with methylation-specific primer set without bisulfite DNA. Lanes 9, 17 = H2O negative control with unmethylation-specific primer set without bisulfite DNA. MT1A lanes 2,3 bronchial tissue (M-U+) fully methylated. MT1A lanes 10, 11 bronchial tissue (M+U+) partially methylated. Rassf1a lanes12, 13 lung tissue (M+U-) fully methylated

Tab.3 (appendices) summarizes the promoter methylation of the genes Rassf1a, p16, DAPK and MT1A. The MSP results show to be fully methylated which means that all the tested CpG positions are methylated (signal only in methylated lane). Similarly, (fully) unmethylated means that all the CpG positions in the amplification are unmethylated (signal only in unmethylated lane). MSP sometimes results in heterogeneous methylation (signals in methylated- and unmethylated lane) as shown in Fig.1. A total of 24 out of 26 samples (92%) displayed CpG island hypermethylation in at least one gene in one tissue type. 8 % (2/26) have no methylation status. There was no association between hypermethylation and the patient's age or sex.

Rassf1a promoter methylation was detectable in 62% (16/26) of the patients. A high methylation ratio of 69% (11/16) was recorded for Rassf1a in tumor tissue. One patient (1/16) (6 %) was solitarily methylated in lung tissue. Methylation of Rassf1a in both types of healthy tissue (LuBr) examined was found in one patient (1 out of 16) (6 %). 3 of 16 (19 %) of were methylated in lung and tumor tissue (LuTu) as shown in (Fig.2).

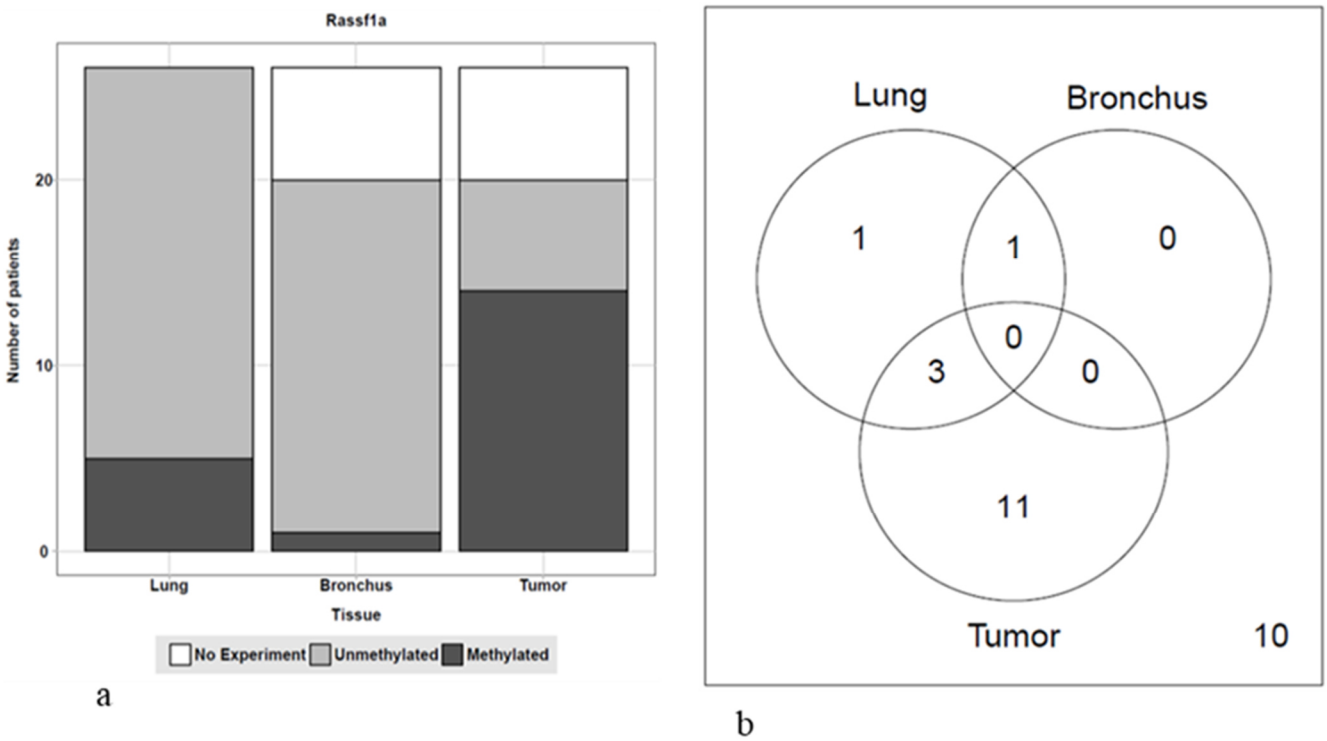

Fig.2. Methylation analysis of Rassf1a gene in 26 lung cancer patients. (a) shows the methylation frequencies of Rassf1a in Lu, Br, and Tu tissues. (b) shows the methylation of Rassf1a gene in more than a tissue type. Methylation frequencies of candidate genes were analysed by Fisher's exact test. Results with $p < 0.05$ were considered statistically significant (one-tailed test).

Methylation frequency of DAPK across tissue types was 12 out of 26 cases (46%). Solitary methylation of DAPK in bronchial epithelial tissue was detected in 2 out of 12 (17%) patients as shown in (Fig.3). Solitary methylation of DAPK in lung was detected in one patient (8%). 25% (3/12) were methylated in all three tissue types, lung tissue or bronchial tissue or tumor tissue (LuBrTu). 42% (5 out of 12) were methylated in lung and tumor tissue (Lu.Tu). One out of 12 patients (8%) was methylated in bronchial epithelial tissue and tumor. This study showed methylation of p16 in 54% or 14 out of 26 cases. (Fig 3).

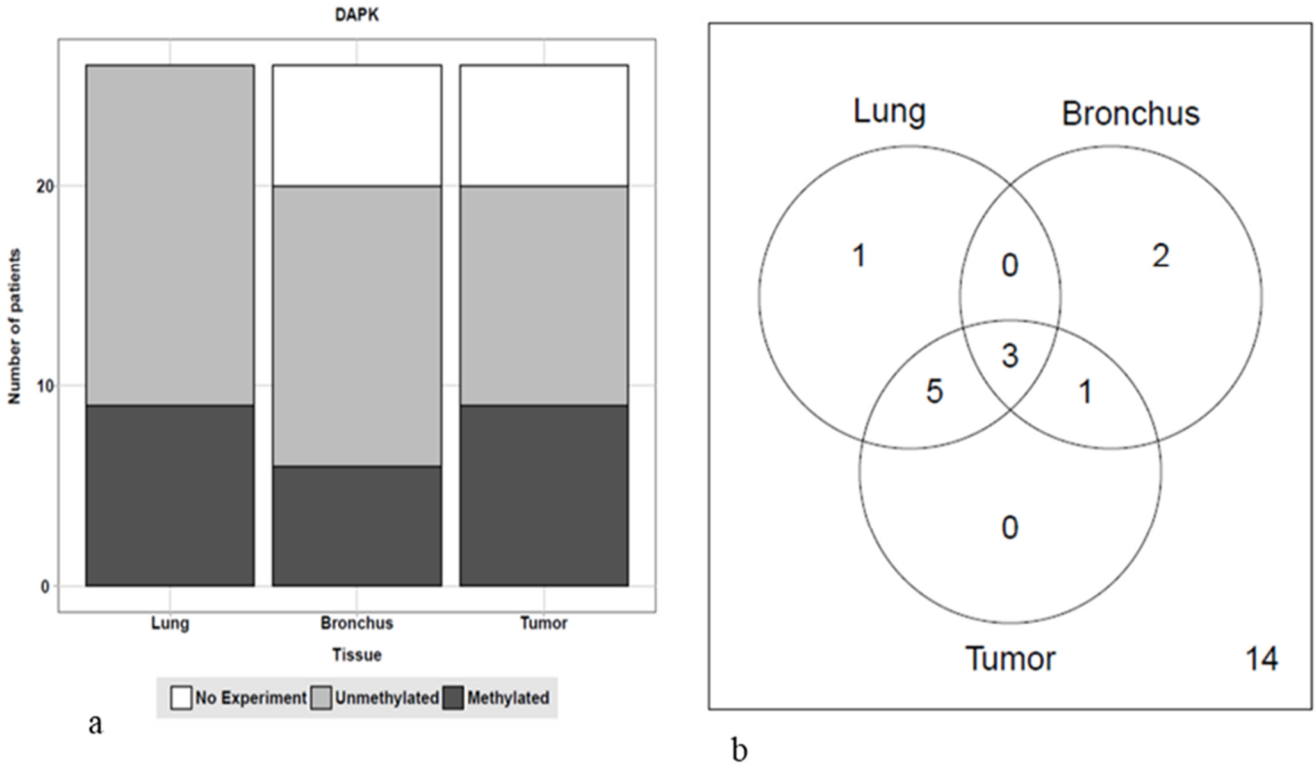

Fig.3. Methylation analysis of DAPK gene in 26 lung cancer patients. (a) shows the methylation frequencies of DAPK in Lu, Br, and Tu tissues. (b) shows the methylation of DAPK gene in more than a tissue type. Methylation frequencies of candidate genes were analysed by Fisher's exact test. Results with $p < 0.05$ were considered statistically significant (one-tailed test).

Promoter methylation of p16 in normal bronchial tissue was detected in 3 out of 14 cases (21%). Solitary methylation of p16 was found in only 1 out of 14 (7%) in normal lung tissue. 2 out of 14 cases (14%) were methylated in both bronchus and tumor tissues (BrTu), and 2 others of 14 (14%) were methylated in both bronchus and lung tissues (BrLu). Moreover 1 of 14 (7%) was methylated in all three sample types (BrLuTu). In 4 others out of the 14 (29%) p16 was methylated in normal lung and tumor tissues (LuTu). Two others of the 14 (14%) were methylated in lung and in bronchial tissues (BrLu). In one case (7%) only tumor tissue was methylated (Fig.4).

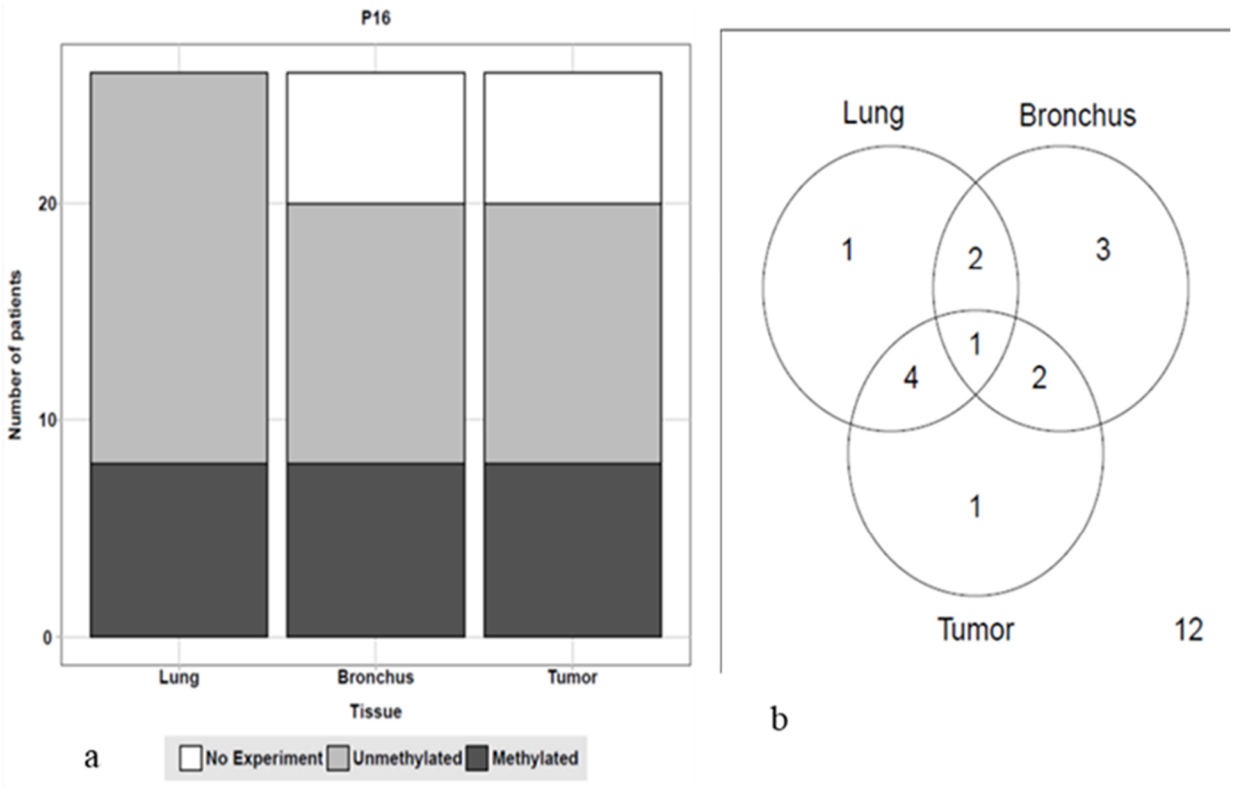

Fig.4. Methylation analysis of p16 gene in 26 lung cancer patients. (a) shows the methylation frequencies of p16 in Lu, Br, and Tu tissues. (b) shows the methylation of p16 gene in more than a tissue type. Methylation frequencies of candidate genes were analysed by Fisher's exact test. Results with $p < 0.05$ were considered statistically significant (one-tailed test).

Methylation of MT1A was observed in 10 patients (39%). 9 out of 10 (90%) were smokers. In 5 out of these 10 patients (50%) MT1A methylation was detected in both, lung and tumor tissue samples (LuTu). 2 out of 10 (20%) were methylated in normal lung tissue, only. 2 other cases (20%) in tumor tissue, only. In only one sample (10%) MT1A methylation was found in bronchial tissue, solitarily (Fig.5).

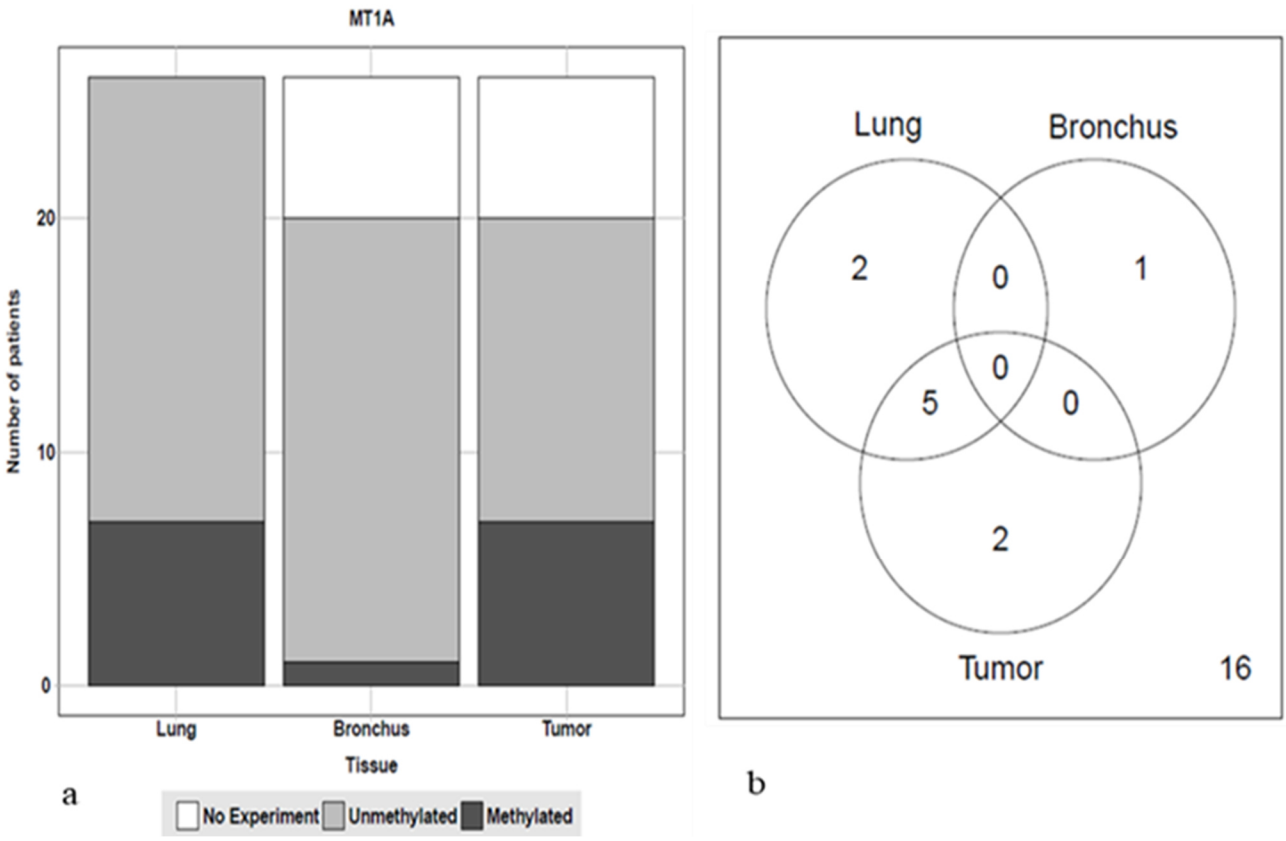

Fig.5. Methylation analysis of MT1A gene in 26 lung cancer patients. (a) shows the methylation frequencies of MT1A in Lu, Br, and Tu tissues. (b) shows the methylation of MT1A gene in more than a tissue type. Methylation frequencies of candidate genes were analysed by Fisher's exact test. Results with $p < 0.05$ were considered statistically significant (one-tailed test).

To assess a possible prognostic value in the clinic the results shown before were investigated with bioinformatics approaches to check for statistically significant associations between methylation status of Rassf1, DAPK, p16, MTIA genes and tissue origin, clinical history, age, sex, smoking and occupational exposure of the patient. First it was checked for a possible association between tissue types [healthy tissue regarded together (BrLu), solitary bronchus (Br), solitary lung (Lu) and tumor (Tu)] and methylation status of the candidate genes (Tab.4), which means if lung or bronchus of the same patient were methylated it was counted single (LuBr). In the test healthy tissues and tumors were compared on a patient specific level. Rassf1a showed no association in Fisher's exact test, whereas MT1A ($p = 0.007$), DAPK ($p < 0.001$) and p16 ($p = 0.005$) showed significant associations, as shown in (Tab.4), (Fig.11 appendices).

Tab.4. Methylation frequency and tissue types.

Gene	p-Value Lu Br vs. Tumor
Rassf1a	0.521
DAPK	< 0.001
P16	0.005
MT1A	0.007

Healthy tissues (Lung & Bronchial tissues) (LuBr) vs. tumor tissue were compared on a patient specific level, if lung and bronchus of the same patient were both methylated it was counted single, using Fisher's exact test.

Checking for an association between gender and methylation status for each gene and each of the three tissue types individually we found no association as shown in (Tab.5). In order to check for a possible effect of age on the methylation status the patients were divided in two groups, one older and one younger than the median (64 years). The result showed no significant association as seen in (Tab. 6). The set of patients analysed consisted of 22 smokers and 4 non-smokers in total (Tab.1).

Tab. 5. The methylation frequency and gender

Gene	Tissue	p-value
Rassf1a	Bronchus	0.15
Rassf1a	Lung	1
Rassf1a	Healthy	1
Rassf1a	Tumor	0.521
MT1A	Bronchus	1
MT1A	Lung	0.546
MT1A	Healthy	0.277
MT1A	Tumor	0.521
DAPK	Bronchus	1
DAPK	Lung	0.263
DAPK	Healthy	0.598
DAPK	Tumor	0.218
P16	Bronchus	0.242
P16	Lung	0.277
P16	Healthy	0.096
P16	Tumor	0.242

Tab.5

Tab. 6. The methylation frequency and age

Gene	Tissue	p-value
Rassf1a	Bronchus	1
Rassf1a	Lung	0.635
Rassf1a	Healthy	0.635
Rassf1a	Tumor	0.336
MT1A	Bronchus	1
MT1A	Lung	0.665
MT1A	Healthy	1
MT1A	Tumor	0.642
DAPK	Bronchus	1
DAPK	Lung	0.683
DAPK	Healthy	1
DAPK	Tumor	0.653
P16	Bronchus	1
P16	Lung	0.401
P16	Healthy	0.695
P16	Tumor	1

Tab. 6

Tab.5. The methylation analysis of Rassf1a, DAPK, p16, MT1A in 26 lung cancer patients. according to the gender in lung, bronchial, and tumor tissue and healthy tissue (lung and bronchial tissue together). P-values of Fisher's exact test.

Tab.6.The methylation analysis of Rassf1a, DAPK, p16, MT1A in 26 lung cancer patients. according to the age in lung, bronchial, and tumor tissue and healthy tissue (lung and bronchial tissue together). P-values of Fisher's exact test.

Checking for an association between smoking history and the methylation status for each gene and each of the three tissue types individually, we found no association as presented in (Tab.7). Checking for an association between chemotherapy and the methylation status for each gene and each of the three tissue types individually we found a significant association in the Rassf1a gene in lung ($p = 0.042$) regarded individually and in healthy tissue, when lung and bronchus were regarded together ($p = 0.042$). Moreover, there was a significant association for DAPK ($p = 0.042$) in bronchial tissue (Tab. 8), (Fig.12 appendices).

Tab. 7. The methylation frequency and smoking.

Gene	Tissue	p-value
Rassf1a	Bronchus	1
Rassf1a	Lung	0.555
Rassf1a	Healthy	0.555
Rassf1a	Tumor	0.521
MT1A	Bronchus	1
MT1A	Lung	0.546
MT1A	Healthy	0.277
MT1A	Tumor	0.521
DAPK	Bronchus	0.521
DAPK	Lung	1
DAPK	Healthy	0.598
DAPK	Tumor	1
P16	Bronchus	1
P16	Lung	1
P16	Healthy	1
P16	Tumor	1

Tab.7

Tab.8. The methylation frequency and Chemotherapy.

Gene	Tissue	p-value
Rassf1a	Bronchus	1
Rassf1a	Lung	0.042
Rassf1a	Healthy	0.042
Rassf1a	Tumor	1
MT1A	Bronchus	1
MT1A	Lung	0.391
MT1A	Healthy	0.216
MT1A	Tumor	0.158
DAPK	Bronchus	0.042
DAPK	Lung	0.218
DAPK	Healthy	1
DAPK	Tumor	0.362
P16	Bronchus	0.648
P16	Lung	1
P16	Healthy	1
P16	Tumor	1

Tab.8

Tab.7. The methylation status in the smoker patients of Rassf1a, MT1A, DAPK and p16 in lung, bronchial and tumor tissue and healthy tissue (lung and bronchial tissue together) in 26 lung cancer patients. P-values of Fisher's exact test.

Tab.8 The methylation status in patients had chemotherapy of Rassf1a, MT1A, DAPK and p16 in lung, bronchial and tumor tissue and healthy tissue (lung and bronchial tissue together) in 26 lung cancer patients. P-values of Fisher's exact test.

We found no significant association, when a correlation was checked between clinical diagnosis in cases of non-small cell lung cancer (NSCLC II vs. III) and the methylation status for each gene and each of the three tissue types individually (Tab 9). When an association between tumor classification (T2 versus T1 and T3 together) and the methylation status was checked, we also found no significant association (Tab 10). Checking for an association between occupational exposure to metals (eg. working as locksmith, mechanic, welder, plumber) and the methylation status for each gene and each of the three tissue types individually we found a significant association (p = 0.03) in healthy tissue for the p16 gene (Tab. 11), (Fig.12 appendices).

Tab. 9. The methylation frequency and clinical diagnosis

Gene	Tissue	p-value
Rassf1a	Bronchus	0.333
Rassf1a	Lung	0.587
Rassf1a	Healthy	0.587
Rassf1a	Tumor	0.242
MT1A	Bronchus	1
MT1A	Lung	1
MT1A	Healthy	1
MT1A	Tumor	1
DAPK	Bronchus	1
DAPK	Lung	0.062
DAPK	Healthy	0.057
DAPK	Tumor	0.282
P16	Bronchus	0.56
P16	Lung	0.613
P16	Healthy	0.642
P16	Tumor	0.329

Tab.9

Tab.10. The methylation frequency and tumor classification

Gene	Tissue	p-value
Rassf1a	Bronchus	0.35
Rassf1a	Lung	0.628
Rassf1a	Healthy	0.628
Rassf1a	Tumor	0.131
MT1A	Bronchus	1
MT1A	Lung	0.635
MT1A	Healthy	0.667
MT1A	Tumor	0.29
DAPK	Bronchus	0.613
DAPK	Lung	0.667
DAPK	Healthy	1
DAPK	Tumor	1
P16	Bronchus	1
P16	Lung	0.667
P16	Healthy	1
P16	Tumor	1

Tab.10

Tab.9. The methylation analysis of patients with a clinical diagnosis (NSCLC II vs. NSCLC III) on methylation state of Rassf1a, MT1A, DAPK and p16 in lung, bronchial and tumor tissue and healthy tissue (lung and bronchial tissue together) in 26 lung cancer patients. P-values of Fisher's exact test.

Tab.10. The methylation status of patients with a tumor class (T2 vs. T1 and T3 together) on methylation state of Rassf1a, MT1A, DAPK and p16 in lung, bronchial and tumor tissue and healthy tissue (lung and bronchial tissue together) in 26 lung cancer patients. P-values of Fisher's exact test.

During data analysis healthy tissues and tumors were compared, but this time on a patient specific level, which means if lung and bronchus of the same patient were both methylated it was counted single (Tab.4). We also checked for general differences in methylation status between tumor and healthy tissues over all samples as shown in (Tab.12), (Fig.13 appendices). Smoking status showed a significant difference in the MT1A ($p = 0.0345$), DAPK ($p = 0.00004$) and p16 ($p = 0.01522$) genes, but this should be evaluated further as only 4 of the 26 patients were smokers. Males showed a significant difference in the MT1A ($p = 0.0345$), DAPK ($p = 0.00004$) and p16 ($p = 0.01522$) genes, but this should be evaluated further as only 4 of the 26 patients were female.

We also found associations between age and methylation status; for patients younger than the median for MT1A ($p = 0.04762$) and p16 ($p = 0.00794$). Patients not exposed to metals showed a significant difference between tumor and healthy tissue in MT1A ($p = 0.00137$), DAPK ($p = 0.00009$) and p16 ($p = 0.03571$) (Tab. 12). In patients with chemotherapy there was a significant difference in methylation of p16 ($p = 0.01786$).

Patients with chronic diseases showed a significant correlation for DAPK (p = 0.00058) and p16 (p = 0.00466). In patients with tumor classification T2 (versus T1+T3) there was a significant difference in methylation state between healthy and tumor tissue for MT1A (p = 0.03297), DAPK (p = 0.00016) and p16 (p = 0.04056) (Tab.12), (Fig.13 appendices).

Tab.11. The methylation status in patients with occupational exposure.

Gene	Tissue	p-value
Rassf1a	Bronchus	1
Rassf1a	Lung	1
Rassf1a	Healthy	1
Rassf1a	Tumor	0.549
MT1A	Bronchus	1
MT1A	Lung	1
MT1A	Healthy	1
MT1A	Tumor	0.587
DAPK	Bronchus	0.613
DAPK	Lung	1
DAPK	Healthy	1
DAPK	Tumor	1
P16	Bronchus	0.161
P16	Lung	0.06
P16	Healthy	0.03
P16	Tumor	0.255

Tab.11

Tabl.12. The methylation status between tumor and healthy tissues.

Category	Gene	p-value subgroup 1	p-value subgroup 2
Gender	Rassf1a	0.51471	1
Gender	MT1A	0.0345	1
Gender	DAPK	0.00004	1
Gender	P16	0.01522	1
AgeMed	Rassf1a	1	0.44444
AgeMed	MT1A	0.15152	0.04762
AgeMed	DAPK	0.00303	0.00794
AgeMed	P16	0.24242	0.00794
Smoking	Rassf1a	0.51471	1
Smoking	MT1A	0.0345	1
Smoking	DAPK	0.00004	0.33333
Smoking	P16	0.01522	0.33333
Metal	Rassf1a	1	0.52857
Metal	MT1A	1	0.00137
Metal	DAPK	0.33333	0.00009
Metal	P16	0.25	0.03571
Therapy	Rassf1a	1	0.49091
Therapy	MT1A	0.125	0.24242
Therapy	DAPK	0.01786	0.00202
Therapy	P16	0.01786	0.24242
Disease	Rassf1a	1	1
Disease	MT1A	0.09491	0.06667
Disease	DAPK	0.00058	0.06667
Disease	P16	0.00466	1
TumorClass	Rassf1a	1	1
TumorClass	MT1A	0.4	0.03297
TumorClass	DAPK	0.1	0.00016
TumorClass	P16	0.1	0.04056

Tab.12

Tab.11. The methylation status in patients with occupational exposure for Rassf1a, MT1A, DAPK, and p16 in lung, bronchial, and tumor tissue and healthy tissue (lung and bronchial tissue together) in 26 lung cancer patients. P-values of Fisher's exact test.

Tab.12. Checking for general findings of methylation status between tumor and healthy tissues (lung and bronchial tissue together) over all samples using Fisher's exact test in 26 lung cancer patients. Gender: subgroup1= male, subgroup2=Female, Age: subgroup1= > 64. Subgroup2 = < 64, Smoking: subgroup1 = yes. Subgroup2= no, Metal exposured: subgroup1= yes. Subgroup2= no, Chemotherapy: subgroup1= yes. Subgroup2= no, Chronic disease: subgroup1= yes. Subgroup2= no, Tumor class: subgroup1= T3 T1. Subgroup2= T2. P-values of Fisher's exact test.

4.2 Role of carcinogenic factors in epigenetic silencing of Rassf1a, DAPK, p16, and MT1A genes in human lung cells in culture

4.2.1 Long-term culture of normal human lung cells

Primary cultures of human lung cells were established from specimens of bronchial epithelium as well as of peripheral lung. The cultures were split and maintained over a relatively long period of time. When modifications of culture conditions are applied, the cultures develop a highly differentiated morphology. Immuno-histochemical staining indicated that the cultures of lung epithelium consist of > 95% of cells of epithelial characteristics (Fig.6). Electron microscopic studies revealed a differentiated phenotype featuring cilial structures. More than 95 % of the lung and bronchial specimens could be used as a raw material for cell cultures. The ability of cells to be maintained by repeated cultivation showed inter-individual variation. 60-70% of the obtained tissue explants could be re-cultivated to obtain higher generations (> 3 generations). 10-15% of the specimens could reach the 5th -7th generation. Generally, the overall duration of cultivation, which ranged between 2-24 weeks, varied with respect to the number of culture generations and the rate of cell growth. These findings draw attention to the importance of individual variation as a determining factor, not only in the rate of cell growth, but also for the survival capability of cells in culture.

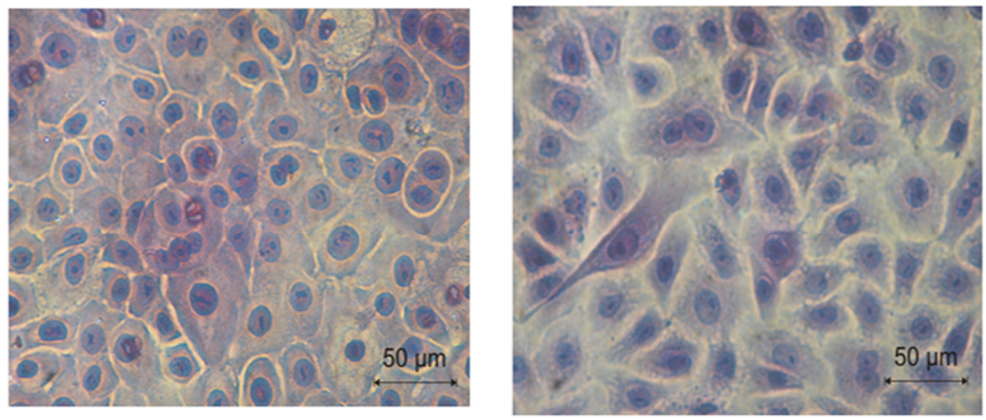

Fig.6 Image of cultured normal human bronchial epithelial cells (NHBEC) and peripheral lung cells (PLC) from human lung explants. Haematoxylin and Eosin (H&E) stain; 40x-fold magnification.

4.2.2 Epigenetic effects of chronic exposure to Cd (II) and CSC

Various compounds may induce epigenetic silencing of tumor suppressor genes, although they act by different mechanisms. In this study we investigated the methylation status of the tumor related genes Rassf1a, DAPK, p16 and the detoxification related gene (metallothionein-1A) in peripheral lung cells (PLC) and normal human bronchial epithelial cells (NHBEC) of 12 lung cancer patients after a long-term exposure to 5µM Cadmium Cd (II)) and 10mg/l cigarette smoke condensate (CSC) [for 14 weeks in NHBEC, and 11 weeks in PLC]. Generally, chronic exposure to cigarette smoke condensate (CSC 10 mg/l) leads to methylation of genes in 7 (NHBEC) and 4 (PLC) of 48 cultures. On the other hand, chronic exposure to Cd (II) resulted in methylation in 13 (NHBEC) and 9 (PLC) of 48 cultures (Tab.13).

Tab. 13. Summary of methylation status of the candidate genes in long term cultures.

ID	Control		Rassf1a				DAPK				**p16**				**MT1A**			
	NHBEC	PLC	NHPEC		PLC		NHBEC		PLC		NHBEC		PLC		NHBEC		PLC	
			Cd	CSC	Cd	CSC	Cd	CSC	Cd	CSC	Cd	CSC	Cd	CSC	Cd	CSC	Cd	CSC
200																■		■
208																		
215							■		■			■		■				
221			■				■		■						■		■	
222			■				■											
223							■		■									
225			■	■			■	■	■					■	■	■	■	
228																		
229			■	■			■	■	■					■				
234			■									■		■			■	
268																		
276																		

The methylated samples of Rassf1a, DAPK, p16 and MT1A genes in long term cultures [14 weeks in NHBEC, and 11 weeks in PLC] of NHBEC, PLC for 12 lung cancer patients. Control are untreated cells. Dark squares represent methylated samples, white squares represent unmethylated. NHBEC: normal human bronchial epithelial cells, PLC: peripheral lung cells. Cd: cadmium (II) (5µM); CSC: cigarette smoke condensate (10mg/l).

4.2.3 Targeting of methylation of specific genes in primary cell cultures following incubation with Cd and CSC

Rassf1a

Hypermethylation of the Rassf1a promoter is one of the most frequent epigenetic inactivation events detected in human cancer leading to the silencing of rassf1a expression. Our results show that no Rassf1a methylation was detected in peripheral lung cells (PLC) after long-term exposure to 5 µM Cd (II) and 10 mg/l CSC [for 14 weeks in NHBEC, and 11 weeks in PLC]. On the other hand, the Rassf1a promoter was methylated in 5 out of 12 cases (42%) in normal human bronchial epithelial cells (NHBEC) incubated with 5 µM Cd (II), whereas treatment of cultures from the same patients with 10 mg/l CSC resulted in methylation of 2 of 12 (Tab.13). Nevertheless, these treatment effects these effects were not statistically significant ($p = 0.185$) as shown in (Tab.14).

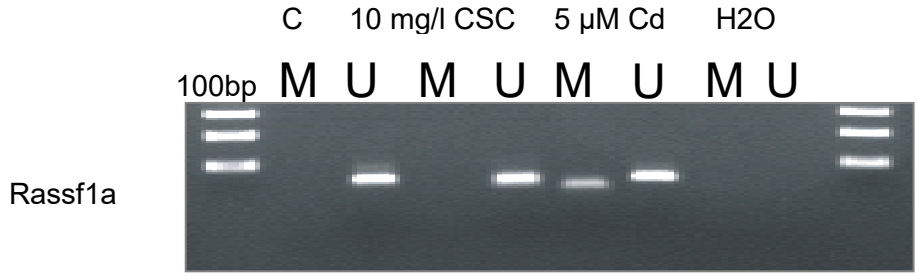

Fig.7. Rassf1a promoter methylation analysis by MS-PCR in normal human bronchial epithelial cells (NHBEC) [ID222]. Lane c untreated cells, lane 5 µM Cd (II) treated cells for long term exposure, lane 10mg/l CSC cigarette smoke condensate (CSC) treated cells for long term exposure. M indicates the presence of methylated Rassf1a, U indicates the presence of unmethylated Rassf1a.

Tab.14. Statistical analysis of methylation frequency of candidate genes after long term exposure.

	NHBEC		*p*-Value	PLC		*p*-Value
	Cd (II)	CSC		Cd (II)	CSC	
Rassf1a	5	2	0,185	0	0	/
DAPK	6	2	0,097	5	0	0,019
p16	0	2	0,239	0	4	0,047
MT1A	2	2	0,705	3	1	0,295
sum	13	8		8	5	

Statistical analysis of methylation frequency of Rassf1a, DAPK, p16, and MT1A in normal human bronchial epithelial cells (NHBEC) and peripheral lung cells (PLC) after long exposure [14 weeks in NHBEC, and 11 weeks in PLC] to cigarette smoke condensate (CSC) and cadmium Cd (II) using Fisher's exact test. $p < 0.05$ was considered statistically significant.

DAPK

We analysed the promoter methylation of the DAP- kinase gene in lung and bronchial cell cultures of 12 lung cancer patients using methylation-specific-PCR (MSP). When cultures were exposed [for 14 weeks in NHBEC, and 11 weeks in PLC] to 5µM Cd (II) in 6 out of 12 (50%), the DAPK gene was partially methylated to different degrees in NHBEC and in 5 out of 12 in PLC. (Tab. 14). Incubation with 10 mg/l CSC induced methylation of DAPK in 2 cultures of NHBEC, but on the other hand it did not result in DAPK methylation in PLC (Tab.13). The methylation frequency of DAPK was statistically significant between Cd (II) treated PLC versus untreated cells ($p = 0.019$). Whereas Fisher's exact test showed no significant for DAPK and CSC treated NHBEC versus untreated cells ($p = 0.097$).

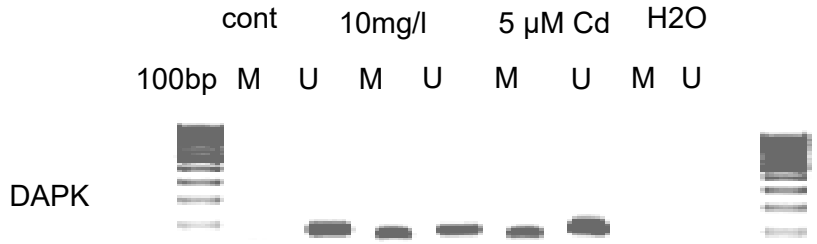

Fig.8. DAPK promoter methylation analysis by MS-PCR in normal human bronchial epithelial cells (NHBEC) [ID225]. Lane cont untreated cells. Lane Cd 5 µM Cd (II) treated cells for long term exposure. Lane 10 mg/l CSC cigarette smoke condensate treated cells for long term exposure. M indicates the presence of methylated DAPK, U indicates the presence of unmethylated DAPK.

p16

p16 is one of the most intensively studied genes in patients with NSCLC. Various studies have shown that methylation of p16 can be detected in the sputum of patients with lung cancer (Tuo et al., 2018). It is consistently suggested to be hypermethylated, especially in cases associated with smoking. We therefore investigated the methylation status of the p16 gene in primary lung cell cultures with long term exposure [for 14 weeks in NHBEC, and 11 weeks in PLC] to 5 µM Cd (II) and 10 mg/l CSC. Aberrant methylation of the p16 gene was detected in PLC form 4 out of 12 patients (33%) after long-term exposure to 10 mg/ml CSC. Cultures from two of these patients were also methylated in NHBEC. On the other hand, long-term exposure to 5 µM Cd(II) did not cause methylation of p16, neither in NHBEC nor in PLC. Checking for an effect of chronic exposure to CSC on methylation status of p16 gene in the different cell types, we found a significant difference between PLC and NHBEC, as CSC treated peripheral lung cells (PLC) showed a significant induction of methylation ($p = 0.047$). Whereas Fisher's exact test showed no difference ($p = 0.239$) in CSC treated normal human bronchial cells (NHBEC).

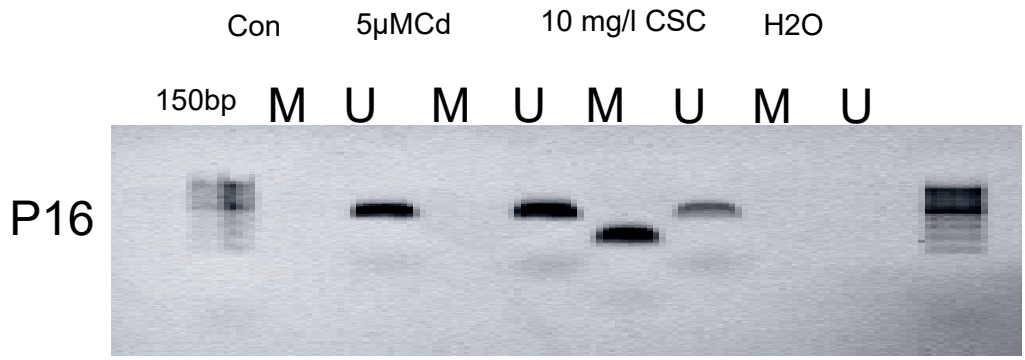

Fig 9. P16 promoter methylation analysis by MS-PCR in normal peripheral epithelial lung cells (PLC) [ID234]. Lane cont untreated cells, Lane 5 µM Cd Cd (II) treated cells for long term exposure. Lane10 mg/l CSC cigarette smoke condensate treated cells for long term exposure. M indicates the presence of methylated p16, U indicates the presence of unmethylated P16.

Metallothionein 1A (MT1A)

Frequent methylation of the heavy metal binding protein MT1A has been reported to be associated with higher tumor stage in prostate cancer, moreover MT3 and MT1G expression are downregulated by methylation in oesophageal cancer and thyroid carcinoma respectively. (Tsou et al., 2007). Maleckaite et al. (2019) reported that the DNA methylation of metallothionein genes is associated with the clinical features of renal cell carcinoma. Only few studies have analysed aberrant DNA methylation of metallothionein in human so far, especially in the lung. We therefore investigated whether Cd (II) and CSC as known inducers of lung cancer can cause MT1A silencing through promoter hypermethylation in primary cultures of lung cells. The methylated status of MT1A was recorded in 4 out of 12 (33%) cases. Incubation with 5 µM Cadmium weeks [14 weeks in NHBEC, and 11 weeks in PLC] resulted in methylation of MT1A in PLC from 3 donors. Cultures of NHBEC from two of these donors showed methylation of MT1A after 5 µM Cd (II) for weeks as well. Treatment with 10 mg/l CSC [for 14 weeks in NHBEC, and 11 weeks in PLC] resulted in methylation of MT1A in cultures of (NHBEC) from two patients; PLC from one of these showed methylations of MT1A as well after this treatment (Tab.13). Fisher's exact test showed no difference between chronic exposure to Cd (II) or CSC and methylation status for the MT1A gene and cell types individually ($p = 0.705$) neither for (NHBEC) versus untreated NHBEC, nor for peripheral lung cells (PLC) versus PLC untreated cells ($p = 0.295$).

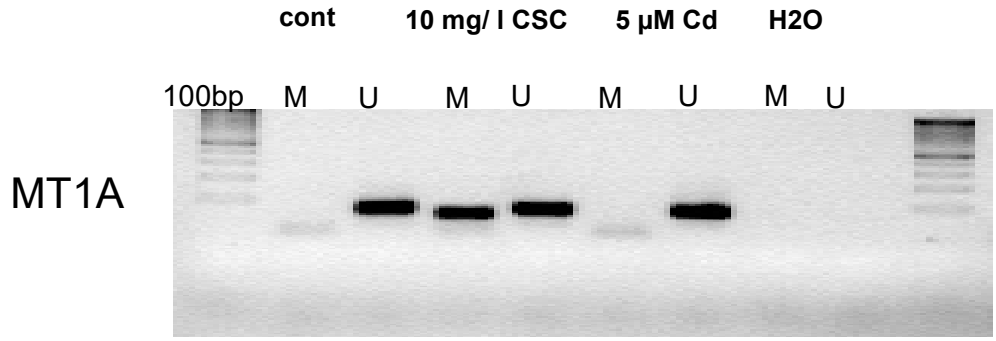

Fig 10. Metallothionein,1A promoter methylation analysis by MS-PCR in normal peripheral epithelial lung cells (PLC) [ID200]. Lane cont untreated cells, lane Cd 5 µM (II) treated cells for long term exposure. Lane 10 mg/l CSC cigarette smoke condensate treated cells for long term exposure. M indicates the presence of methylated MT1A, U indicates the presence of unmethylated MT1A.

4.3 Differences in methylation status of Rassf1a, DAPK, p16, and MT1A, and their expression in primary cultures of human lung cells

To investigate the relation between promoter methylation and expression of mRNA for Rassf1a, DAPK, p16 and MT1A mRNA- expression of these genes analysed in methylated samples. Long term exposure to CSC [for 14 weeks in NHBEC, and 11 weeks in PLC] induced methylation (Tab. 15) and led to a significant down-regulation of Rassf1a gene transcription in 2 of 12 NHBEC samples [(p = 0.022);] compared to controls. On the other hand, incubation with Cd (II) did not cause a change in mRNA expression of the Rassf1a gene in NHBEC (p = 1.0) despite methylation.

DAPK mRNA was significantly (p < 0.001) up-regulated to 181% in methylated NHBEC samples after long-term exposure to 5 µM Cd (II), and in the methylated PLC samples (159%) (p = 0.001), compared to controls. In cigarette smoke condensate (CSC)- treated NHBEC, DAPK- mRNA was up-regulated significantly (p = 0.002) to 120%. We therefore suggest that the long exposure to Cd (II) and CSC could induce DAPK expression despite of the methylation status. Long term exposure [for 14 weeks in NHBEC, and 11 weeks in PLC] to CSC led to significant down-regulation of p16 gene transcription (70%) in methylated NHBEC (p = 0.022), and in methylated PLC [85 %; (p = 0.002)]. The mRNA of the MT1A gene showed significantly increased expression levels in NHBEC [181%; (p = 0.022)], and in PLC [293%; (p = 0.006)] exposed to Cd (II) [for 14 weeks in NHBEC, and 11 weeks in PLC], even though these samples

showed increased methylation of MT1A. In cigarette smoke condensate (CSC)-treated NHBEC cells, MT1A mRNA was also increased significantly [151%; (p = 0.002)].

Tab.15. mRNA expression of the methylated samples in NHBEC and PLC after long term exposure

ID	Control		Rassf1a\GAPDH				DAPK\GAPDH				p16\GAPDH				MT1A\GAPDH			
	NHBEC	PLC	NHPEC		PLC		NHBEC		PLC		NHBEC		PLC		NHBEC		PLC	
			Cd	CSC	Cd	CSC	Cd	CSC	Cd	CSC	Cd	CSC	Cd	CSC	Cd	CSC	Cd	CSC
200	100	100														150		165
215	100	100					160		144			70		80				
221	100	100	100				170		130						180		250	
222	100	100	100				188											
223	100	100					151		169									
225	100	100	100	80			177	120	180					80	182	151	218	
229	100	100	100	82			119	119	170					84				
234	100	100	100									71		81			250	

mRNA-expression of Rassf1a, DAPK, p16 and MT1A genes in methylated samples after long term cultures of NHBEC and PLC from lung cancer patients. Control is untreated cells. NHBEC: normal human bronchial epithelial cells, PLC: peripheral lung cells. Cd: Cd (II) (5µM); CSC: tobacco smoke condensate (10mg/l). Black squares: no mRNA test performed.

5. Discussion

The Role of DNA hypermethylation of Rassf1a, DAPK, p16, and Metallothionein1A in normal and tumor tissues of lung cancer patients

Tumor suppressor genes can have various functions; for example, they can direct expression of proteins that are part of the system that regulates cell division. When mutated or the structure of the chromatin is altered without changing the nucleotide sequence of the DNA (DNA hypermethylation), a tumor suppressor gene is unable to do its job, and as a result, uncontrolled cell growth may occur. This may contribute to the development of a tumor, e.g. when losing p16/INK4A/CDKN2A, which is required for the control of cell growth in many and perhaps most cell types (Agarwal et al., 2013). Silencing of tumor suppressor genes by methylation of the CpG islands in the promoter regions is a major event in the initiation of many cancers (Kalari et al., 2010). We investigated frequencies of aberrant CpG island methylation for several tumor-associated genes: Rassf1a, DAPK, p16 and the stress gene Metallothionein IA in 26 lung carcinomas, since inactivation of these genes directly interferes with cell cycle and cell growth regulation, apoptosis, and detoxification of carcinogens. We used methylation-specific PCR (MSP) to determine methylated and unmethylated CpG sites of the four genes. The cellular composition of the tissue specimen that is tested for DNA methylation may also play a crucial role. In MSP fully methylated means that all the CpG positions in all tested cells are methylated. Similarly, (fully) unmethylated means that all the cells analysed are unmethylated. Sometimes a homogeneous mixture of cells may contain both unmethylated and fully methylated alleles (Mikeska et al., 2007). Therefore, it is possible that there are positive signals in both reactions for one DNA sample. This may also be owing to the heterogeneity of cells in the samples because our samples from lung and bronchus consist of different cell types. Moreover, there are often remaining non-malignant cells in tumor specimens. On the other hand, even in a pure cell population, there may be heterogeneous methylation patterns (partial methylation) in a given CPG island, which may also result in a mixed MSP result (Galm et al., 2005). In our study, samples were obtained from tumor and corresponding adjacent normal lung and bronchial tissue located at least 2 cm away from the site where the tumor was sampled. The Rassf1a gene is located at chromosome 3p21.3 and functions as a tumor suppressor gene involved in cell apoptosis, genomic and microtubule stability and cell cycle regulation. This gene codes for two major transcripts, rassf1a and rassf1c, by alternative splicing. The promoter methylation of the Rassf1a gene has been shown to be associated with gene silencing in lung cancer (Marsit et al., 2006).

In lung cancers, methylation of Rassf1a was present more frequently in non-small cell lung cancer tumors (18 of 42; 43%) and cell lines (15 of 30; 50%) than in small cell lung cancer cell lines (6 of 30; 20%; $p = 0.03$) (Toyooka et al., 2001). Our data shows that Rassf1a promoter methylation was generally detected in 16 of the 26 Patients (62%) (Fig.2). We observed that the highest methylation frequency in tumor tissue was detected for Rassf1a (69%) (11/16) compared to the three other candidate genes. The study of Dammann, et al., 2000 is in agreement with our study, as the promoter of Rassf1a was methylated in 24 of 60 (40%) primary lung tumors, moreover, 4 of 41 tumors analysed carried missense mutations that suggest Rassf1a may function as a lung tumor suppressor. In one patient (1/16) (6 %) only the lung tissue sample was positive for methylation. Methylation of Rassf1a in both types of healthy tissue lung and bronchial (LuBr) examined was found in one patient (1 out of 16) (6 %). 3 of 16 (19 %) were methylated in lung and tumor tissue (LuTu) as shown in (Fig.2). In contrast to these findings, a study by Belinsky et al., 2002 showed, that promoter methylation was not seen in bronchial epithelium (lung cancer) cases n = 52, and controls n = 89. Only two sputum samples were positive for Rassf1a methylation suggesting that inactivation of this gene could be a later event in malignant transformation. This might be supported by our results including two patients actually suffering from NSCLC, but not showing methylation of Rassf1a in their respective tumor samples. There are many hints to an inactivation of Rassf1a during lung cancer development. Dammann et al., 2000 demonstrated that one of the major transcripts of this gene, Rassf1 is frequently inactivated by promoter hypermethylation in tumors. Re-expression of the gene in lung cancer cell lines suppressed the malignant phenotype. These results indicated that loss of heterozygosity (LOH) from chromosome 3p21.3 and CpG island methylation of Rassf1a are one of the common and earliest identified events in the pathogenesis of carcinoma which first were found in lung cancer. Our results were investigated after that by using bioinformatics approaches to check for statistically significant associations using Fisher's exact test between methylation status of Rassf1a, clinical history, age, sex, smoking, chemotherapy and occupational exposure of the patients. We first analysed the association between Rassf1a gene and the three tissue types on a patient-specific level, which means if lung or bronchus of the same patient were methylated it was counted single. Rassf1a showed no association (Tab.4). In contrast to these results (Endoh et al., 2003) suggest that methylation of Rassf1a may have prognostic importance for the patients with early stage disease, but further studies are needed to confirm this (Endoh et al., 2003). In our study, 12/26 patients have been subjected to chemotherapy. Checking for an association between chemotherapy and the methylation status for each gene and each of the three tissue types on a patients specific level, we found a

significant association in the Rassf1a gene in lung (p = 0.042) regarded individually and in healthy tissue, when lung and bronchial tissues were regarded together (p = 0.042). No significant association of the Rassf1a and chemotherapy was found when bronchial was regarded individually, which is also due to the fact that we had only one sample from a patient without chemotherapy that showed methylation of Rassf1a in bronchus. Nevertheless, multiple studies have shown that inactivation of tumor suppressor genes might result in tumors that are unresponsive to chemotherapy (Hamilton et al., 2006). Reactivation of epigenetically silenced tumor suppressor genes has been suggested as a therapy in cancer treatment. In particular, natural compounds isolated from herbal extracts have been tested for their capacity to induce Rassf1a in cancer cells, through demethylation with supplements (e.g., methyl donors, vitamins and polyphenols) (Damann et al., 2017).

We also checked for general differences in all samples in methylation status of Rassf1a between tumor and both healthy tissues regarded together as shown in (Tab.12). Smoking and non-smoking patients showed no significant difference in methylation status of Rassf1a. In contrast to Kim et al., 2003, suggesting that starting cigarette smoking at an early age resulted in hypermethylation in 32% of 204 primary non-small lung cancer Patients. Wu et al., 2014 supported the idea that Rassf1a gene hypermethylation is associated with cigarette smoking-induced lung cancer. Various studies have investigated the association between cigarette smoking and Rassf1a gene promoter hypermethylation in lung cancer patients, but a unanimous conclusion could not be reached (Kim et al., 2003). DAPK is a cytoskeleton-associated protein kinase that is important in cytokine-induced apoptosis. DAP kinase was initially isolated as a positive mediator of apoptosis induced by interferon γ by using a strategy of functional cloning (Inbal et al., 1997). Several studies have suggested that loss of DAPK expression or the methylation of its associated CpG islands may be characteristic of highly invasive or metastatic tumors (Narayan et al., 2003). In the study of DAPK in cervical cancer by Zhao et al., 2008, it was found that the methylation rate of the DAPK gene was significantly higher in cervical squamous cell carcinomas than in adenocarcinomas. We observed that the methylation frequency for DAPK was 46 % (12/26) in 26 patients. Solitary methylation of DAPK in bronchial epithelial tissue was detected in 2 out of 12 (17%) patients as shown in (Fig.3). Solitary methylation of DAPK in lung tissues was detected in one patient (8 %). 25% (3/12) were methylated in all three tissue types (LuBrTu). Moreover, 42 % (5 out of 12) were methylated in lung and tumor tissues (LuTu). One patient (1/12) (8 %) was methylated in bronchus and tumor (BrTu) (Fig.3). No patient was methylated in tumor, only. In contrast to

that, a study by Brabender et al., 2009 found that methylation of DAPK in tumor tissues was significantly higher compared to matching normal oesophageal tissues.

Also, Licchesi et al., 2008 demonstrated that DAPK hypermethylation was not detectable in normal lung specimens, but in invasive adenocarcinoma. In agreement with these findings the study by Zhao et al., 2008 observed that no methylation of the DAPK gene was found in normal cervical tissues. 17 % and 8 % of the patients in our study were methylated in bronchial and lung tissues, respectively. These results were subsequently investigated by using bioinformatics approaches using Fisher's exact test to check for statistically significant associations between methylation status of DAPK, clinical history, age, sex, smoking, chemotherapy and occupational exposure of the patients. We first analysed the association between the DAPK gene and the three tissue types on a patient-specific level, which means if lung or bronchus of the same patient were methylated it was counted single. For DAPK we found a significant association ($p < 0.001$) (Tab.4) that patients with unmethylated DAPK in healthy tissue had unmethylated DAPK in tumor as well and vice versa: those with methylated DAPK in healthy tissue also had methylated DAPK in tumor. Checking for an association between chemotherapy and the methylation status for each gene and each of the three tissue types on a patient's specific level, there was a significant association for DAPK ($p = 0.042$) in bronchial tissue (Tab. 8). Various studies have reported an association between DAPK methylation and chemotherapy. A study by Kato et al., 2008 showed that methylation of the apoptosis-related gene DAPK causes resistance to anticancer drugs and is associated with a poor prognosis in patients with gastric cancer. Another study found that the response rate to chemotherapy in gastric cancer was significantly lower in patients with methylation of either DAPK or BNIP3, or both, than in those without methylation ($p = 0.003$) (Sugita et al., 2011). Our study is agreement with both these studies, suggesting that DAPK methylation might predict prognosis and response to chemotherapy also in lung cancer cases. In our statistical analysis (Tab. 12), general differences in all samples in methylation status of DAPK between tumor and healthy tissues were checked. Male sex was significantly associated with methylation of DAPK ($p = 0.0004$). While the study of Hu et al., 2019 found no association between hypermethylation of DAPK and other parameters such as gender. Neither Kiyohara et al., 2002 nor Liu et al., 2007 found associations between methylation of DAPK and smoking. When we checked the influence of smoking on methylation of DAPK in healthy vs. tumor tissue, we found that the proportion of smokers with methylated tumor is significantly higher ($p = 0.00004$), when the healthy tissue is also methylated and vice versa (the proportion of smokers with unmethylated tumor is significantly higher, when the tumor is not methylated as well).

Nevertheless, our results should be validated in further experiments, as we only had tumor samples of three non-smokers. While it is accepted that smoking increases cancer incidence in individuals, the final molecular mechanism underlying this effect remains unknown. Moreover, our results show a significant association (p = 0.00009) between occupational exposure to metals (eg. working as locksmith, mechanic, welder, plumber) and methylation of DAPK. In this case there is a significant association for simultaneous methylation of DAPK in tumor and healthy tissues of the same patient for people not working with metals. An association for patients with chronic diseases was recorded for DAPK methylation in all samples (p = 0.00058) (Tab.12). In these patients there was a significant association that those with unmethylated DAPK in healthy tissue had unmethylated DAPK in tumor as well and vice versa: those with methylated DAPK in healthy tissue also had methylated DAPK in tumor. Nevertheless, the chronically ill group was very heterogeneous; it comprised of diseases with different path-mechanisms, like chronic hypertensive disease (CHD), chronic obstructive pulmonary disease (COPD), diabetes mellitus (DM), percutaneous transluminal coronary Angioplasty (PTCA), non-small cell lung carcinoma (NSCLC) and chronic abuse of alcohol (Tab. 1). For patients with tumor classification T2, compared to those with tumor classification T3 and T1 together, we found a significant association between methylation of DAPK in tumor and healthy tissue. Unfortunately, there are only few studies to illuminate the relationship between epigenetics and chronic diseases as well as tumor classification. P16 is a cell cycle regulator involved in the inhibition of G1 phase progression. Methylation of CpG islands silences the transcription of the p16 gene (Luo et al., 2005). Aberrant methylation of the p16 gene can be an early event in lung cancer (Belinsky et al., 1998), it was reported to occur frequently in multiple human lung cancers and oesophageal adenocarcinoma (Merlo et al., 1995; Seike et al., 2000). Aberrant methylation of 5' CpG islands of p16 was also present in 50 % of hepatoblastoma cases (Shim et al., 2003). We have detected methylation of p16 in 54% (14 of 26) of our cases. We first analysed the association between p16 gene and the three tissue types on a patient-specific level. p16 showed a significant association (p = 0.005) (Tab.4). Exclusive promoter methylation of p16 in normal bronchial- or lung tissue was detected in 21% (3 of 14) and 7% (1of 14), of the patients, respectively. One of 14 (7%) samples was methylated in tumor tissue, only. Several patients showed promoter methylation of p16 in more than one tissue, simultaneously, 14% (2 of 14) in bronchial and lung (BrLu), 29 % (4 of 14) in lung tissue and tumor tissue (LuTu), 14 % (2 of 14) in bronchial and tumor (BrTu), 7% (1 of 14) in bronchial, lung and tumor (BrLuTu) (Fig 4). The study by Kim et al., 2001 showed that smoking was associated with methylation of the p16 promoter region but not the DAPK gene promotor. In a study by Soria et al., (2002)

p16 methylation was higher in former smokers with a history of previous cancer than in former smokers without a history of cancer.

In another study, p16 methylation was detected in the sputum of 4% of heavy smokers without cancer compared to 32% of patients with lung cancer (Destro et al., 2004). All these results found that an exposure to tobacco smoke increases the likelihood of inactivation of p16 by methylation. In our study of 22 smoking and 4 non-smoking lung cancer patients we checked for general differences in all samples in p16 methylation between healthy tissue vs. tumor, smoking showed a statistically association ($p = 0.01522$). Kim et al., 2001 observed the same trend in their study of 172 smoking and 13 non-smoking lung cancer patients from the Massachusetts General Hospital ($p = 0.05$). Furthermore, the authors showed that the rate of methylated p16 gene promoter was significantly associated with pack-years smoked ($p = 0.007$). Liu et al., 2006 also, suggested an association between tobacco smoking and an increased incidence of aberrant promoter methylation of the p16 and MGMT genes in non-small cell lung cancer. In our study also gender was significantly associated with methylation of p16 ($p = 0.01522$), there was a significant association that male patients with unmethylated p16 in healthy tissue had unmethylated p16 in tumor as well and vice versa: those with methylated p16 in healthy tissue also had methylated p16 in tumor as well. Nevertheless, these results should be investigated further as we had only three samples from females. Patients with an age below the median of the study (64 years) ($p = 0.00794$) showed the same pattern of methylation A study of Vaissière et. al (2009) found no association between methylation status of p16 gene and age or histologic subtypes of the tumor in lung cancer patients. Accumulating evidence clearly shows that toxic metal exposure leads to induction and alteration of epigenetic marks in experimental and epidemiological studies (Martinez-Zamudio et al., 2011). To contribute to the knowledge about the epigenetic mechanisms underlying the molecular modes of action of metal effects, we tried to highlight the relationship between exposure to metals and methylation of the four genes covered by our work. In our results p16 methylation was significantly associated twice. First p16 was recorded ($p = 0.03$) in patients who worked with metals (working as locksmith, mechanic, welder, plumber) on a patient's specific level in healthy tissue (LuBr) (Tab.11). Secondly, we checked for general differences over all samples in methylation status of p16 in healthy tissues vs. tumor tissue, patients without metal exposure showed a significant association ($p = 0.03571$) (Tab.12). Our results are in agreement with Hou et al., (2011), as the authors showed that DNA methylation levels of p16 were significantly higher in post-exposure samples after contact with ambient air particulate matter (PM) ($p = 0.006$). From our results we hypothesize that there might be an association between methylation

of the p16 and DAPK gene, as in 6 out of 26 patients (23 %) neither the DAPK nor the p16 gene were methylated in any tissue. In contrast to our study, Kim et al., 2001 consider the DAPK gene independent from p16 methylation.

Metallothioneins (MTs) are a group of small cysteine-rich antioxidant and metal binding proteins. They are implicated in zinc homeostasis and heavy metal detoxification (Hunziker et al., 1985). In humans, the MT genes are located on chromosome 16 in a cluster and involve 16 identified genes, from which five are pseudogenes. Although the MT-II, MT-III and MT-IV proteins are encoded by a single gene, the MT-I protein comprises many subtypes encoded by a set of 13 MT-I genes. The known active MT-I genes are MT-IA,-IB, -IE, -IF,-IG, -IH,-IM and -IX. The rest of the MT-I genes (MT-IC,-ID,-II,-IJ and IL) are pseudogenes that are not expressed in humans (Moleirinho et al., 2011). MTs are involved in the protection against oxidative damage, metal homeostasis, cell proliferation and apoptosis (Theocharis et al., 2004). Therefore silencing of these genes should have an adverse effect in cells. The basic expression of the MT-I and MT-II genes is relatively low in most tissues except the brain and testes, but it can be induced by a variety of agents that include heavy metals, steroid hormones, interleukins, interferons, restraint stress, radiation and other agents that produce oxygen intermediates or free oxygen radicals (Kägi et al, 1991; Ghoshal et al, 2002; Takahashi et al., 2012). Compere et al., (1981) observed that MT-I and MT-II genes are silent in some lymphoid derived tumor cell lines, W-7 and S-49, but can be induced by heavy metals after treatment with the chemotherapeutic agent 5-azacytidine (5-AzaC). Some metals are required for physiological functions, such as the formation of blood (Iron), and the control of physicochemical processes, like intercellular signalling and DNA-repair (Zink). The metallothionein-I (MT-I) gene is silenced by the methylation of CpG islands in mouse lymphosarcoma P1798 cells (Majumder et al., 1999). Also, the metallothionein (MT) promoter was described to be methylated in rat hepatoma and in mouse lymphosarcoma cells by methylation (Jacob et al., 2001). The study by Ghoshal et al., 2001 of MT-I in a mouse model showed that inhibitors of DNA methyltransferase (Dnmt) and histone deacetylases (HDAC) synergistically activate the methylated metallothionein I gene (MT-I) promoter in mouse lymphosarcoma cells. Downregulation of MT synthesis in hepatic tumors may be related to hypermethylation of the MT-promoter or mutation of other genes, such as the p53 tumor suppressor gene. A methylation profiling analysis indicated that the MT1-promoter is methylated in the majority of hepatocellular carcinoma tumors examined (Mao et al., 2012).

Similarly, Faller et al., 2010 observed DNA methylation in MT1E in malignant melanoma, taken together these findings suggest that MT1E is a potential tumor suppressor gene. Downstream effects of MT overexpression are modulation of transcription of both tumor suppressor protein p53 and nuclear transcription factor NF-κB. A major effect of MT overexpression is free radical scavenging. All these MT effects influence cell survival, cell growth, drug resistance, and differentiation. Many studies have mentioned that methylation profiles are modified in lung cancer (Daniels et al., 2005). On the other hand, there have been only a few studies on the methylation of metallothionein1A in patients with lung cancer. The following question should be addressed: could promotor hypermethylation of metallothionein1A also be a candidate for representing a new biomarker for lung cancer diagnosis, or not? The mechanism facilitating induction or growth of tumors could be through inactivation of MT protein in some of the patients by promoter methylation of the gene in lung. In order to find out if our working hypothesis is generally valid, we analysed MT1A promoter methylation in normal and the corresponding tumor tissues of the lung of 22 smokers and 4 non-smokers using bisulfite-methylation specific PCR. Methylation of MT1A was observed in 10 of 26 patients (39%). Methylation of MT1A was detectable in (5 out of 10) patients 50% in lung and corresponding tumor tissues together (LuTu). (2 out of 10) 20% of the patients were positively methylated in lung tissue (Lu) only, as well as (20%) 2 out of 10 in tumor tissue (Tu) only. One sample (10%) was recorded for solitary methylation of MT1A in bronchial tissue (Br). When we analysed a possible association between the MT1A gene and the three tissue types on a patient-specific level, MT1A showed a significant association ($p = 0.007$) (Tab.4). In samples of smoking patients, we found a statistically significant overrepresentation of patients having unmethylated MT1A genes ($p = 0.0345$) in healthy tissue (BrLu) and tumor (Tabl.12). Nevertheless, these results should be evaluated further as only 4 of the 26 patients were non-smokers. (Belinsky et al., 2005) hypothesized, that inactivation of key genes through promoter hypermethylation is one mechanism by which tobacco smoke promotes the development of lung cancer. Smoking was also found to be associated with an accelerated methylation change in men; it is therefore possible that male lifespan may be prolonged by improving unhealthy lifestyles at or before middle age. Lung cancer is of prime interest for gender research, as it is pathogenesis, as well as progression, are strongly influenced by hormones and the mutational status of their receptors. Various other factors might also influence cancer risk and development. Therefore, we checked for general differences in all samples in methylation status between tumor- and healthy tissues (Tab. 12). In Patients not exposed to metals at work the proportion of persons with non-methylated MT1A in both healthy and tumor

tissue was significantly increased (p = 0.00137). Also, in male patients MT1A significantly (p = 0.0345) showed this pattern of distribution. Nevertheless, these findings should be evaluated further, as we had only three complete sets of samples from females. Patients with < 64 age showed a significant (p = 0.0476) association between tumor and healthy tissues for simultaneous methylation of MT1A. The study by Xiao et al., (2018) found that the sex-biased methylation changes occur in middle-aged men in an acceleration manner. These sexually dimorphic methylation changes were significantly overrepresented in genes associated with cardiovascular disease (CVD), which may impact the potential activation of disease expression, moreover higher prevalence of drinking and smoking in males might have some contribution to the sex-based methylation patterns during aging. In patients with tumor class T2 we found a significant over-representation of those with un-methylated MT1A in tumor and healthy tissue when we compared them to tumor classes 1 and 3 together. Jacob et al., 2002 suggest that the MT1A gene could be a potential tumor suppressor gene. Nevertheless, our data do not support this hypothesis, as we found several conditions, were unmethylated MT1A in tumor and healthy tissue of the same patients was significantly overrepresented or with significant associations for simultaneous methylation of MT1A in tumor and healthy tissues of the same patient.

Role of carcinogens factors in epigenetic silencing of Rassf1a, DAPK, p16, genes and MT1A genes in human lung cells in culture

Lung cancer is the most common cause of cancer-related death worldwide. It is acknowledged that prognosis and treatment outcomes in lung cancer might be improved by increasing the effectiveness of early-stage diagnosis. Harmful chemicals and multiple etiological factors, including genetics and smoking largely increase a person's risk of developing lung cancer. There is also increasing evidence for a major role of epigenetic aberrations in lung cancer and many other cancers (Bowman et al., 2006). Methylation is the most extensively studied category of epigenetics, leading to gene suppression. Hypermethylation in cancer seems to be a tissue-specific event. Some genes are commonly methylated in a variety of tumours, like p16 INK4a (Zulueta et al, 1995; Merlo et al., 1995) and Rassf1a (Pfeifer et al., 2005). These findings, indicate that methylation of certain genes may be a useful biomarker for early detection, prognosis, disease recurrence and lung cancer risk assessment. It is well known that smoking is the major risk factor for lung cancer and about 85-90% of all lung cancers occur in smokers (Heller et al., 2010) and have been related to DNA damage and an impaired expression of genes (Zhang et al, 2010; Zheng et al., 2003). We therefore tried to view the effects of cigarette smoke condensate and cadmium on DNA methylation of the tumour suppressor genes Rassf1a, p16,

PAPK and the detoxification gene MTIA in human lung using normal human bronchial epithelial cells (NHBEC) and peripheral lung cells (PLC) as models for inhalation toxicology. Normal human lung cells from the bronchial epithelium and peripheral lung tissue explants were cultivated under serum-free conditions in order to expand the cultures of normal bronchial epithelial cells (NHBEC) and peripheral lung cells (PLC) for experimental purposes.

The explants could be repeatedly transferred onto new culture dishes in order to gain monolayers of up to 15 generations and a culture time of up to 120 days. The cultures can be expanded and maintained over a relatively long time period. Cells were treated with (5µM, 10mg/l) non-toxic concentrations of cadmium (Cd) and cigarette smoke condensate (CSC), respectively. These doses were pre-checked for cellular toxicity in the MTT assay in order to guarantee that non-toxic conditions were applied. Tumour suppressor genes play an essential role in cell cycle control and apoptosis. The silencing of these genes in normal cells through hypermethylation is an alarm signal indicating the threat of developing lung cancer. (Vaid et al., 2009). The Rassf1 gene is located at the 3p21.3 tumour suppressor locus (Vos et al., 2004). RASSF1A methylation has been reported in the majority of lung, kidney, neuroblastoma, nasopharyngeal, prostate, bladder and gastric tumours (Honorio et al, 2003; Dammann et al., 2001). The methylation of Rassf1a appears to be common in non-small cell lung cancer (NSCLC) and an inactivation of Rassf1a was found in 30 to 38% of primary NSCLC, but in none of the non-malignant lung tissues (Dammann et al, 2000; Agathanggelou et al, 2001; Burbee et al., 2001). In this study, the Rassf1a gene was unmethylated in peripheral lung cells after a long-term exposure to 5 µM Cd (II) and 10mg/l CSC. This is in contrast to the findings of Dammann et al., 2001, where the promoter was highly methylated in 24 of 60 (40%) primary lung tumours. On the other hand, the Rassf1a promoter was methylated in 5 out of 12 (41.6%), in normal human bronchial epithelial cells (NHBEC) treated with 5µM cadmium (II). In contrast to our findings (Belinsky et al., 2002) reported that no Rassf1a inactivation was detected in the bronchial epithelium and was only seen in 2 out of 66 (3%) of sputum controls. The extract isolated from cigarette smoke (CSC) has been shown to increase the intracellular level of S-adenosylmethionine in A549 cells (Panayiotidis et al., 2004). This fact is of interest, as gene silencing was shown to be induced by S-adenosylmethionine in muscle differentiation (Fuso et al., 2001). The mechanism by which the heavy metal Cd influences the epigenetics has not been fully understood, yet (Beyersmann., et al 2008). We thought that inactivation of Rassf1a and other candidate tumour suppressor genes which may happen as a result of smoking and exposure to the heavy metal cadmium could differ between the proximal and distal regions

of the respiratory tract. In our study exposure to cigarette smoke condensate (CSC) 10 mg/l, however, did not have an effect on the methylation status of Rassf1a in peripheral lung cells (PLC). On the other hand, 10 mg/l CSC led to methylation of Rassf1a in cultures of NHBEC from 2 out of 12 (17 %) patients. Cultures from the same two patients also showed methylation after treatment with Cadmium (II) in NHBEC (Tab.3). Nevertheless, these effects were not statistically significant (p = 0.185) as shown in (Tab.4).

The death-associated protein kinase (DAPK) is a pro-apoptotic serine/threonine protein kinase that is dysregulated in a wide variety of cancers. The mechanism by which this occurs has largely been attributed to promoter hypermethylation, which results in gene silencing (Michie et al., 2009). The study of (Tada et al., 2002) suggests that the hypermethylation of DAP-kinase might be useful as a prognostic marker for disease recurrence in superficial bladder cancers. It is well known that smoking is the most common cause of bladder cancer. Cigarette smoke induces oxidative stress and is also one of the major causes of non-small lung cancer (NSCLC) (Michie et al,2009; Jarmalaite et al., 2010). In this study we investigated the methylation status of the DAPK gene in PLC and NHBEC after a long-term exposure [for 14 weeks in NHBEC, and 11 weeks in PLC] to 5 µM Cd (II) and 10mg/l cigarette smoke CSC. In NHBEC exposure to 10 mg/l CSC for 14 weeks caused aberrant methylation of DAPK in cultures of 33% (2 out of 6) patients (p = 0.097). Methylation of DAPK was not detected in PLC after long-term exposure [11 weeks] to 10 mg/l cigarette smoke condensate. These results are in agreement with (Kim et al., 2001) who found that cigarette smoke condensate did not cause DAPK methylation in normal peripheral lung cells. DAPK promoter methylation in cultures treated with 5 µM Cd (II) was slightly more frequent in NHBEC with 6 out of 12 (50%) compared to PLC with 5 out of 12 (40%). The effect of Cd (II) on methylation frequency of DAPK was statistically significant in PLC (p = 0.019). These findings are in contrast to (Takiguchi et al., 2003), who described a general reduction of DNA-methylation in rat liver cells treated with Cd in culture (Takiguchi et al., 2003). The p16 tumour suppressor gene plays a key role in cell cycle regulation. It´s alpha mRNA transcript codes for a protein (INK4A) that inhibits cyclin D kinases CdK4 and CdK6, which normally phosphorylate serine and threonine residues of the retinoblastoma (Rb) protein (Sherr et al, 1994; Weinberg et al., 1995). Inactivation of the p16 gene by aberrant methylation of its promoter region is believed to be a pathway to tumorigenesis (Merlo et al, 1995; Hermann et al., 1995). Zhang et al., 2011 speculated that p16 hypermethylation could be an early marker for cancer diagnosis. Müller et al., 2002 found that p16 methylation was detected in sputum samples from cancer-free chronic smokers.

The results here showed that promoter methylation of the p16 gene was detected in 4 out of 12 (33%) cultures of PLC exposed to 10 mg/l of CSC. Two of these samples also give positive signals for methylation in cultures of NHBEC as well. This is in agreement with (Soria J et al., 2002) who found, that in former cigarette smokers p16 was methylated in bronchial brush samples. Checking for an effect of chronic exposure to CSC on methylation status of p16 gene in the different cell types, we found significant difference between PLC and NHBEC, as CSC treated PLC showed a significant induction of methylation ($p = 0.047$), whereas Fisher's exact test was not significant ($p = 0.239$) in CSC treated NHBEC. There is very little research on the role of cadmium in the inactivation of tumour suppressor genes by hypermethylation. No methylation status of p16 gene was found in our study in the peripheral lung cells (PLC) treated with cadmium (5µM) (Tab.3, Interestingly (Yuan et al., 2013) suggested that chronic exposure to low dose Cd could induce hypermethylation of the p16 promoter and hence suppress p16 expression and then promote lymphocyte cell proliferation, which might contribute to Cd-induced carcinogenesis. Metallothioneins (MTs) are a group of low-molecular weight, cycteine-rich, metal-binding proteins, which are encoded by a family of genes located at chromosome 16q13. This family of proteins consists of 10 functional isoforms in humans, with MT-1A and MT-2A being the predominant forms (Cherian et al., 2003). It has been shown that aberrant expression of MTs is related to tumour type and different stages of tumour development and progression (Klaassen et al., 1999). Epigenetic inactivation of Metallothioneins via promoter hypermethylation was observed in acute myeloid leukaemia cell lines AML and paediatric AML samples (Tao et al., 2014). Ghoshal et al., 2000 suggested that lack of MT expression may promote the growth of some tumours like renal cell carcinoma (RCC), and breast cancer. It is therefore logical to conceive that silencing of MT may be advantageous to at least some highly proliferating cells. Ji et al., (2014) suggested that Metallothionein 1G and 1M might be used as potential biomarkers for the non-invasive detection of hepato cellular carcinoma (Ji et al, 2014; Sakamotl et al., 2010) demonstrated a strong association between MT1G methylation level and a poor outcome of patients with hepatoblastoma. MT1G hypermethylation is associated with a higher tumour stage in prostate cancer (Henrique et al., 2005). Methylation of MT-1A and MT-2A in malignant mesothelioma was shown to be associated with tumour grade histology and lymph-node involvement (Jacob et al., 2002). as Also in germ cell carcinoma, the expression of MT is closely related to the tumour grade and proliferative (Cheriana et al., 2003). MT protein stained positively in lung adenocarcinoma, but was absent in small cell lung carcinoma (Theocharis et al., 2002) suggesting that MT expression in the lung is tumour type-specific. However, further research

is required to determine the underlying molecular details. Long-term studies could be helpful in this progress. For this reason, a further objective of this study was to determine the promoter methylation status of MT1A in NHBEC and PLC of lung cancer patients. For this purpose PLC and NHBEC of early passages (2nd passage; P2) and late passages (P8–P15) were harvested from twelve lung cancer patients. After long-term exposure to test substances Cd (II) (5 µM) and CSC (10 mg/l) DNA methylation of MT1 promotor regions was analysed. Incubation with 5 µM Cadmium [for 14 weeks in NHBEC, and 11 weeks in PLC] resulted in methylation of MT1 in cultures of PLC from 3 of 12 donors. PLC.

Cultures of NHBEC from two of these patients were also methylated after comparable exposures. Incubation with 10 mg/l CSC resulted in methylation of MT1 in cultures of NHBEC from 2 of 12 patients, in PLC from one of these MT1 was methylated as well after exposure to CSC (Tab. 3). Both results were not significant according to Fisher's exact test ($p = 0.705$) for NHBEC, and ($p = 0.295$) for PLC. DNA methylation, especially 5′-CpG methylation, is an important mechanism in silencing the expression of genes (Nie et al., 2001). On the other hand, partial methylation of promoters still leads to transcription and also translation of genes in NHBEC and PLC cultures. Another interpretation for the expression of mRNA despite of hypermethylation is that in MSP it is possible that there are positive signals in both reactions for one DNA sample. This could be due to the heterogeneity of cells in the samples, leading to both signals from cells bearing either the unmethylated or the methylated promoters of the respective gene. On the other hand, even in a pure cell population there may be heterogeneous methylation patterns (partial methylation) in a given CpG island, which may also result in a mixed MSP result (Galm et al., 2005). In our study, samples were obtained from adjacent normal lung and bronchial tissue located at least 2 cm away from the site, where the tumour was sampled. In addition to DNA methylation profiles of Rassf1a, DAPK, p16, and MTIA m RNA expression of these genes was examined by real time PCR as well. Long term exposure [14 weeks in NHBEC, and 11 weeks in PLC] to cigarette smoke condensate (CSC 10 mg/l) led to a significant down-regulation of Rassf1a gene transcription in NHBEC ($p = 0.022$) compared to the controls. The study by (Ye et al., 2007) found that expression of Rassf1a was markedly reduced to an abnormal level or completely lost in primary gastric cancer compared to adjacent normal tissue, and this correlated to hypermethylation of the promoter of the Rassf1a gene. On the other hand, exposure to cadmium (II) (5 µM) did not cause a change in mRNA expression level of Rassf1a in NHBEC ($p = 1.0$) in our study. DAPK is activated following a variety of stimuli including TNFα, Ceramide, Interferon (IFN-γ) (Cohen et al, 1999; Pelled et al., 2002).

In order to determine the impact of epigenetic modifications on mRNA expression-PCR was used to investigate the effect of long-term exposure to Cd (II) and CSC on DAPK expression. Our data shows that long -term exposure to cadmium caused partial methylation of DAPK in both NHBEC and PLC and also affected DAPK mRNA expression. DAPK mRNA had increased significantly in our study after long-term exposure to 5 µM Cd in the methylated samples of NHBEC ($p < 0.001$), and in the methylated samples of PLC, ($p = 0.001$) compared to controls. Samples were tested by RT-PCR (tab.4). In cigarette smoke condensate (CSC)-treated NHBEC, DAPK mRNA increased significantly ($p = 0.002$) compared to controls (Tab.4).

In cigarette smoke condensate (CSC)-treated NHBEC, DAPK mRNA increased significantly ($p = 0.002$) compared to controls. Wethkamp et al., (2006) also demonstrated that hypermethylation in renal cell carcinoma may not downregulate the DAPK mRNA expression during tumour progression. (Ivanovska et al., 2014) showed that DAPK overexpression is associated with aggressiveness of tumours and an unfavourable outcome. This interesting finding of DAPK overexpression as a tumour suppressor gene has to be further evaluated. More genes show an inverse correlation between DNA methylation and gene transcription in both normal and malignant cells, illustrating that DNA methylation is only one possible regulator of gene expression (Nakayama et al., 1998). After exposure to CSC mRNA of the p16 gene was down-regulated significantly in NHBEC ($p = 0.022$), and in PLC ($p = 0.002$) as well. These results show that in our study hypermethylation of the promoter region of the p16 gene could not block its mRNA expression completely in NHBEC or PLC. These findings are in contrast to (Fujiwara et al., 2008) who suggested that p16 promoter methylation suppresses p16 gene expression in oesophageal squamous cell carcinomas (ESCC). Also (Foster et al, 1998; Huschtscha et al., 1998) showed that epithelial cells in culture lost expression of p16 by methylation of the corresponding CpG islands in the promoters. However, in-vitro long-term exposure of PLC and NHBEC to (5 µM) Cadmium showed neither effects on methylation status nor an increase of p16 mRNA expression compared to control cell cultures. The anti-apoptotic, antioxidant effect of metallothionein (MT)-I+II has resulted in an increased focus on their role in oncogenesis, tumour progression, therapy response and patient prognosis. Studies have reported an increased expression of MT-I+II mRNA and protein in various human cancers, such as breast, kidney and lung (Pedersena et al., 2008). Oxidative stress plays a crucial role in the development of age-related diseases including arthritis, diabetes, dementia, cancer, atherosclerosis, vascular diseases, obesity, osteoporosis, and metabolic syndromes (Tan et al, 2015; Liu et al., 2017).

With increasing age, the cumulative burden of oxidative stress damage is expected to grow. The overexpression of MT may mean pathological indications, like drug-resistance, as well as having an antioxidant effect and regulatory function in inflammation and apoptosis. Exposure to Cd (II) led to significant induction of MT1A-mRNA in the methylated samples in NHBEC ($p = 0.022$), as well as in PLC ($p = 0.006$). In CSC- treated NHBEC MT1A mRNA upregulated significantly, as well ($p = 0.002$). Whereas mRNA of MT1A after long term exposure to CSC showed no significantly induction. In this study, the methylation status of MT1A by Fisher's exact test showed no difference between chronic exposure to Cd (II) or CSC and methylation status for MT1A gene and cell types individually ($p = 0.705$) for normal human bronchial cells (NHBEC) versus NHBEC untreated cells , and ($p = 0.295$) for peripheral lung cells (PLC) versus PLC untreated cells.

6 Summary and Conclusions

Despite a better understanding of its molecular pathogenesis and advances in developing new treatment strategies, lung cancer is still one of the leading causes of cancer-related deaths worldwide. The development of treatments for lung cancer could be supported by a better understanding of genetic and epigenetic alterations in tumor suppressor genes. Moreover, knowledge about the epigenetic silencing process is of fundamental importance for elucidation of the etiology of lung cancer. We investigated the role of tumor suppressor genes and the detoxification protein MT1A in tumors, adjacent normal lung, and bronchial tissues. In further experiments we tried to specify the role of risk factors in epigenetic silencing of these genes in relation to the etiology of lung cancer through the use of cell cultures exposed to cigarette smoke condensate (CSC) and Cd (II) for a long time. The following paragraphs summarize the most important results.

1) The Role of DNA hypermethylation of Rassf1a, DAPK, p16, and MT1A in normal and tumor tissues of lung cancer patients

In search of biomarkers for early lung cancer detection and cancer risk assessment, we investigated frequencies of inactivation by aberrant CpG island methylation for several tumor-associated genes like Rassf1a, DAPK, p16 and the detoxification protein metallothionein IA. Samples were taken from 26 lung cancer patients and included tumours, corresponding adjacent normal lung and bronchial tissue located at least 2 cm away from the tumour site. Genomic DNA from tumor and paired normal tissues was extracted, bisulfite modification was done and then methylation-specific PCR was performed. The frequencies of methylation over all samples were: 61.5% for Rassf1a, 46.2 % for DAPK, 53.8% for p16 and 38.5% for Metallothionein IA. In more detail, these were the most important results that we got in the screening of the tumor-associated genes Rassf1A, DAPK, p16 and the stress gene MT1A in tissue specimens of lung cancer patients:

1) First, we checked for a possible association between tissue types - bronchus, lung, both healthy tissues regarded together and tumor and methylation status of the candidate genes. Rassf1a showed no association, whereas MT1A ($p = 0.007$), DAPK ($p < 0.001$) and p16 ($p = 0.005$) showed significant associations. On the other hand, we checked for general differences in methylation status of candidate genes between tumor and healthy tissues over all samples on a patient specific level. The results showed significant associations for DAPK ($p < 0.001$), p16 ($p = 0.005$) and MTIA ($p = 0.007$), whereas Rassf1a showed no association, again.

2) Inhalation is one of the major routes of human exposure to carcinogenic agents. In addition to environmental background exposure sources of carcinogens are lifestyle factors such as occupational exposure and smoking, which are important in the etiology of NSCLC. In patients who had occupational exposure to metals (working as locksmith, mechanic, welder, plumber), We found a significant association ($p = 0.03$) in healthy tissue [Lung and Bronchus regarded together (LuBr)] for methylation of the p16 gene (Tab. 11).

3) The lifestyle factors cigarette smoking is strongly related to lung cancer. Smoking patients in our study showed significant differences in the methylation status of DAPK ($p = 0.00004$), p16 ($p = 0.01522$) and MTIA ($p = 0.0345$). As chronic exposure of humans to cigarette smoke is not uncommon, therefore it also was a pivotal step in our further studies to investigate the effect of prolonged exposure to CSC on tumor suppressor gene silencing in primary cultures of human lung cells.

4) Chemotherapy is the main treatment for small cell lung cancer that has started to spread beyond the lungs. In patients with chemotherapy, we found a significant association in the Rassf1a gene in the lung ($p = 0.042$) regarded individually, and in healthy tissue lung and bronchial regarded together ($p = 0.042$) when each gene and each of the three tissue types individually were tested. Moreover, there was a significant association for DAPK ($p = 0.042$) in bronchial tissue.

5) Patients younger than the median age also showed significant associations for MT1A ($p = 0.04762$) and p16 ($p = 0.00794$) in tumor tissue when healthy tissue was methylated as well and vice versa. Nevertheless, these results should be evaluated further as they originate from as small number of patients.

II. Role of cigarette smoke condensate and Cd(II) in epigenetic silencing of the candidate genes in normal human bronchial epithelial and peripheral lung cell cultures

Smoking and some environmental carcinogens are associated with neoplasia in the proximal and distal regions of the respiratory tract. The current study is aimed to determine whether long term exposure to cigarette smoke condensate (CSC) and cadmium Cd (II) could trigger aberrant promoter methylation and silence specific genes (Rassf1a, DAPK, p16, MT1A) in primary cultures of normal human bronchial epithelial cells (NHBEC) and peripheral lung cells (PLC). In conclusion, these were the most important results that we got after long term exposure of NHBEC and PLC cultures to 5 µM Cd and 10 mg/l CSC [14 weeks in NHBEC, and 11 weeks in PLC]:

1) In this study, we have obtained evidence that Cadmium (II) significantly induces aberrant methylation of the tumour suppressor gene DAPK in PLC ($p = 0.019$), in NHBEC ($p = 0.097$).

2) Long term exposure to CSC led to significant induction of p16-methylation in PLC ($p = 0.047$). Consequently, it also led to down-regulation of p16 gene transcription in the methylated PLC samples [($p = 0.002$) 85 %] and in in methylated NHBEC samples [($p = 0.022$); 70%].

3) On the other hand, long term exposure to CSC led to a significant upregulation of the mRNA levels of DAPK and MT1A. In cigarette smoke condensate (CSC)- treated NHBEC, DAPK mRNA was up-regulated (120%) significantly ($p = 0.002$). DAPK mRNA was up-regulated (181%) significantly ($p < 0.001$) in methylated NHBEC samples after long-term exposure to 5 µM Cd(II) , and in the methylated PLC samples it was increased 159% ($p = 0.001$) compared to controls (Tab.5). The mRNA of the MT1A gene showed high expression levels in NHBEC (181%) significantly ($p = 0.022$), and in PLC mRNA was (239%) significantly ($p = 0.006$) after long term exposure to Cd (II) compared to controls. In cigarette smoke condensate (CSC)-treated NHBEC cells, MT1A mRNA was also increased (151%) significantly ($p = 0.002$) compared to controls. We therefore suggest that the long exposure to Cd (II) and CSC could induce the tumor suppressor genes DAPK and MT1A despite of the methylation status, this is may be caused by the partial methylation of these genes.

4) Human lung cell cultures as in-vitro models enable a long term follow up in studies on the effects of environmental carcinogens. PLC and NHBEC were shown to be suitable models to study responses to inhalation of repeated low doses of cadmium and cigarette smoke condensate and can help to elucidate the contribution of DNA-methylation of certain genes to mechanisms of tumor development in the lung.

7 References

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Theses

1) A total of 24 out of 26 samples (92%) taken from lung cancer patients displayed CpG island hypermethylation in at least one of the genes tested (Rassf1a, DAPK, p16 or (MTIA)) in one tissue type (tumor, corresponding normal lung and bronchial tissue). 8% (2/26) have no methylation status.

2) Rassf1a promoter methylation was detectable in 62% (16/26) of the patients. A high methylation ratio of 69% (11/16) was found for Rassf1a in tumor tissue. Methylation frequency of DAPK across tissue types was 12 out of 26 cases (46.2%). This study showed methylation of p16 in 54% or 14 out of 26 cases. Methylation of MT1A was observed in 10 patients (39%).

3) When healthy tissues and tumors were compared on a patient specific level. Rassf1a showed no association, whereas MT1A ($p = 0.007$), DAPK ($p < 0.001$) and p16 ($p = 0.005$) in the healthy tissues and tumors showed significant associations.

4) A significant association ($p = 0.03$) for the p16 gene was found in healthy tissue in patients who had occupational exposure to metals (working as locksmith, mechanic, welder, plumber).

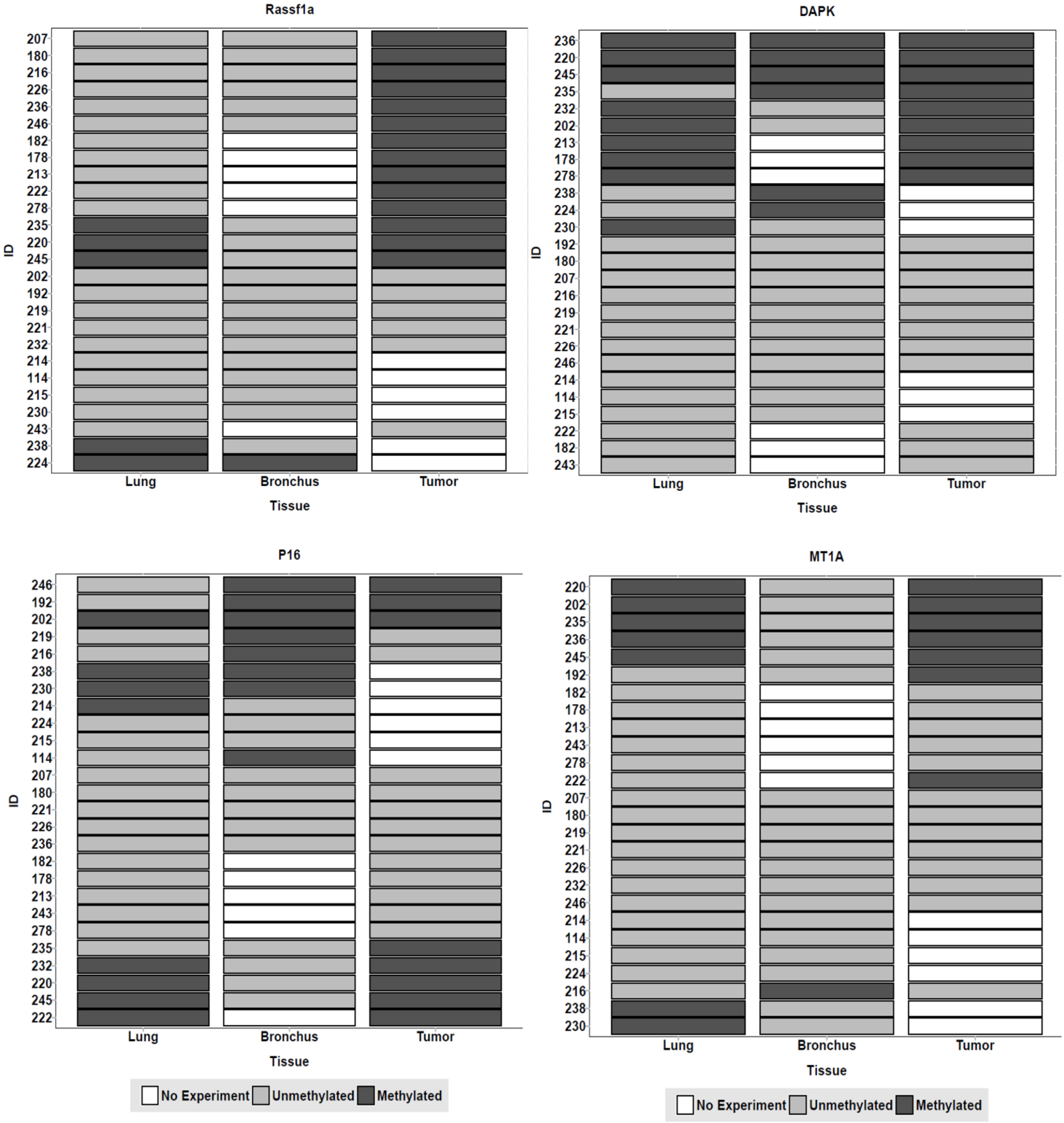
5) Chemotherapy had a significant influence on methylation of p16 ($p = 0.01786$). Patients with chronic diseases showed a significant correlation for methylation of DAPK ($p = 0.00058$) and p16 ($p = 0.00466$).

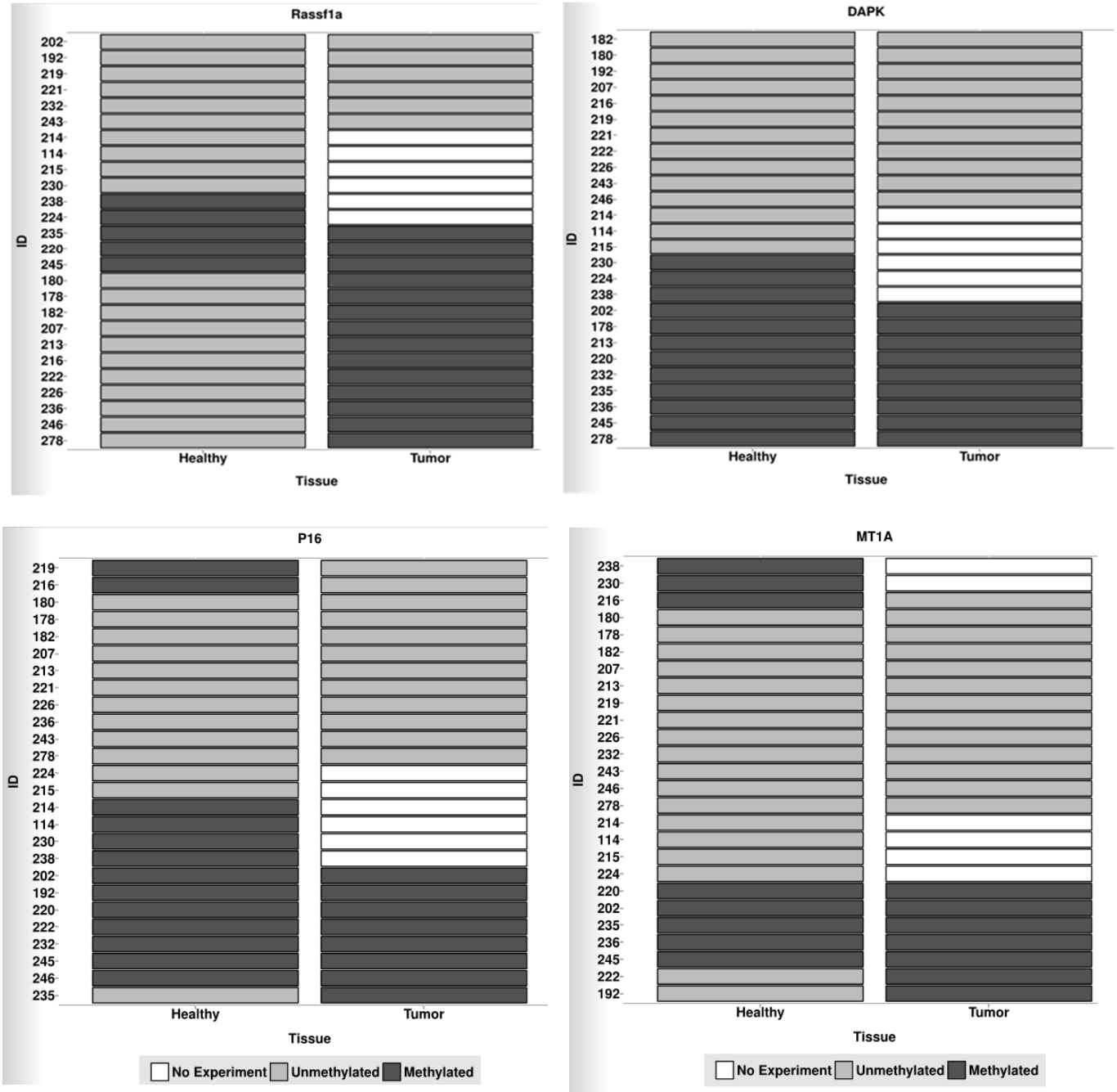
6) Explants of normal human bronchial epithelial cells (NHBEC) and peripheral lung cells (PLC) from samples of 12 lung cancer patients can be cultured over a time period of up to five generations, which equals 12 weeks. Cell monolayers of explant cultures can be passaged over at least 5 passages and keep normal morphology of epithelial cells over that time determined by general histological techniques.

7) When NHBEC and PLC are exposed to CSC (10 mg/l) or Cd (II) (5µM) [14 weeks in NHBEC, and 11 weeks in PLC] Cadmium (II) significantly induces aberrant methylation of the tumor suppressor gene DAPK in PLC ($p = 0.019$) and in NHBEC ($p = 0.097$).

8) CSC leads to significant methylation of p16 in (PLC) ($p = 0.047$). On other hand long term exposure to CSC leads to down-regulation of p16 gene expression to 70% in the methylated NHBEC samples of p16 ($p = 0.022$), and to 85% in methylated PLC samples of p16 ($p = 0.002$).

Tab..3. Frequency methylation of Rassf1a, DAPK, p16, MT1A genes in lung cancer patients





Dark squares represent signals in methylation-specific PCR. Grey squares represent no signal in the methylation-specific PCR. White squares represent no tissue was available. M = MSP using specific primer for methylation. U = MSP using specific primer for unmethylated. Healthy = normal lung and tumor tissues, (n =3).

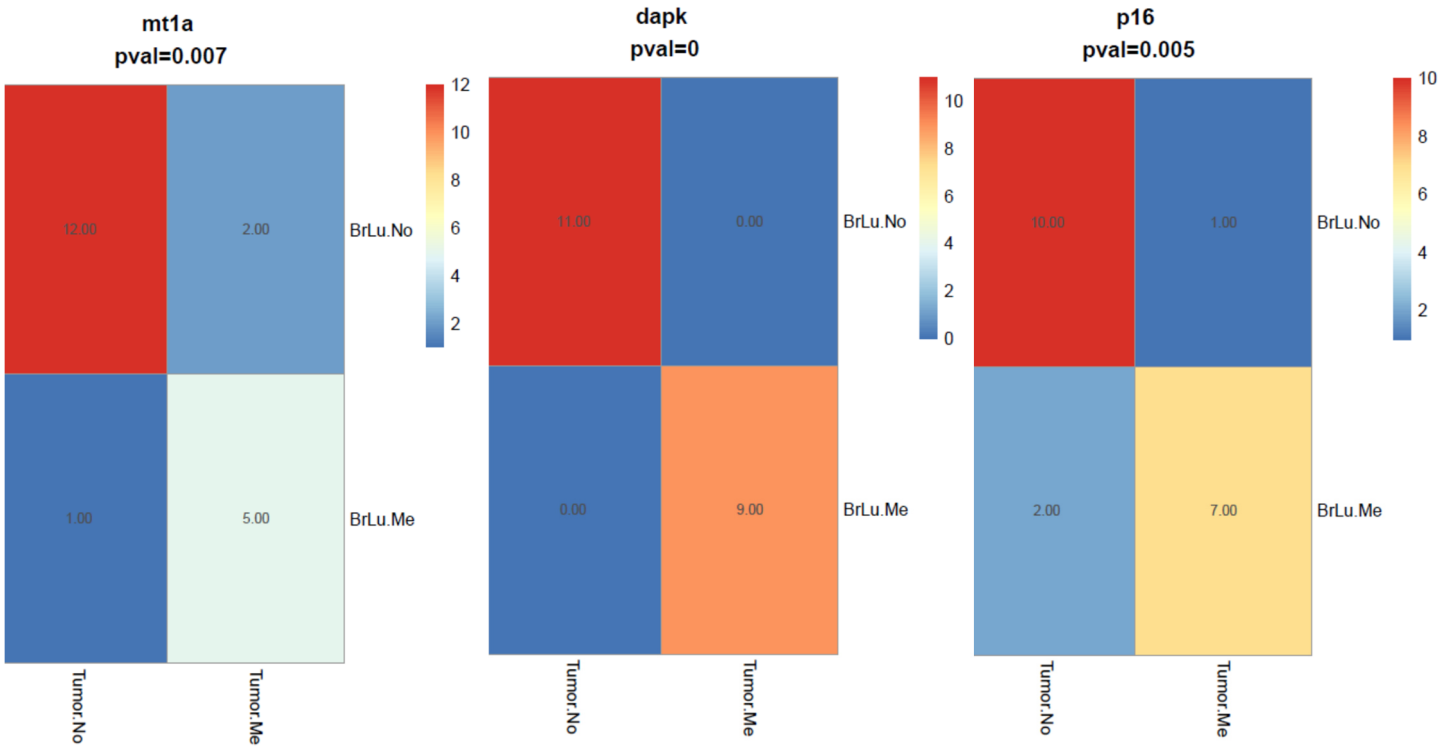


Fig.11. Methylation frequency of MT1A, DAPK, p16 genes and tissue types. Healthy tissues (Lung & Bronchial tissues) (LuBr) vs. tumor tissue were compared on a patient specific level, if lung and bronchus of the same patient were both methylated it was counted single, using Fisher's exact test.

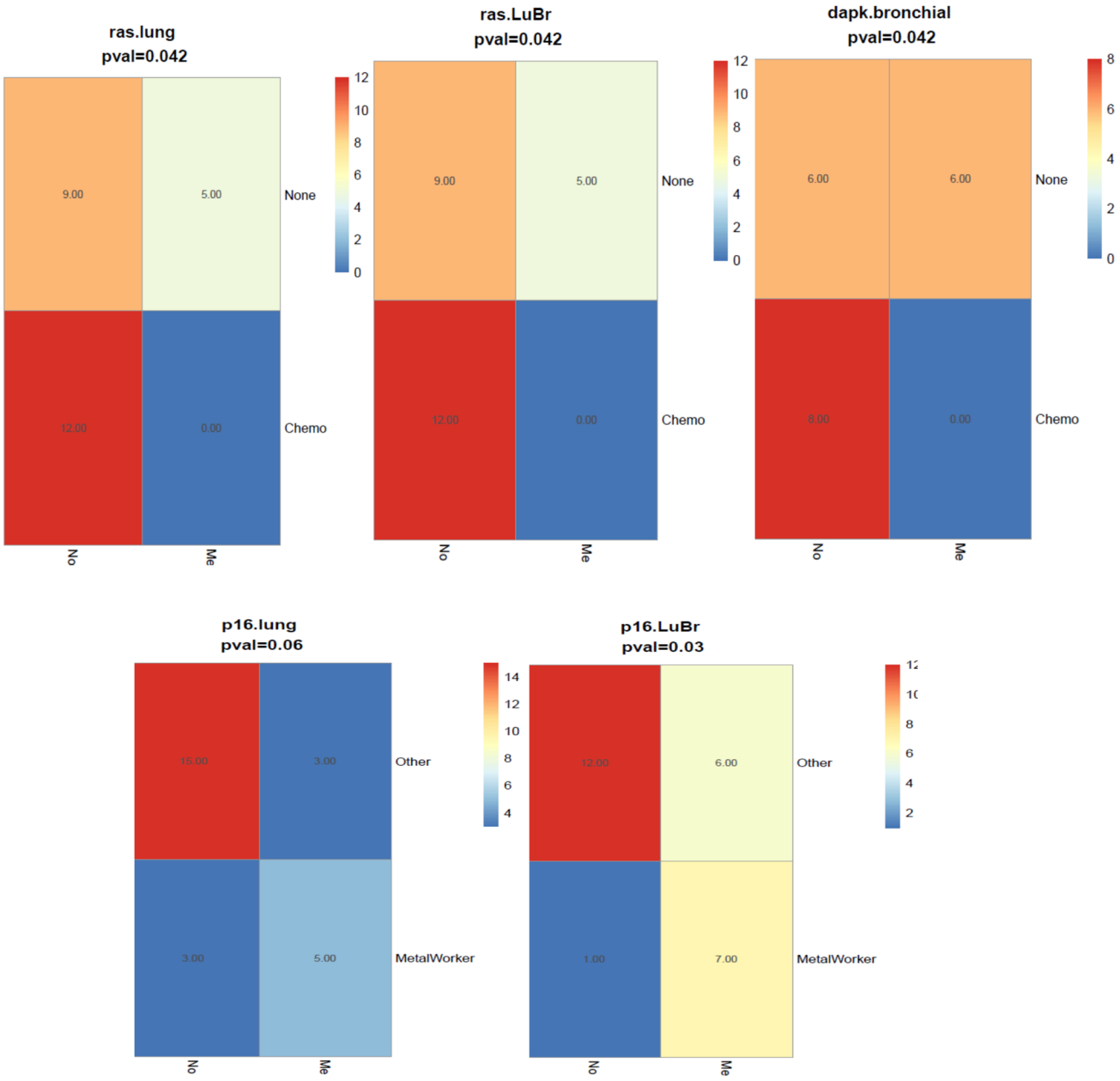
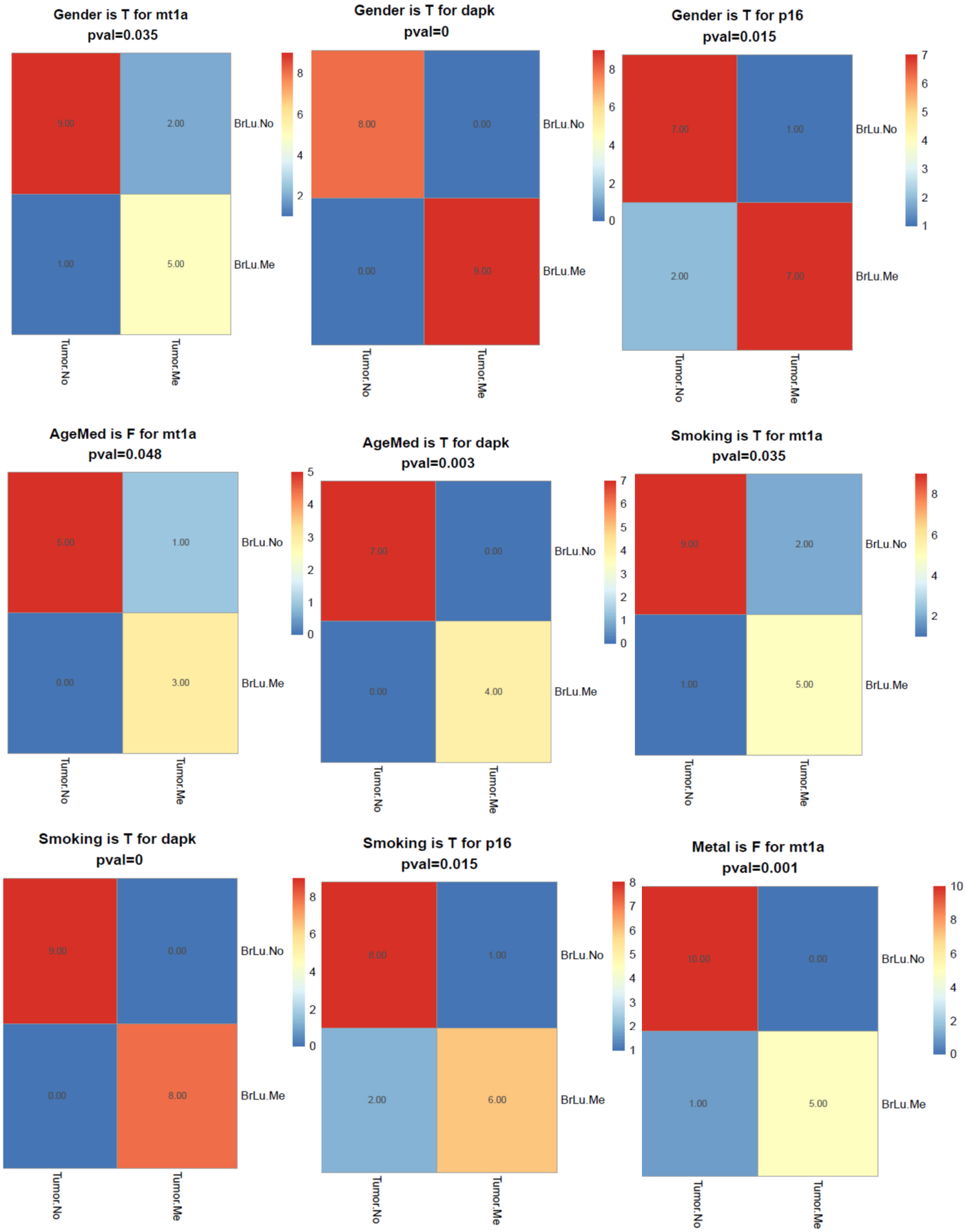


Fig.12. The significant methylation frequencies of Rassf1a, DAPK, p16 genes of healthy tissues in human lung cancer patients treated with chemotherapy, and worked with metals as shown in Tab.8 & Tab.11.

(Fig.13)


Gender is T for mt1a
pval=0.035
9.00
2.00
1.00
5.00
BrLu.No
BrLu.Me
Tumor.No
Tumor.Me
Gender is T for dapk
pval=0
8.00
0.00
0.00
9.00
Gender is T for p16
pval=0.015
7.00
1.00
2.00
7.00
AgeMed is F for mt1a
pval=0.048
5.00
1.00
0.00
3.00
AgeMed is T for dapk
pval=0.003
7.00
0.00
0.00
4.00
Smoking is T for mt1a
pval=0.035
9.00
2.00
1.00
5.00
Smoking is T for dapk
pval=0
9.00
0.00
0.00
8.00
Smoking is T for p16
pval=0.015
8.00
1.00
2.00
6.00
Metal is F for mt1a
pval=0.001
10.00
0.00
1.00
5.00

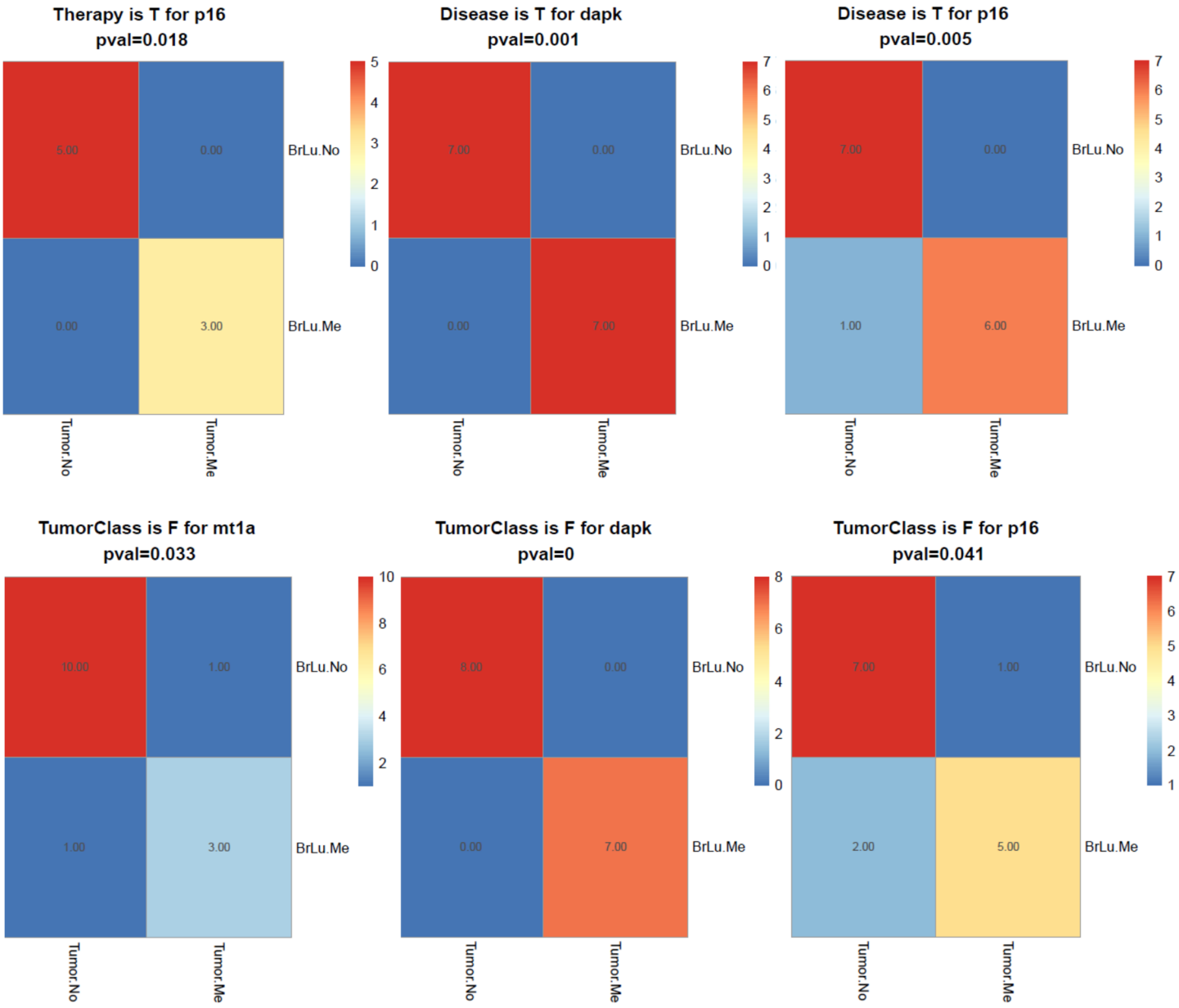


Fig.13. The significant methylation frequencies of Rassf1a, DAPK, p16, MT1A genes in healthy tissues (bronchial & lung).vs tumor tissue. Related to the study group description as shown in Tab.12. Gender: T= male, Age T= > 64. F = < 64, Smoking: T = yes. F= no, Metal exposured: T= yes. F= no, Chemotherapy: T= yes. F= no, Chronic disease: T= yes. F= no, Tumor class: T= T3 T1. F= T2.

Selbständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe Dritter angefertigt habe. Daten die aus anderen Quellen direkt oder indirekt übernommen wurden, sind unter Angabe der Quelle gekennzeichnet.

Henda Gheit

Erklärung über Promotionsversuche

Hiermit erkläre ich, dass ich bisher keine früheren Promotionsversuche mit dieser oder einer anderen Dissertation unternommen habe.

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