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„Epigenetic changes of p16, IL-6 and LINE-1 in buccal cells after exposure to particulate matter“

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3 List of abbreviations

5-hmC	…	5-hydroxymethylcytosine
ADP	…	Adenosindiphosphat
ANOVA	…	analysis of variance
ARF	…	alternate open reading frame
BED	…	biologically effective dose
BMI	…	Body mass index
Bp	…	base pairs
C	…	control group
CpG	…	cytidine-phosphate-guanosine
DNA	…	deoxyribonucleic acid
DNMT	…	DNA methyltransferase
dNTPs	…	deoxynucleoside triphosphates
G1	…	Gap 1
GIT	…	gastrointestinal tract
gp130	…	glycoprotein 130
HAT	…	histone acetyltransferase
HDAC	…	histone deacetylase
HW	…	high exposed welders
IL	…	Interleukin
IL-6Rα	…	IL-6 receptor α
INF	…	Interferon
INK4	…	inhibitors of CDK4 kinase
JAK	…	Janus kinase
LINE-1	…	long interspersed nuclear element
LW	…	Low exposed welders
MAPK	…	mitogen activated protein kinase
MBD	…	methyl binding domain
MAG	…	metal active gas
mRNA	…	messenger RNA
miRNA	…	microRNA

NK cells	…	natural killer cells
NF- κB	…	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NKT cells	…	natural killer T cells
NSCLC	…	non-small cell lung carcinoma
ORF	…	open reading frame
PM	…	ambient particulate materials
Rb	…	retinoblastoma protein
RNA	…	ribonucleic acid
ROS	…	reactive oxygen species
SAM	…	S-Adenosylmethionin
SD	…	standard deviation
sIL-6Rα	…	soluble IL-6 receptor α
SINE	…	short interspersed nuclear element
STAT	…	signal transducer and activators of transcription
TET	…	ten-eleven translocation
TIG	…	tungsten inert gas
TNF	…	tumour necrosis factor

4 Zusammenfassung

Umweltgifte können in der Promoterregion von Genen zu epigenetischen Veränderungen in der Methylierung sowie zu Kernanomalien führen, wie zum Beispiel Studien über Emissionen aus dem Straßenverkehr zeigen. Die Frage, wie verschiedene Substanzen aus der Umwelt wie Feinstaub die epigenetischen Prozesse auf zellulärer Ebene als auch in Bezug auf komplexe Krankheiten, insbesondere Krebs, beeinflussen, ist von steigendem Intersse.

In dieser Studie wurden die Auswirkungen auf die DNA-Methylierung durch den beim Schweißen produzierten Rauch in Promoter CpGs untersucht. Folgende Gene wurden hierbei analysiert: IL-6, eines der am besten charakterisierten kanzerogenen Cytokine, das Tumor-Suppressor-Gen p16, sowie die in der DNA oft wiederkehrende LINE-1 Sequenz. Diese drei Gene spielen eine wichtige Rolle bei der Krebsentstehung und werden durch epigenetische Prozesse reguliert. In dieser Arbeit wurde die DNA von Schweißern aus Abstrichen der Mundschleimhaut entnommen und mit Hilfe von Bisulfitumwandlung sowie Pyrosequenzierung analysiert.

Für IL-6 zeigte sich eine Methylierung zwischen 1 und 10 %. Dieser Messbereich konnte bereits in anderen Studien beobachtet werden und zeigt eine allgemeine Abnahme der IL-6-Promoter-Methylierung bei den Schweißern. Es konnte eine signifikante Abnahme der Methylierung in einem CpG beim Vergleich der Schweißer mit der Kontrollgruppe beobachtet werden, was mit einer erhöhten Entzündungsreaktion in den Mundschleimhautzellen korrelieren könnte. Die durchschnittliche Methylierung für p16 fand sich im Bereich von 10 %. CpG 1 und 2 der Schweißer zeigten eine erhöhte Methylierung, wie bereits in anderen Studien beobachtet. Da p16 eine Schlüsselrolle bei der Regulation des Zellzyklus einnimmt, führt eine Hypermethylierung der Promoter-Region in diesem Gen zu einer Stilllegung des Gens und ist demzufolge mit Tumorwachstum assoziiert. Die Werte von LINE-1 lagen in allen Gruppen im gleichen Bereich. Dies unterstützt die Rechtfertigung der Verwendung von LINE-1 als Marker für die globale DNA-Methylierung. Insbesondere die Hypermethylierung der Promoterregion von p16 könnte ein Biomarker für die Früherkennung bei Veränderungen des Zellwachstums sein, da diese frühzeitig in der Krebsentstehung auftritt.

Diese Ergebnisse geben Hinweise, dass Änderungen in der DNA-Methylierung durch Umweltgifte verursacht werden können. Epigenetische Veränderungen sollten daher weiter untersucht werden, um die damit verbundenen Krankheiten frühzeitig erkennen zu können.

5 Summary

Environmental toxins such as from traffic pollution have been shown to change epigenetic methylation in the promoter region of genes and to induce nuclear anomalies. There is growing interest on how environmental substances, such as particulate matter, can influence epigenetic processes on cellular level and in regard to complex diseases, including cancer.

The study investigated effects from welding fumes on epigenetic methylation in promoter CpGs of IL-6, one of the best-characterized pro-tumourigenic cytokines, in the tumour suppressor gene p16 and in the repetitive DNA sequences LINE-1. These three genes play an important role in carcinogenesis and are regulated by epigenetic processes. DNA of buccal cells of welders were analysed using DNA bisulfite conversion and pyrosequencing.

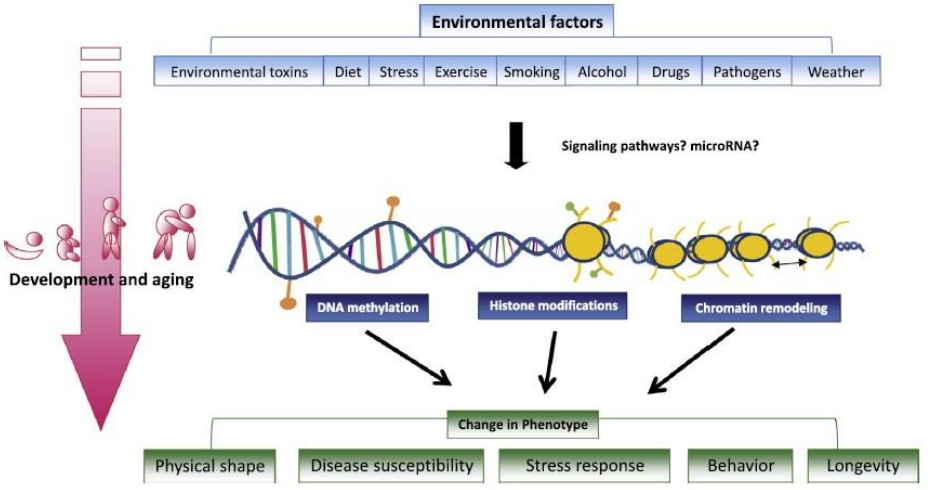
For IL-6 a methylation rate between 1 and 10 % was measured, this range was already observed in other studies. Hereby a general decrease of methylation could be shown for welders in the IL-6 promoter. Comparing welders with the control group a significant decrease was observed in one of the CpGs. This may correlate with increased inflammatory responses in buccal cells. Average methylation for p16 was about 10 %. The CpG 1 and 2 of welders showed an increased methylation as already before observed in other studies. As p16 plays a key role in the regulation of the cell cycle, hypermethylation of this gene in the promoter region is an important mechanism of gene silencing and therefore associated with tumour growth. LINE-1 values were in the same range in all groups. This supports the justification of using LINE-1 as a marker for global DNA methylation. Especially hypermethylation of the promoter region of p16 could be a potential biomarker for early diagnosis of alteration in cell growth, for as it occurs as an early event in cancer.

These results add evidence that modification in DNA methylation caused by environmental toxins should be further investigated and associated diseases can be early detected.

6 Introduction

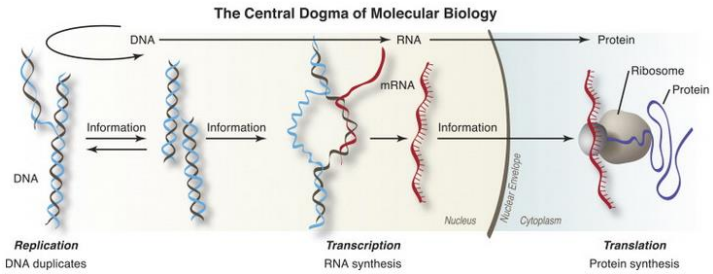
The term “epigenetics” was coined in 1942 by Conrad Hal Waddington who linked the two fields of epigenesis and genetics to describe how genes and their products interact to produce the diversity of phenotypes. Since then, the definition remains controversial but the most commonly used term describes heritable changes in gene expression that do not involve alterations in DNA sequence [Aliberti and Barile, 2014; Tallen and Riabowol, 2014]. Epigenetic can be grouped into DNA methylation, covalent histone modification, chromatin remodelling and microRNAs (miRNAs). Although these are discrete mechanism they interact closely in order to regulate gene expression [Liloglou et al., 2014; Aliberti and Barile, 2014]. Different environmental factors such as toxins, diet, stress, smoking, alcohol or pathogens can influence the epigenetic process throughout lifetime. Epigenetic changes can result in an alteration of gene expression, which is associated with different downstream effects including changes in disease risk, stress response and metabolism [Tammen et al., 2013].

Figure 1: Influence on phenotypes by epigenetic mechanism [Tammen et al., 2013]



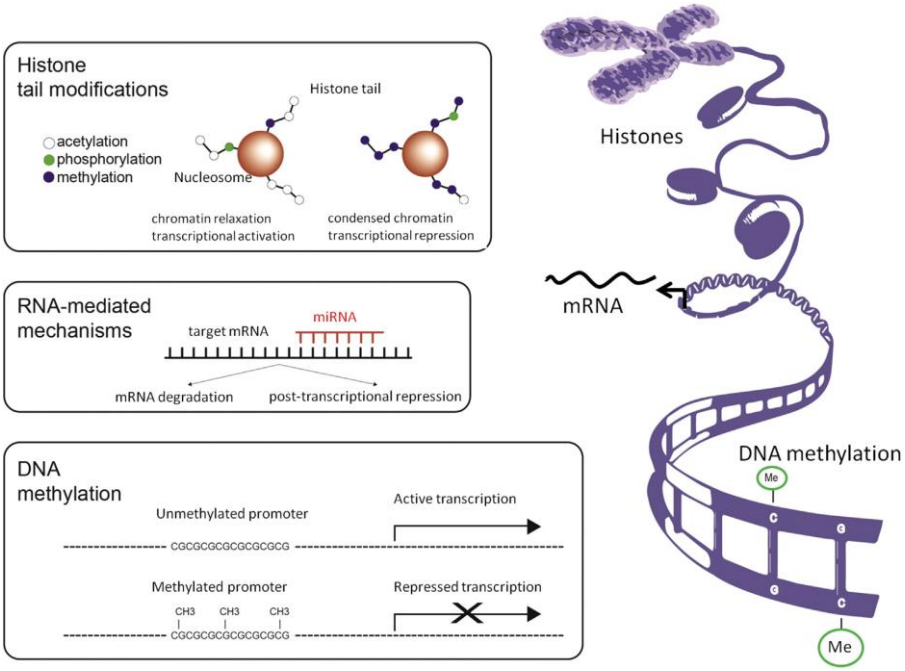
There are tree main steps of genetetic information starting from DNA going to RNA and to protein. The expression of proteins can be controlled through transcription and translation, these are key mechanism of gene expression. The processes are mediated by DNA and RNA, whereby DNA information is copied into messenger RNA (mRNA) through transcription process. The builded mRNA synthesizes proteins through translation processs [Alberini and Klann, 2014].

Figure 2: Protein building and gene expression [Alberini and Klann, 2014]



Gene transcription can be affectively altered through epigenetic modification in response to environmental factors [Tammen et al., 2014]

Figure 3: Overview of the main epigenetic mechanism [Stoccoro et al., 2013]

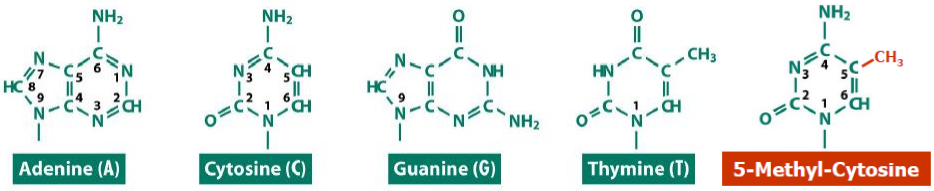


6.1 DNA methylation

DNA methylation is currently the most studied form of epigenetic programming and describes the covalent addition of a methyl group to the cytosine of CpG dinucleotides, more precisely a cytosine-5'phosphodiester bonded to guanine, also known as cytidine-phosphate-guanosine [Liloglou et al., 2014]. 5-methylcytosine results as a product of methylation of the 5' position of the pyrimidine ring of cytosine within the genome by the enzymatic family of DNA methyltransferases (DNMTs). The cofactor

S-Adenosylmethionin (SAM), a modified amino acid which is produced in the folate metabolism, donates the methyl group in this reaction why DNA methylation reactions depend on SAM availability. The produced 5'methylcytosine, often called the "fifth-base", is capable to pair with guanine like cytosine, without compromising DNA integrity [How Kit et al., 2012; Stoccoro et al., 2013; Tammen et al., 2013].

Figure 4: The five bases in DNA [© Garland Science, 2007]



Maintenance and *de novo* methylation are two different types of DNA methylation, which are now widely accepted. During cellular replication patterns of DNA methylation are passed on from the parental strand to the daughter cells. Maintenance methylation involves the conservation of existing methylation patterns during DNA replication and ensures programmed DNA methylation patterns to remain through cellular generation. De novo methylation occurs on new established patterns of DNA methylation and is producing new DNA methylation marks [Tammen et al., 2013; Aliberti and Barile 2014]. As mentioned before DNMTs catalyse the transfer of a methyl group from SAM to cytosine thereby forming 5-methyl-cytosine. DNMT can be distinguished in DNMT1 which is primarily involved in the maintenance of DNA methylation patterns during development and cell division and DNMT3a and DNMT3b. The latter are the de novo methyltransferases which establish DNA methylation patterns during early development. These two might also play a role in the maintenance of DNA methylation by correcting errors left by DNMT1 [Stoccoro et al., 2013].

DNA methylation is also the most prevalent genetically programmed DNA modification in mammals. 5-methylcytosine accounts for ~1 % of the cytosine bases within a human genome and varies slightly depending on cell type. Almost all DNA methylation occurs within CpG dinucleotides. The human genome contains about 30 million CpG dinucleotides that exist in a methylated or unmethylated state, whereby the majority (75 %) of CpG dinucleotides throughout mammalian genomes is methylated. Regions

rich in CpG dinucleotides are called CpG islands and occur throughout the genome. Especially methylation of CpG islands in the promoter region of a gene is usually inversely associated with transcription of that gene and can block the transcription [How Kit et al., 2012; Tammen et al., 2013].

DNA methylation can affect the molecular function in further various ways. The gene expression can be affected either by directly inhibiting the binding of transcription factors or indirectly through the recruitment of MBD (methyl binding domain) proteins and other chromatin factors. Consequently this will induce a closed state of the chromatin (heterochromatin) and abolish transcription. DNA methylation has been found to implicate X-chromosome inactivation, silencing of repetitive elements, genomic imprinting as well as disease processes, particularly tumour genesis which is probably the best studied disease with a strong epigenetic component. In tumour both phenomena can be observed, a global decrease in DNA methylation (hypomethylation) linked with transcriptionally activation of the genome and gene-specific increase of methylation (hypermethylation) in the normally unmethylated promoter-associated CpG islands, which is often associated with transcriptional silencing of the associated genes [How Kit et al., 2012; Tallen and Riabowol, 2014]. A loss of the physiological balance, which can be achieved due to imbalances in genes determining the phenotype of different cells which are permanently turned on, while others are permanently turned off, often results in cancer. This is linked with aberrant transcription of growth regulatory genes and dysregulated signalling pathways which are indicators of cancer [Tallen and Riabowol, 2014].

DNA methylation is a stable, highly conserved, non-mutational modification and therefore – at least in principle – reversible [Aliberti and Barile, 2014; How Kit et al., 2012]. DNA demethylation can be promoted by converting 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC) via the 5-methylcytosine hydroxylase enzyme TET (ten-eleven translocation), proposed as a process of removing the methyl group from cytosine bases and returning the cytosine to its unmodified form [Stoccoro et al., 2013; Tammen et al., 2013]. Due to the potential regulatory role in gene transcription, 5-hmc is also called the “sixth base” [Tammen et al., 2013]. DNA demethylation processes are

currently a growing topic in epigenetic research but further investigations are required [Stoccoro et al., 2013; Tammen et al., 2013].

Figure 5: Proposed mechanism of active demethylation through hydroxymethylation by TET [Tammen et al., 2013]

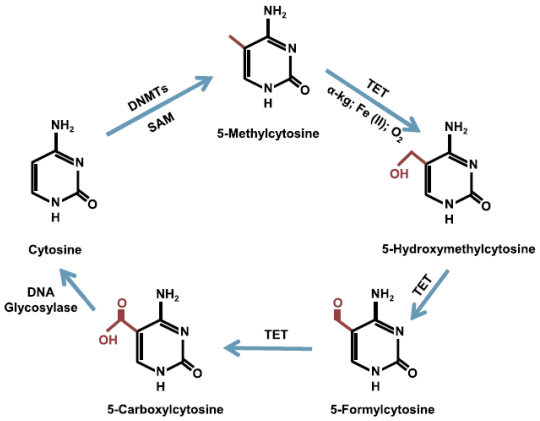
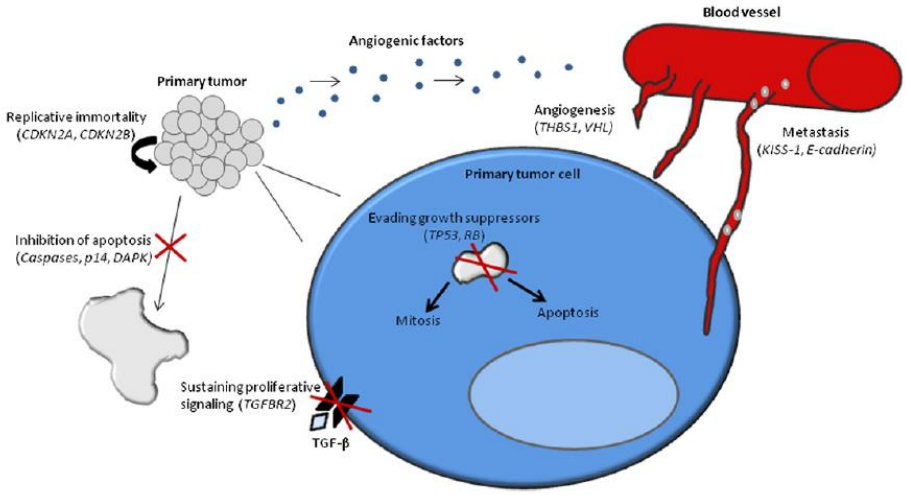


Figure 6: Impact of epigenetic silencing as a hallmark of cancer [How Kit et al., 2012]



There are several checkpoints to ensure proper cell division in normal cells and an error of any of these essential pathways is linked with an increased risk of a transformation into a cancer cell. Therefore it comes to a change of the phenotype if any of the tumour suppressor genes is not capable to fulfil its normal role in the cell. The CDKN2A, also known as p16 is given here as an example [Figure 6]. The cancer cell has not only the ability to inhibit apoptosis it also initiate angiogenesis, evades from growth suppressors, sustains proliferative signalling and may even metastasize. Genes that may be

inactivated in different pathways due to epigenetic processes are put in brackets [How Kit et al., 2012].

6.2 Histone modification

Histones are approximately 147 base pairs, which are weaving around proteins to compact the DNA and forming a DNA-protein complex, known as a nucleosome. There are four core histones, namely H2A, H2B, H3 and H4, whereby each nucleosome consists of an octamer of two copies of these. Changes in epigenetic caused by histone modification can result due to various post-translational modifications such as histone acetylation, methylation, phosphorylation, ubiquitination, ADP-ribosylation and biotinylation on the n-terminal histone tails which is linked with an altered regulation of transcription [Tammen et al., 2013]. These posttranslational modifications influence DNA structure and activity due to different interaction between histones, DNA and other proteins [Aliberti and Barile, 2014].

Chromatin of eukaryotic cells can be grouped in heterochromatin and euchromatin. Heterochromatin is condensed and the closed form of chromatin which is transcriptionally silent compared to the opened, less condensed euchromatin, which allows active and silent gene transcription and is therefore important in DNA repair and replication. Low levels of histone acetylation and high levels of histone methylation are commonly in heterochromatin. The active transcription of euchromatin is associated with high levels of histone acetylation and tri-methylation whereas the transcriptionally inactive form of euchromatin indicates low levels of histone acetylation, methylation and phosphorylation [Aliberti and Barile, 2014].

On the contrary histone acetylation changes either the ionic charge of the histone tail or is functioning as a binding platform for other proteins. Acetyl groups are transferred onto the amino group of a lysine via the enzyme histone acetyltransferase (HATs) and leads to a neutralisation of the charge of lysine. This results in a weakened interaction between the histone tail and DNA. Contrary to this reaction acetyl groups can be removed from lysines and restore the positive charge via histone deacetylase (HDACs). HATs act generally as transcriptional activators and HDACs as transcriptional repressors. Phosphorylation acts like acetylation and changes the charge and ionic

properties of histones with changes in structure of the local chromatin environment [Tammen et al., 2013].

Histone methylation can occur on residues of the two amino acids lysine or arginine. The transcription can be activated or repressed by the methylation of lysine, depending on the site and the degree of methylation [Aliberti and Barile, 2014]. Activation of transcription is linked with opening of the chromatin structure which enables transcriptional enzymes and other factors to bind to the DNA [Vo and Millis, 2012]. Methylation on arginine is commonly linked with activation of transcription [Aliberti and Barile, 2014].

It is still uncertain, if histone modification can be reproduced by following DNA replication and transmitted from one cell generation to the next. DNA methylation in comparison is stably inherited between cell division [Tammen et al., 2013].

6.3 Chromatin remodelling

Regulation of gene transcription at the chromatin level can be modified if the flexible euchromatin is tightly packed into the nucleosome-rich heterochromatin. Resulting in gene silencing as regions of DNA can no longer be transcribed by transcription factors and RNA polymerases [Tammen et al., 2013].

6.4 MicroRNAs

MicroRNAs (miRNAs) are short, non-coding forms of RNAs with a length of about 20 to 30 nucleotides that control the expression of their target genes through influenced messenger RNA stability and translational rate [Liloglou et al., 2014; Tammen et al., 2013]. It is a relatively new area of epigenetic research and it is still not agreed if miRNA can be classified as an epigenetic phenomenon [Tammen et al., 2013]. Up to 30 % of human genes may be regulated by miRNA wherefore gene expression may be the main spot for epigenetic changes in this connection. In several types of cancer including lung cancer expression of specific miRNAs could be shown with tumour invasiveness and metastatic potential [Liloglou et al., 2014]. It could be also discovered that aberrant miRNA expression in cancer cells can be caused by changes in DNA methylation which supports the evidence of feedback between miRNA expression DNA

methylation and histone modification. These two can regulate the transcription of miRNA, and in return some miRNAs can alter the expression of DNA methyltransferases (DNMT3A and DNMT3B) [Tammen et al., 2013].

6.5 Cancer-Facts

Cancer is one of the leading cause of deaths worldwide, with 8.2 million deaths in 2012. Lung, liver, stomach, colorectal and breast cancer are those types of cancer causing the most cancer deaths each year. Lung cancer is on the top of the list with 1.59 million deaths worldwide of which 71 % are caused by tobacco use. Tobacco use is therefore the single most important risk factor for cancer with about 22 % of global cancer deaths.

The WHO recommends avoiding key risk factors such as tobacco use, urban air pollution and indoor smoke from household use of solid fuels, whereby more than 30 % of cancer deaths could be prevented.

In the year 2012, 14 million annual cancer cases were accounted and it is expected that the number will rise to 22 million within the next two decades [WHO, 2014].

One of the main problems is the lack of effective screening tools for early detection of lung cancer and it will be the biggest challenge in future establishing sensitive and reliable methods for early diagnosis of lung cancer. Alteration in normal DNA methylation patterns of cancer cells and hypermethylation in the promoter regions of tumour suppressor gene associated with an epigenetically mediated gene silencing are observed as common features in human carcinomas. A number of studies established that several genes, including p16 are hypermethylated in lung cancer cells, leading to the loss of gene expression [An et al., 2002].

6.6 DNA methylation as a biomarker

There is a great potential of using DNA methylation as a nucleic acid based biomarker for clinical implementation in cancer medicine and a number of recent studies highlighted the prospective utility of this biomarker in lung cancer prognosis and predicting response to therapy. DNA is the most stable biological macromolecule and the methyl groups on the cytosines are part of the covalent structure of the DNA which enables a relatively easy transformation from a research laboratory setting into routine

diagnostic. However, the fact that some epigenetic changes are due to environmental influences as well as accumulation of DNA methylation at some promoters during aging makes the use of these biomarkers also challenging. Biomarkers with sufficient sensitivity and specification are needed but so far no single gene has been found that is always methylated in a certain type of cancer. Therefore most likely panels of DNA methylation based biomarkers will be used in future [How Kit et al., 2012; Liloglou et al., 2014].

6.6.1 Best suited regions for DNA methylation based biomarkers

Changes in DNA methylation barely occur within regions that seem to be directly implicated in carcinogenesis like altered regulation of gene expression or influenced genomic stability. The main focus of research is the hypermethylation of promoter-associated CpG islands which is inversely correlated to their transcriptional activity. Nevertheless the presence of methylation does not necessarily induce silencing of nearby genes. A modification of expression is likely when a specific core region of the promoter, which is often the transcription start site, becomes hypermethylated. The methylation status at specific CpG dinucleotides in the core region, which can be in some cases as small as a single CpG dinucleotide, might therefore be a better marker for the expression of the gene than the overall methylation level of the entire CpG island. Even more vulnerable for aberrant methylation in various cancers are CpG islands which are found outside the promoter regions than methylation in the respective promoter sequences. However, a methylation of these CpG islands does not usually reduce transcription [How Kit et al., 2012].

6.6.2 Detection of DNA Methylation

There are three categories for the detection of DNA methylation, which are based on the principle of discrimination between 5'-methyl cytosine and cytosine:

Sodium bisulphite conversion

> The most widely used method consists of the chemical modification of genomic DNA with sodium bisulphite, inducing a conversion of cytosine, but not 5'methyl cytosine to uracil. Epigenetic modification can be effectively translated into sequence differences, which can be detected by standard methods.

Methylation-Specific Restriction Enzymes (MSRE)

With the use of specific restriction enzymes methylated and unmethylated DNA sequences can be identified. This is a simple and cost effective method, without the need of special instrumentation but with a limitation to specific restriction sites as only CpG sites found within these sequences can be analysed.

Chromatin immunoprecipitation (ChIP)

This method is using anti-5'methyl cytosine or methyl binding domain MBD complexes and is analysed on microarrays [How Kit et al., 2012; Liloglou et al., 2014].

6.7 Epigenetic and environmental chemicals

Most of the environmental factors such as nutrition or toxicants have no influence promoting genetic mutations or altering DNA sequences. Nevertheless, these factors have the capacity to alter the epigenome. There is growing interest how environmental chemicals can influence epigenetic processes on cellular level and in regard to complex diseases, including cancer [Stoccoro et al., 2012].

The definition of genotoxicity can be subdivided in primary genotoxicity which is directly related to the exposure of the 'substance' and in secondary genotoxicity. The latter is defined as a result of the 'substance' interacting with cells or tissues and releasing factors with the consequence of causing adverse effects such as inflammation and oxidative stress [Arora et al., 2012].

Nanoparticles are basically defined as particles around a threshold dimension of 100 nm [Donaldson and Poland, 2013]. According to the definition the term "nanoparticles" applies only to engineered particles (including metals oxides, carbon nanotubes etc.). Particles produced as by-products of other processes such as welding fumes, fire smoke, or carbon black under 100 nm do not fall under the term "nanoparticles" [Arora et al., 2012]. These small particles are referred as ambient particulate materials or simple, particulate matter (PM). It is a complex and heterogeneous mixture and can diversify in time and space. PM encompasses a variety of chemical components and physical characteristics and most of these substances have the potential to contribute toxicity. PM also include particulate pollution from both natural and man-made sources. The former implies wind-blown dust, sea salt pollens, fungal spores, oxidation of biogenic reactive gases, products of forest fire among others. Whereas man-made sources indicate fossil fuel combustion mainly produced by vehicles and power plants; industrial processes such as metal producing, construction work, cigarette smoke, and wood stove burning. The main source of PM in urban areas is from motor vehicles particularly from diesel exhaust, which produces more particles than gasoline engines [Kelly and Fussell, 2012]. It has been assumed that particles from traffic origin are the most toxic components and are associated with a variety of adverse health outcomes [Baccarelli et al., 2009]. A furthers subdivision can be made between primary and secondary particles. Primary particles are released directly from their source into the

atmosphere, mainly by combustion, whereas secondary particles are a result of chemical reactions and are subsequently formed within the atmosphere. An example therefore is the forming of sulphates and nitrates by the oxidation of sulphur dioxide. This chemical process is relatively slow and persists prolonged in the atmosphere compared to primary particles [Kelly and Fussell, 2012].

$PM_{2.5}$ can be defined as particles with a diameter of between 0.1 and 2.5 µm in combination with ultra-fine mode, the latter is mainly produced by combustion or formed by coagulation and condensation.

Ultrafine particles, a further subdivision of sizing, have a diameter of equal to or less 0.1 µm ($PM_{0.1}$) which are in addition by far the greatest number of particles. The main origin of these small particles arises from primary combustion emissions and gas-to-particle conversion processes. These small particles are characterized as unstable and may form into larger particles due to coagulation and condensation [Kelly and Fussell, 2012].

Due to the varying size of these particles ranging from a few nanometers to ten micrometers the behavior in the atmosphere and within the human respiratory system is not fully determined [Kelly and Fussell, 2012]. Also the different origins of these particles and the influence of emission sources and metrological conditions results in varying ambient level and composition, and makes it a complex mixture [Lu et al., 2014].

6.7.1 Intake into the human body

The intake of nanoparticles and PM into a living system can result in an uncountable number of interactions with the surrounding system regardless of size. Recently there is growing interest in the interaction of DNA and nanoparticles due to their molecular bindings and biochemical reactions. In particular how the nanoparticle-DNA binding could vary DNA molecular structure and its bioactivities [An and Jin, 2012].

The human body has three main routes of direct substance exchanges with the environment including skin, respiratory tract and gastrointestinal tract (GIT). Wherefore the respiratory system serves as a major portal for ambient particulate materials. More

and more attention is paid of the pathogenic effects and pathology of inhaled manufactured nanoparticles, especially $PM_{2.5}$ as this can reach the alveoli and also accumulate in the alveolar region. After absorption across the lung epithelium it comes to a distribution throughout the entire body, because the nanomaterial can enter the blood and lymph to reach cells in the bone marrow, lymph nodes, spleen and heart [Arora et al., 2012]. An association between inhaled ambient ultrafine particles and cardiovasculare events was verified by different studies and could be of great importance [Arora et al., 2012; Kile et al., 2013]. But also sensory nerve endings in the airway epithelial as well as ganglia and central nervous system via axon may be affected by the inhalation of ultrafine particles [Arora et al., 2012]. It is proposed that larger particles up to 10 µm will deposit primarily in the primary bronchi wherefore the nasopharynx will be the deposition for much larger particles of up to 100 µm. [Kelly and Fussell, 2012].

There are two ways for nanomaterials to reach the GIT, via clearance from the respiratory tract through the nasal region or direct ingestion such in food, water and drugs but there are only limited studies of toxic effects of nanomaterials of post oral ingestion [Arora et al., 2012].

The skin is the largest primary organ for protection in our body and has direct contact with many toxic agents. One of the most important sources of nanoparticles in conjunction with harmful health effects are cosmetic products. Particularly nanocrystalline magnesium oxide and titanium dioxide (TiO_2) are of great importance and are under suspicion to cause e.g. cancer [Arora et al., 2012].

6.7.2 Metals and Human health

Broadly speaking metals are found throughout the environment whereat certain metals are indispensable for living, others are toxic with mutagenic and genotoxic effects. However most metals and the majority of environmental factors do not interact with DNA and do not promote genetic mutations but have the capacity to induce epigenetic changes which account for their carcinogenic activity [Davidson et al., 2007; Stoccoro et al., 2012]. The outcome of in vitro, animal and human studies have identified several classes of environmental chemicals which are associated to modify epigenetic marks,

including metals (cadmium, arsenic, chromium, nickel and methylmercury) peroxisome proliferators (trichloroethylene, dichloroacetic acid and trichloroacetic acid), air pollutants (particulate matter, black carbon and benzene) and endocrine-disrupting/reproductive toxicants (diethylstilbestrol, bisphenol A, persistent organic pollutants and dioxin) [Stoccoro et al., 2012]. Most studies connected to this topic investigated changes in DNA methylation and have reported changes in global and gene specific DNA methylation [Arita and Costa, 2011]. Only little research has been done on the influence of environmental chemicals relating to histone modifications and non-coding RNA [Stoccoro et al., 2012].

Particle toxicology defines the dose by mass or concentration of particles either per unit tissue or per number of cells or surface area of cells in cell culture. Mass is the unit for measuring particles in workplace and the environment for risk management purpose. Donald and his colleague have defined the biologically effective dose (BED) in particle toxicology as "*the entity within any mass dose of particles that drives a critical pathophysiologically relevant form of toxicity in tissue, such as inflammation, genotoxicity or cellular proliferation*" [Donaldson and Poland, 2013]. There are several physiochemical factors of nanoparticles including size, surface, charge, solubility and reactivity leading to biological responses and make an evaluation with a standard toxicity screening almost impossible [Stoccoro et al., 2012].

6.7.3 Welding processes and health risk caused by particulate matter

More than 800 000 workers are employed full time as welders worldwide and even a higher number are welding as part of their job duties. This group of workers inhale a number of hazardous compounds. The welding process produces a complex mixture of gases, aerosols and particulate matter, caused by high temperatures. The product of this molten mixture condenses to $PM_{2.5}$ which can be easily inhaled. Epidemiological studies investigated the effects of chronic exposure of welding fumes and showed that these fumes are associated with respiratory health effects, such as asthma, bronchitis, lung function changes and an increased risk of lung cancer. Epigenetic changes especially altered DNA methylation could be shown in previous studies in combination with different metals from welding fumes. Kile et al. (2013) hypothesized that exposure to particulate matter generated from welding activities can alter DNA methylation [Kile

et al., 2013]. More and more studies investigated the cytotoxic effects of nanoparticles and it could be shown that the intracellular uptake of unmodified nanoparticles is size and shape dependent. A 10 nm nanoparticle is multiple smaller in size compared to a single cell, which has a size range of tens of microns. Therefore nanoparticles can penetrate the cell membrane which can lead to cell damaging and altered enzymatic, metabolic and genomic activities. The main mechanism for the uptake of nanoparticles into the human cells is via endocytosis. Hence nanoparticles are able to reach the nucleus through different barriers and can bind to DNA which could lead to long-term or chronic mutations [An and Jin, 2012].

6.7.4 Particulate matter and DNA methylation

Exposure to PM, including welding fumes, are linked to a variety of adverse biological effects, and different studies indicated that epigenetic changes are not excluded [Kile et al., 2013]. Hence, animal and human studies have shown that air particles or air particle components are linked to induce changes in global and promoter specific DNA methylation levels. These induced changes may represent a mechanism by which human health is affected, as alteration in DNA methylation levels are associated with cancer and cardiovascular disease [Arita and Cost, 2011]. Furthermore it is proven that air particles can increase the production of reactive oxygen species, perhaps in a catalytic way via redox cycling. Oxidative DNA damage can result in hypomethylation due to disturbed ability of methyltransferase-interaction with DNA. Also altered gene-expression belonging to DNA methylation is related to reactive oxygen species, caused by components of airborne particulate matter, such as metals [Baccarelli et al., 2009].

This topic is already of great interest and will be even more important in future especially because an exposure to particulate matter may be minimized but hardly turned off.

6.8 LINE-1, IL-6 and p16

6.8.1 LINE-1

Approximately half of the human genome consists of repetitive elements [Guo et al., 2014]. One type of those repetitive DNA sequences is LINE-1 (Long Interspersed

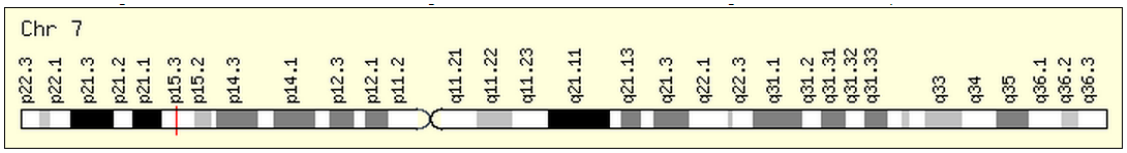
Nuclear Element) which is approximately 17 % dispersed throughout the entire genome [Kitkumthorn et al., 2012; Pobsook et al., 2011]. LINE-1 and short interspersed nuclear elements (SINESs) form the biggest fraction of human interspersed repeats. SINEs have a length of 100 to 400 bp, while LINE-1 has an average length of 6 to 8 kb (6 000 to 8 000 bp) [Smit, 1996]. LINE-1 encodes a reverse transcriptase and other proteins necessary for retrotransposition, such as the open reading frame ORF1p and ORF2p which are both strictly required for retrotransposition [Smit, 1996; Wallace et al., 2008]. The non-coding SINE, Alu, requires enzymatic assistance from LINE-1 as it needs the LINE-1-encoded ORF2p for its own amplification. Alu contributes to approximately 11 % of human genome sequence mass [Wallace et al., 2008]. LINE-1 and other repetitive elements may encode proteins which are involved in their replication and insertion into new locations within the genome and influence genome transcriptional output and aberrant epigenetic alteration of the neighbouring genes [Guo et al., 2014].

Repetitive elements, including LINE-1, are normally heavily methylated, however it has been shown that the level of methylation of LINE-1 can differ in each locus of genome [Turcot et al., 2012; Kitkumthorn et al., 2012]. Whereas promoter hypermethylation of specific genes is associated with cancer, genome wide DNA hypomethylation is observed in most types of cancer. Genome-wide DNA hypomethylation occurs particularly in repetitive DNA sequences and is produced by the reduction of 5-methyldeoxycytosine at CpG-sites throughout the whole genome [Kitkumthorn et al., 2012; Guo et al., 2014]. DNA methylation of repetitive elements is required to maintain gene silencing [Guo et al., 2014]. LINE-1 hypomethylation is not only correlated with genetic changes during carcinogenesis furthermore it can lead to genomic instability, hypermethylation and mutation of tumour suppressor genes, alternate transcription of oncogenes and deregulation of cancer cells. These properties could be utilised using LINE-1 methylation as a universal tumour marker for the detection of cancer DNA. At present, most investigations, studying the methylation levels of LINE-1 is based on comparing DNA from tumour tissue to DNA from histologically normal tissues of the same original cell type [Kitkumthorn et al., 2012]. Further studies are necessary to improve non-invasive methods, as histological studies which require surgical biopsy is still the “gold standard” for diagnosis [Subbalekha et al., 2009]. Epidemiological studies

observed moreover some associations between alteration in LINE-1 methylation and other diseases, including ischemic heart disease and stroke. The knowledge about altered LINE-1 methylation in complex diseases are increasing, an emerging literature uses CpG methylation of repetitive DNA sequences, such as LINE-1 in disease association analyses. LINE-1 can be used as a marker of the global DNA methylation of the genome [Turcot et al., 2012]. This can be supported by the fact that LINE-1 is the most prevalent repetitive sequence in the human genome. Also other repetitive elements, such as Alu repeats are sometimes used to evaluate global DNA methylation.

6.8.2 IL-6

Figure 7: Genomic location of IL-6 Gene (GeneCards)



Size: 6 119 bases

Orientation: plus strand

IL-6 (Interleukin-6) is located on chromosome 7p21 and plays a complex role in inflammation because of its anti-inflammatory as well as its pro-inflammatory properties [Fisher et al., 2014]. Inflammatory responses play important roles in cancer development and IL-6 is one of the best-characterized pro-tumourigenic cytokines [Taniguchi and Karin, 2014]. Although we investigated only the methylation of IL-6, the function of other cytokines should be briefly mentioned. Cytokines are proteins mediating the immunological balancing act by regulating the survival, growth, activation, differentiation and suppression of the innate and adaptive immune responses. They are grouped in interleukins, interferons (INF), tumour necrosis factor (TNF), lymphokines and chemokines. INF are subdivided in INF-α, β, γ, λ whereby INF- α/β have antiviral activities as they are induced in many cell types by a wide variety of exogenous and endogenous pro-inflammatory stimulants. INF-γ is produced by T-lymhocytes, NK cells and NKT cells and is the main immunoregulatory product and co-inducer of the TH1 cytokine pathway. This type of INF has only minimal antiviral activity. The third type of INF, INF- λ, has antiviral and immunostimulatory activities.

The TNF-family involves 19 cytokines and was discovered in serum as a cytotoxic factor for tumours. TNF can cause apoptosis but also acts as a proinflammatory cytokine, mediating acute and chronic inflammatory responses to bacterial infections [Oppenheim, 2014]. TNF-α is one of the major mediators of inflammation as it can be induced by a variety of pathogenic stimuli. TNF-α, itself is in a position to induce other inflammatory mediators and proteases that produce inflammatory response. It is also involved in tumourigenesis and can act as an endogenous tumour promoter [Aggarwal et al., 2006]. Lymphokines are produced by T cells and can induce the adaptive immune response with the ability to start specific reactions to invasive organism and/or damaged cell products. Chemokines are responsible for organogenesis of lymphoid and other tissues. These chemotactic cytokines have only minimal immune cell activating effects in general but play an important role in the migration of inflammatory and noninflammatory cells [Oppenheim, 2014].

Interleukin

Two groups of cytokines were established in 1979, it was thought that all the activities of cytokines could be attributed to two molecules, the monocyte/macrophage-derived interleukin-1 (IL-1) and the lymphocyte derived cytokines interleukin-2 (IL-2). This assumption could not persist and by now 38 interleukins and many more cytokines are established, hence a new classification was necessary [Oppenheim, 2014].

IL-6 is produced by the bone marrow stroma and can be further enhanced though an NF- κB-dependent mechanism (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells). IL-6 is of great importance in the JAK/STAT pathway as it has the ability to directly activate the signal transducer and activators of transcription (STAT) factors STAT1 and STAT3 [Hodge et al., 2005]. JAKS (Janus kinase) and STATS are critical components of cytokine receptor systems and regulate growth, survival, differentiation, and pathogen resistance. These cellular events are among others, crucial for the immune development [Rawlings et al., 2004]. Furthermore IL-6 is able to act through several classic protein kinase cascades including mitogen activated protein kinase (MAPK), serving to drive for example proliferation [Hodge et al., 2005].

Regulation of the impact/signalling of IL-6

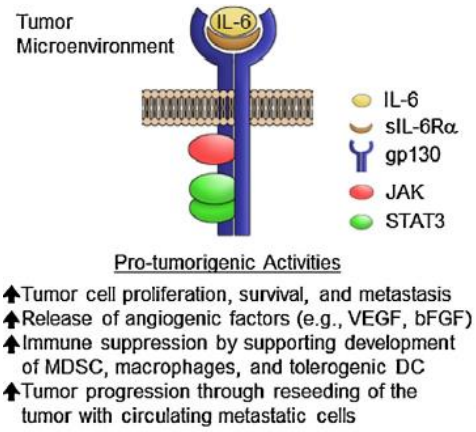
There are two types of IL-6 signalling, the classic- and the trans-signalling. The classic signalling is initiated through the binding of IL-6 to a membrane-bound IL-6 receptor α (IL-6Rα), forming an IL-6/IL-6Rα complex which binds the signal transducing subunit glycoprotein 130 (gp130). The second form of signalling mechanism is characterized thru binding of IL-6 on a soluble form of the IL-6Rα, released from cells (sIL-6Rα). The IL-6/sIL-6Rα binds to membrane-anchored gp130. The restricted expression of IL-6Rα mainly to hepatocytes and some leukocytes limits the types of cells that can respond to IL-6. Therefore the trans-signalling is essential for those cells which do not express IL-6Rα on cell surface [Fisher et al., 2014; Taniguchi and Karin, 2014]. It is considered that these two ways of signalling indicate differences in the mediation of anti- and pro- inflammatory responses. Whereas the classic signalling is relevant for the regenerative or anti-inflammatory activities pro-inflammatory responses are mediated by trans-signalling [Taniguchi and Karin, 2014].

Inflammation and cancer

The influence of inflammation as a risk factor for most types of cancer is approved. Several steps which are involved in tumourigenesis are associated with chronic inflammation, such as transformation, promotion, survival, proliferation, invasion, angiogenesis and metastasis [Aggarwal et al., 2006]. The link between inflammation and cancer has a potential for therapy and prevention, parameters of inflammation are a prognostic factor. Especially the IL-6-family of cytokines can be used as important mediators, as IL-6 and IL-11 are highly up-regulated in many cancers. Therefore IL-6 is one of the best-characterized pro-tumourigenic cytokines and has special attention because of its central role in physiological and pathophysiological processes [Taniguchi and Karin, 2014]. It is assumed that IL-6 drives tumour initiation and subsequent growth and metastasis [Fisher et al., 2014]. Elevated IL-6 has been shown in previous studies in different types of cancer, including skin, lung and liver cancers [Taniguchi and Karin, 2014]. However beside its impact as a critical driver in cancer IL-6 is also involved in anti-tumour immunity by mobilizing T-cell responses. Specifically this phenomenon was investigated in the study of Fisher et al. (2014) *"The two faces of IL-6 in the tumour microenvironment"* [Fisher et al., 2014]. In addition to their assumption Hodge et al. (2005) mentioned detrimental effects of IL-6 such as resistance to

chemotherapeutic drugs as well as the ability to protect cells from "byproducts" of inflammation, such as ROS (reactive oxygen species) and free-radical damage [Hodge et al., 2005].

Figure 8: Protumourigenic activities in IL-6 in the tumour microenvironment [Fisher et al., 2014]

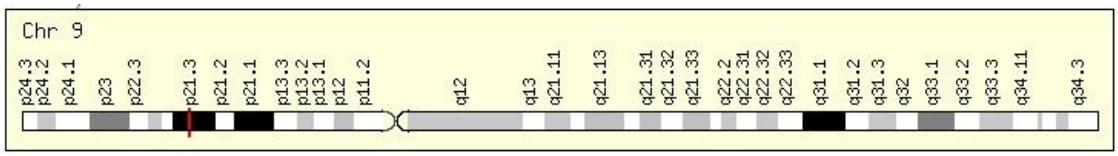


IL-6, as a pro-inflammatory cytokine, mediates chronic inflammation and may play an important role in inflammation-driven oral carcinogenesis. Inflammatory cells and tumour cells are releasing IL-6 continuously. Patients suffering on oral squamous cell carcinoma, a multifactorial disease, primarily associated with chronic tobacco and alcohol use, often show elevated IL-6 levels in their saliva and blood. Numerous studies observed that DNMTs may mediate IL-6-induced hypermethylation and gene silencing. Thus could be approved by a study, showing that IL-6 mediated inflammation induces global hypomethylation of LINE-1 sequences as well as hypermethylation of tumour suppressor genes [Gasche et al., 2011]. The mechanism whereby DNA methylation leads to gene repression by directly blocking the binding of transcription factors to the gene promoter region is already well established. This could also be observed in a study demonstrating that DNMT1 was responsible for IL-6 expression. It can be assumed that IL-6 expression is regulated by promoter demethylation which can be induced by down-regulation of DNMT activity [Tang et al., 2011].

Summarizing the results it is suggested that IL-6 induced inflammation and carcinogenesis may be in part driven via epigenetic changes [Gasche et al., 2011].

6.8.3 p16

Figure 9: Genomic location of p16 (GeneCards)



Size: 27 550 bases

Orientation: minus strand

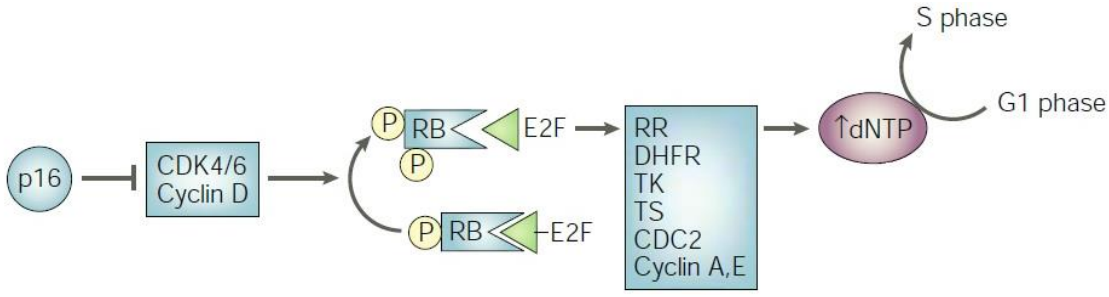
Cancer associated genes can be generally classified in oncogenes, tumour suppressor genes and genes responsible for maintaining stability. Oncogenes promote overexpression of cellular proteins which are involved in cell growth and proliferation, leading to unbreaked tumour growth. Oncogenes gain their function due to genetic and epigenetic mechanism [Brown and Hinds, 2015]. Tumour suppressor genes operate as negative regulators of oncogenes by encoding proteins that are functionally integrated into pathways that prevent unscheduled cell proliferation, stimulate apoptosis, or trigger the induction of permanent cell cycle arrest [Brown and Hinds, 2015; Guérillon et al., 2014]. The loss of gene function of tumour suppressor genes makes the involvement of these genes in the tumourigenic process visible. Deletion, nonsense and missense mutations and methylation-mediated gene silencing are known as mechanism inactivating tumour suppressor genes. Cancer associated genes which are involved in cellular processes maintaining basal levels of genomic or chromosomal stability can be classified as third group cancer associated genes. The proficiency of a cell is depending on an accurately repair mechanism for acute genomic damage such as breaks of DNA strands or mobilizing specific enzymatic complexes to site of DNA damage. If genes of this process are inactivated the rates of spontaneous mutations are consequently increasing and may impact the accumulation of mutations in oncogenes and tumour suppressor genes [Brown and Hinds, 2015].

The INK4a/ARF locus on chromosome 9 encodes two tumour suppressor genes, which play a key role in the regulation of two important cell cycle regulatory pathways, the p53 pathway and the Rb (retinoblastoma protein) pathway. The two proteins, namely p14 (ARF) and p16 (INK4a) uses unique first exons (exon 1α for p16 and 1β for p14) and common exons 2 and 3. Despite of shared exons but utilizing different reading frames they are totally unrelated proteins. p14 regulates the p53 pathway by binding the p53-stabilizing protein MDM [Robertson and Jones, 1999].

The tumour suppressor gene p16, also known as CDKN2A, INK4a and CDK4I (cyclin dependant kinase 4 Inhibitor) is located on chromosome 9p21. It is a member of the INK family of cyclin dependant kinase (CDK) inhibitors [Zainuddin et al., 2011].

p16 plays an important part in the pathway, regulating cell-cycle entry and progression by blocking the CDK4-CDK6-cyclin-D complex, formed by the cyclins D1, D2 and D3 with the CDK4 and 6 in G1. This complex increases the phosphorylation state of Rb [Beasley et al., 2003; Chiocca, 2002]. The tumour-suppressor gene Rb regulates the cell cycle by inducing cell cycle arrest at G1. The hypophosphorylated form of Rb can constitute a stable complex with E2F1 leading to G1 arrest which is linked with inhibition of transcriptional activities. Hyperphosphorylated Rb results in releasing the transcriptionfactor E2F [Beasley et al., 2003]. E2F can trigger G1-S transition and consequently the progression of cell cycle by the mediation of transcription of several cellular genes that are involved in G1/S progression and increase the production of deoxynucleoside triphosphates (dNTPs) [Chiocca, 2002].

Figure 10: p16/Rb pathway [Chiocca, 2002]



An overexpression of cyclin D1 is linked with persistent hyperphosphorylation of Rb. Thus circumvent the cell cycle arrest and shortens the phase length of G1. CDK4 and CDK6 activity can be inhibited by p16 due to replacing cyclin D1 in the binary

CDK-cyclin D1 complex, whereby the phosphorylation of Rb can be regulated and therefore the progression of the cell cycle. If p16 is inactivated, phosphorylation of Rb cannot be inhibited and the cell cycle gets out of control [Beasley et al., 2003]. Different studies reported an association between abnormalities in the p16/cyclin D1/Rb pathway and most human cancers [Beasley et al., 2003; Brown and Hinds, 2015]. Loss of p16 or cyclin D1 overexpression occurs more often in non-small cell lung carcinomas (NSCLCs) than the direct loss of Rb [Beasley et al., 2003].

It is known that the regulation of expression of many genes including p16 is controlled by extent methylation of cytosine. Mainly cytosine-rich sequences, known as CpG islands, especially in the promoter regions are affected [Chanda et al., 2006]. Hypermethylation in this region can lead to gene silencing of p16 and is reported in various tumours in human [Fujiwara-Igarashi et al., 2014]. Aberrant hypermethylation has great potential to be used as a biomarker as it occurs as an early event in lung cancer. Early diagnosis of lung cancer is still a big challenge, as effective screening tools are still missing. Therefore sensitive and reliable methods have to be established [An et al., 2002].

7 Objective

The aim of the present study was to find out if there are changes in DNA-methylation due to higher exposure of toxic fumes in the promoter region of LINE-1, p16 and IL-6. These three genes play an important role in carcinogenesis and are regulated by epigenetic mechanism. Methylation analysis of buccal cells of welders and controls should give information about the methylation levels in different individuals by bisulfite-pyrosequencing. Different methylation levels should help to understand the impaired regulation of genotoxic effects of toxic fumes produced by welding relating to inflammation and cancer. The level of methylation could be used as a biomarker for early detection of cancer. To exclude age related methylation levels in p16 and IL-6, the global methylation was analysed by LINE-1.

8 Material and Methods

8.1 Study design

For the study three different groups of men between the ages of 30 to 63 years were examined. Two of the three groups included welders. Demographic data, such as age, height, weight and smokers were measured and collected with questionnaires and are listed in Table 1. Due to the type of welding process and thereby the degree of exposition two groups differed: low exposed (LW) and high exposed welders (HW). The difference between these two groups is the type of welding process. One group works as so called factory mechanics and operates with manual metal arc welding only with steel, which has no carcinogenic effect, and labeled with LW. The other group of welders was involved mainly in two types of welding processes, tungsten inert gas (TIG) welding and Gas metal arc welding process, more precise Metal active gas (MAG) welding, labeled with HW. This group worked with high-grade alloy steel and different metals. The HW had to work in confined space with some more breaks but nevertheless with an exposure of eight hours a day whereby the LW had a daily exposure to the toxic fumes of only five to six hours. The third group was a control group which was matched by gender, age and body mass index (BMI), labeled with C.

The study included a total of 61 men, namely 20 participants in the LW and the control group and 21 in the HW group.

Table 1: Demographic data of the study participants

Parameters	**Low exposed Welders (LW)**	**High exposed Welders (HW)**	**Control (C)**
Age	44.3 ± 8.7	44.8 ± 9.8	43.0 ± 9.1
BMI	26.0 ± 3.2	28.2 ± 3.9	23.6 ± 2.4
Smoker	6 current smokers (30%) 3 occassionally smokers (15%)	21 smokers (100%)	1 current smoker (5%) 2 former smokers (10%)

Hight and body weight to calculate the BMI were measured before sampling, other characteristics were collected by questionnaires.

8.2 Inclusion criteria for the two welding groups

All welders are working in Austria and are under health control of the occupational physician Dr. Georg Wultsch in Graz.

8.3 Sample collection

On the last day of a working week exfoliated buccal cells were collected from the participants of both welding groups. The participants had to rinse their mouth twice with tap water immediately before sample taking to diminish unwanted contamination. Buccal cells were collected from both cheeks by the participants themselves by use of wooden spatulas (Paul Hartmann AG, Heidenheim, Germany). The samples of each group were collected on the same day, except of the participants of the control group, where the sampling took place at different point of times and at any day of the week. All samples were stored at -20 °C.

8.4 DNA isolation and bisulfite conversion

For DNA extraction QuickExtract™ DNA Extraction Solution (Epicenter, Chicago, USA) was used according to the manufactures' protocol (see Appendix).

Bisulfite conversion was necessary before the analysis of DNA methylation for all samples. Therefore the EpiTect® Bisulfite Kit (Qiagen, Hilden, Germany) was used according to the the manufactures' instruction (see Appendix). The principle of this kit is the sodium bisulfite induced conversion of unmethylated cytosines residues into uracil while remaining methylated cytosines unchanged.

The DNA was measured after bisulfite conversion using the Pico100 (Picodrop Limited, Hinxton, UK).

All bisulfite converted samples were stored at -20 °C.

8.5 PCR conditions and gel electrophoresis

Primer design software (Qiagen, Hilden, Germany) was used to find adequate primers for the bisulfite converted DNA in addition to amplify certain regions of p16 and IL-6 genes. For LINE-1 all essential Primer information were assumed from

Bollati et al. (2007). For the following pyrosequencing it is necessary to use biotinylated reverse primers.

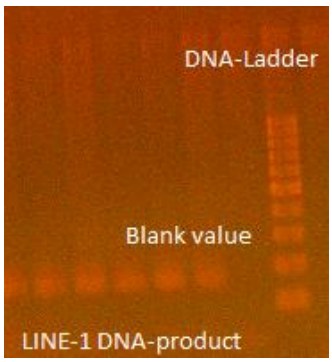
PCR (polymerase chain reaction) amplification of template DNA was done by use of PyroMark PCR kit (Qiagen, Hilden, Germany) (see Appendix).

The PCR was carried out in a reaction mix with a total volume of 25 µl, containing 12.5 µl PyroMark PCR Master Mix, 2.5 µl CoralLoad Concentrate (Qiagen, Hilden, Germany), 8 pmol of each primer (p16), 5 pmol of each primer (IL-6) and 25 pmol of each primer (LINE-1) and 10 ng of converted DNA for p16, 10 ng of converted DNA for IL-6 and 10 ng of converted DNA for LINE-1.

The PCR conditions for p16 were 95 °C for 15 minutes, 45 cycles of 94 °C for 30 seconds, 58.5 °C for 45 seconds and 72 °C for 45 seconds, and a final elongation at 72 °C for 10 minutes. The PCR conditions for IL-6, as well as for LINE-1 were 95 °C for 15 minutes, 45 cycles of 94 °C for 30 seconds, 50 °C for 45 seconds and 72 °C for 45 seconds, and a final elongation at 72 °C for 10 minutes.

After the PCR, a gel electrophoresis was followed to check each product, inclusive the blank value without a sample, on a 2 % agarose gel. For this purpose an amount of 3 µl of the 25 µl were used. Different criteria had to be fulfilled to proceed with Pyrosequencing, such as identical DNA bands without any byproducts and no DNA band for the control sample. The remaining 22 µl of PCR products were subsequently used for Pyrosequencing.

Figure 11: Image detail: Gel- electrophoresis of LINE-1 [by Carina Fechner]



8.6 Quantitative gene methylation analysis by pyrosequencing

For further investigation the remaining 22 µl of PCR product, as mentioned above, were mixed with 3 µl streptavidin-coated Sepharose® beads (GE Healthcare, Vienna, Austria), 40 µl PyroMArk binding buffer (Qiagen, Hilden, Germany) and 15 µl high purity water to reach a total volume of 80 µl.

Pyrosequencing was done using the PyroMark® Q24 System (Qiagen, Hilden, Germany). This method calls for a biotinylated primer in the PCR reaction, as the biotinylated end of the amplicon can bind to the streptavidin-coated Sepharose® beads. The formed complex can be sucked by PyroMark® Vacuum Workstation.

The PCR products were purified and denaturized by using 70 % ethanol, 0.2M NaOH solution and washing buffer. The purified single-stranded DNA was released into the annealing buffer (Qiagen, Hilden, Germany), containing a corresponding pyrosequencing primer (8 pmol for p16, 5 pmol for IL-6 and 25 pmol for LINE-1). This mixture was used for pyrosequencing.

The assay for pyrosequencing was designed with the PyroMark Q24 Software (Qiagen, Hilden, Germany). Each assay included controls to verify complete sodium bisulfite DNA conversion in terms of non-CpG cytosines.

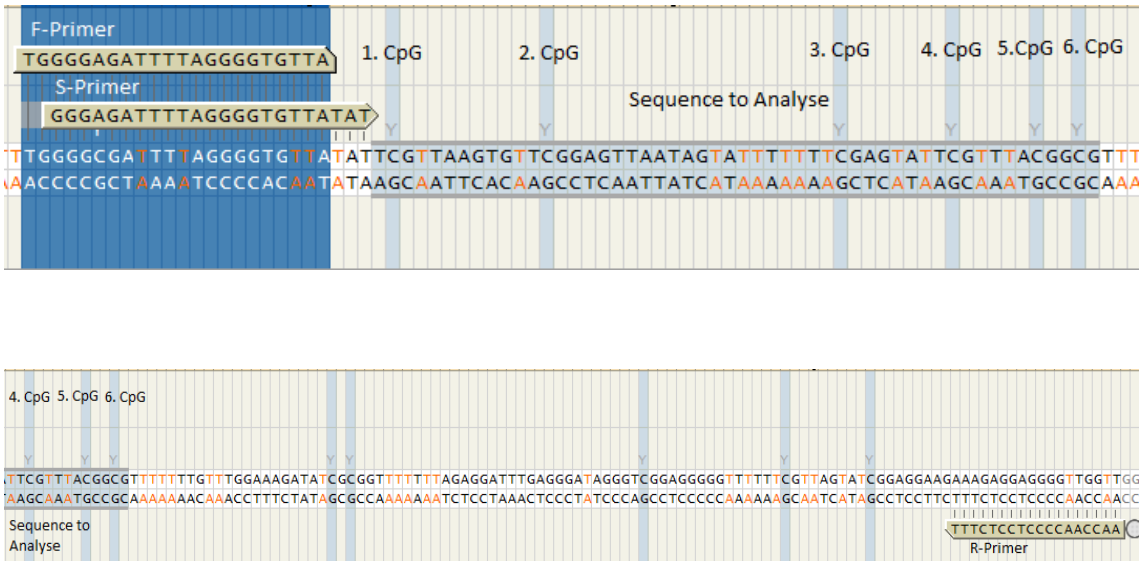
Table 2: Primers for PCR and Pyrosequencing

Name of primer	Sequence 5'-3'	Size	Annealing Temperature
LINE-1 (F)	TTT TGA GTT AGG TGT GGG ATA TA	23	50 °C
LINE-1 (R)	Biotin-AAA ATC AAA AAA TTC CCT TTC	21	50 °C
LINE-1 (S)	AGT TAG GTG TGG GAT ATA GT	20	
IL-6 (F)	AAA TGT GGG ATT TTT TTA TGA	21	50 °C
IL-6 (R)	Biotin-AAT TCC AAA ACT AAA AAT TTC CT	23	50 °C
IL-6 (S)	ATG TTT GAG GTT TAT TTT GTT	21	
p16 (F)	TGG GGA GAT TTT AGG GGT GTT A	22	58.5 °C
p16 (R)	Biotin-AAC CAA CCC CTC CTC TTT	18	58.5 °C
p16 (S)	GGG AGA TTT TAG GGG TGT TAT AT	23	

Table 3: Sequence to analyse of p16, IL-6 and LINE-1

Assay	Sequence to analyse 5'-3'	Number of CpGs	Size
p16	TCGTTAAGTGTTCGGAGTTAATAGTATTTTTTTCGAGTA TTCGTTTACGGCG	6	52
IL-6	TTCGAGTTTATCGGGAACGAAAGAG	3	25
LINE-1	TTCGTGGTGCGTCGTTT	3	17

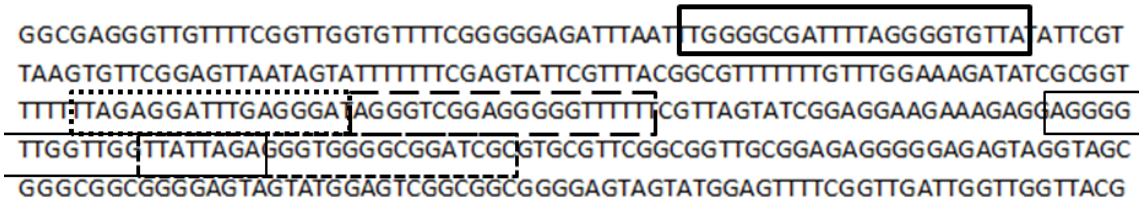
Figure 12: Designing the p16 primer



For designing the p16 primer, the primer design software (Qiagen, Hilden, Germany) was used.

The following figure shows used forward-primers for p16 in already published studies by comparison with the new designed p16 forward-primer.

Figure 13: Used forward-primers for p16 in already published studies



The order of appearance in the figure above is related to following authors starting with the for this study new designed p16 primer:

1. New designed p16 forward primer
2. Balog et al., 2002
3. Umetani et al., 2005
4. Lee et al., 2012
5. Guzmán et al., 2007

8.7 Statistical analysis

IBM® SPSS® Statistics Version 20 (IBM, Armonk, NY) was used for quantitative methylation analysis of LINE-1, IL-6 and p16. Normal distribution of data was tested with the Kolmogorov-Smirnov test. Statistical significance was defined as a p-value <0.05 and was tested with the student's two tailed paired t-test and one-way analysis of variance (ANOVA) with the Tukey post hoc correction. All data shown are mean ± standard deviation (SD).

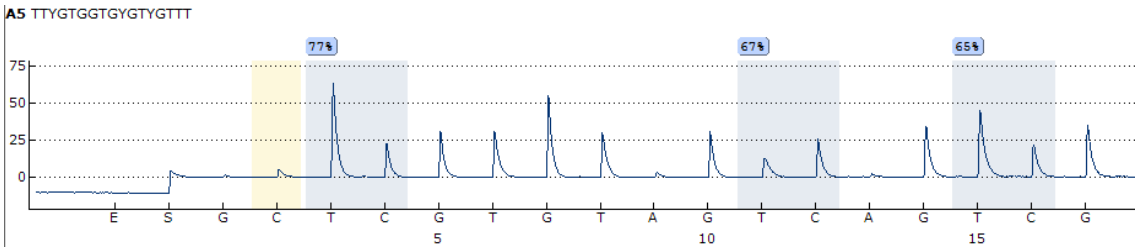
9 Result

We analysed the percent of methylation in the promoter region in three different genes, namely LINE-1, p16 and IL-6 in a total of 62 participants. Afterwards, a comparison between the groups, respectively, a comparison between the welders in total and the control group was carried out.

9.1 LINE-1

Methylation patterns of LINE-1 were analysed of all participants. 3 CpGs were evaluated in the promoter region by pyrosequencing. One bisulfite treatment control at position 2 was used to guarantee successful bisulfite conversion.

Figure 14: LINE-1-pyrogramm of a high exposed welder



9.1.1 Mean methylation and methylation level of single CpGs

Mean methylation and methylation levels of all 3 CpGs in LINE-1 are shown in Table 4 and 5.

CpG 1 shows the highest methylation in all three groups whereas the methylation of CpG 2 and 3 indicate comparable values. Nevertheless the methylation of LINE-1 indicated very consistent methylation between the three groups. High exposed welders show the highest value for mean methylation with 70.33 % and low exposed welder, the lowest value with 68.38 %. By comparing all welders in total with the control group almost no difference can be observed in the level of mean methylation (69.35 % for welders and 69.48 % for the control group). Furthermore no significant difference could be determined in LINE-1 in any of the three measured CpGs. These outcomes support the assumption of LINE-1 as an indicator of the overall methylation [Turcot et al., 2012].

Table 4: LINE-1: Methylation of each CpG and Mean Methylation of all CpGs of all 3 groups

Group	**CpG 1**	**CpG 2**	**CpG 3**	**Mean of all 3 CpGs**
LW	79.70 ±5.24	61.35 ±3.10	64.09 ±3.39	68.38 ±3.23
HW	79.41 ±8.20	65.00 ±7.79	66.62 ±10.04	70.33 ±8.13
C	77.38 ±9.51	64.71 7.26	65.31 ±7.77	69.13 ±8.28
Significance				
LW- C	0.611	0.227	0.864	0.868
HW – C	0.686	0.992	0.846	0.919
LW – HW	0.992	0.168	0.530	0.636

Table 5: LINE-1: Methylation of each CpG and Mean Methylation of all CpGs of all welders in total and controls

Group	**CpG 1**	**CpG 2**	**CpG 3**	**Mean of all 3 CpGs**
LW+HW	79.55 ±6.80	63.15 ±6.13	65.36 ±7.51	69.35 ±6.19
C	77.38 ±9.51	64.71 ±7.26	65.31 ±7.77	69.48 ±8.28
Significance				
LW+HW - C	0.077	0.138	0.346	0.055

Figure 15: LINE-1: Mean Methylation of all CpGs of all groups

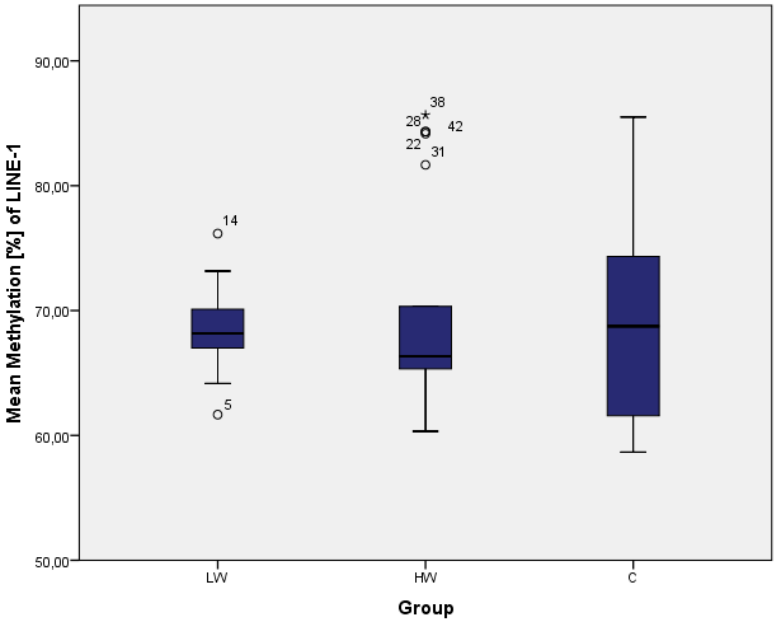
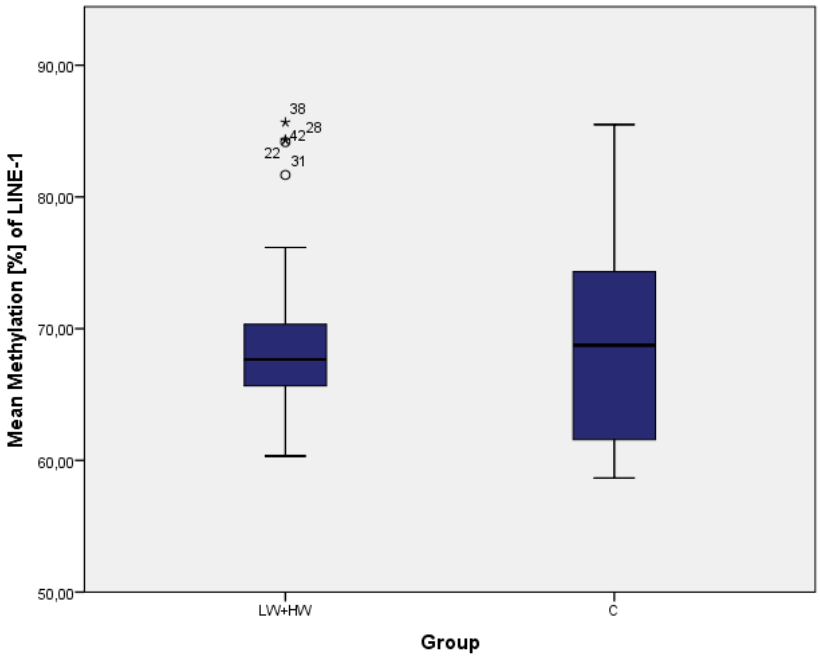


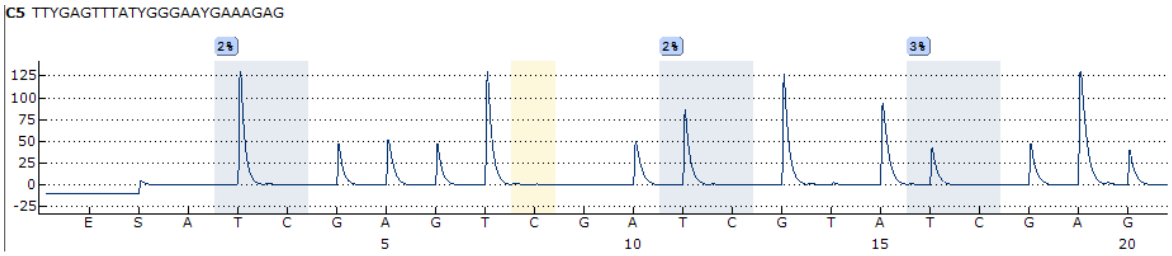
Figure 16: LINE-1: Mean Methylation of all welders in total versus control



9.2 IL-6

In IL-6 three CpGs of the promoter region were analysed in all three groups. One bisulfite treatment control on position 8 was set to assure successful bisulfite conversion.

Figure 17: IL-6-pyrogramm of a control person



9.2.1 Mean methylation and methylation level of single CpGs

Mean methylation and methylation levels of all 3 CpGs in IL-6 are shown in Table 6 and 7.

Both groups of welders show a tendency of decreased values of methylation in the different CpGs as well as in the mean value. Taking a closer look on the differences between the three groups at single CpGs it is shown that the highest value was measured in CpG 3 in the control group with 4.2 % and was significantly increased (p= 0.018) when compared with all welders.

Table 6: IL-6: Methylation of each CpG and Mean Methylation of all CpGs of all 3 groups

Group	**CpG 1**	**CpG 2**	**CpG 3**	**Mean of all 3 CpGs**
LW	1.56 ±1.21	3.31 ±3.18	1.75 ±1.31	2.21 ±1.22
HW	2.58 ±1.36	1.08 ±0.58	2.58 ±1.77	2.08 ±0.89
C	2.00 ±0.61	1.50 ±0.79	4.20 ±5.01	2.57 ±1.44
Significance				
LW – C	0.783	0.332	0.308	0.859
HW – C	0.683	0.946	0.621	0.783
LW – HW	0.253	0.170	0.850	0.979

Table 7: IL-6: Methylation of each CpG and Mean Methylation of all CpGs of all welders in total and controls

Group	CpG 1	CpG 2	CpG 3	Mean of all 3 CpGs
LW+HW	2.00 ±1.33	2.36 ±2.63	2.11 ±1.52	2.15 ±1.05
C	2.00 ±0.61	1.50 ±0.79	4.20 ±5.01	2.57 ±1.44
Significance				
LW+HW –C	0.146	0.177	0.018*	0.762

*p<0.05

Figure 18: IL-6: Methylation of CpG 3 of all welders in total versus control

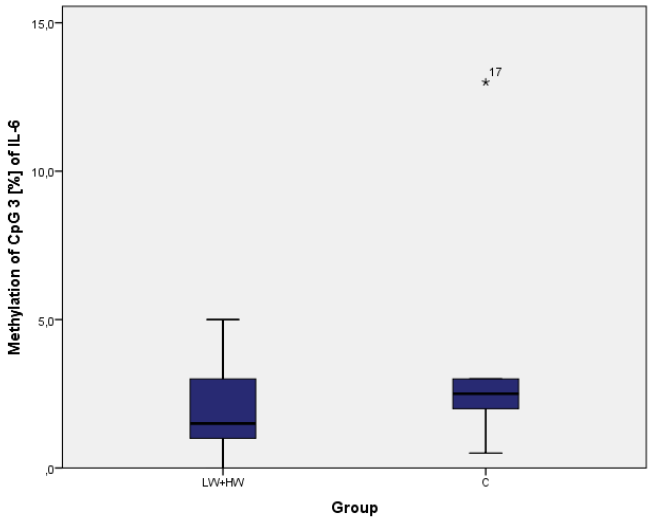
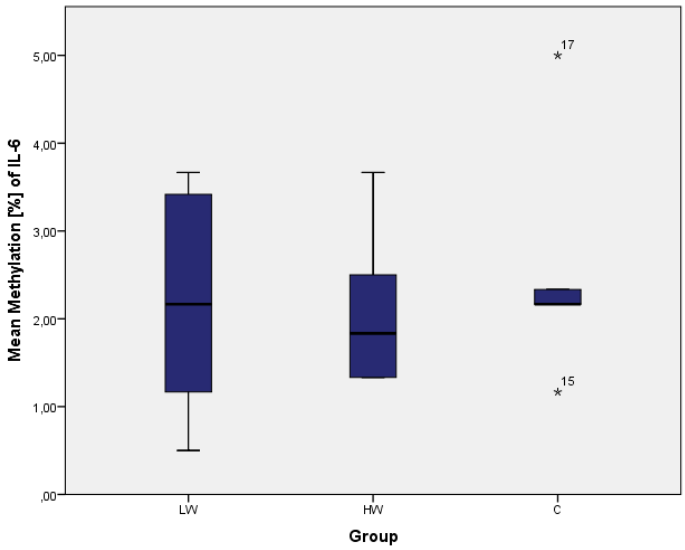


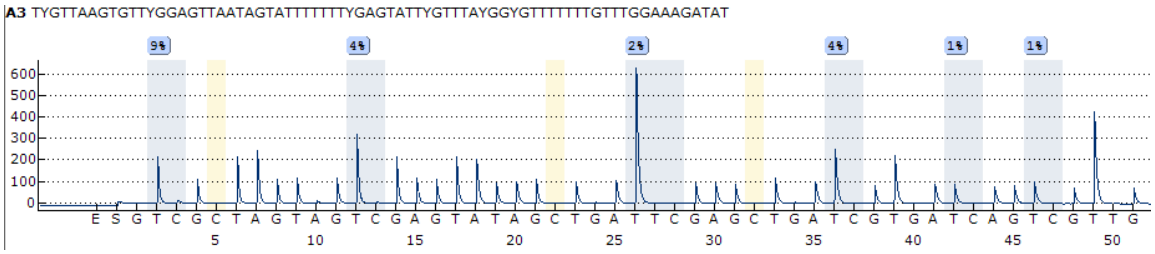
Figure 19: IL-6: Mean Methylation of all CpGs of all groups



9.3 p16

Methylation patterns of p16 were analysed of all three groups. During pyrosequencing six CpGs in the promoter region of p16 were evaluated. To guarantee successful bisulfite conversion three bisulfite treatment controls were used on position 5, 22 and 32.

Figure 20: p16-pyrogramm of a low exposed welder



9.3.1 Mean methylation and methylation level of single CpGs

Mean methylation and methylation levels of all 6 CpGs in p16 are shown in Table 8 and 9.

For mean methylation of all six CpGs almost no differences between the two exposed groups were shown (LW: 7.50 % and HW: 7.34 %). For the control group we measured a value of 9.47 % for mean methylation. Similar values were therefore shown by

comparing the mean methylation of all exposed subjects with the mean methylation of the control group.

Only on closer consideration CpG 1 and 2 showed increased methylation in both exposed groups when compared with the control group. The methylation in CpG 2 was 64.3 % higher in welders as in the control group but the difference did not reach statistical significance. In all groups at each site average percentage of methylation was between 2.6 % and 12.3 %.

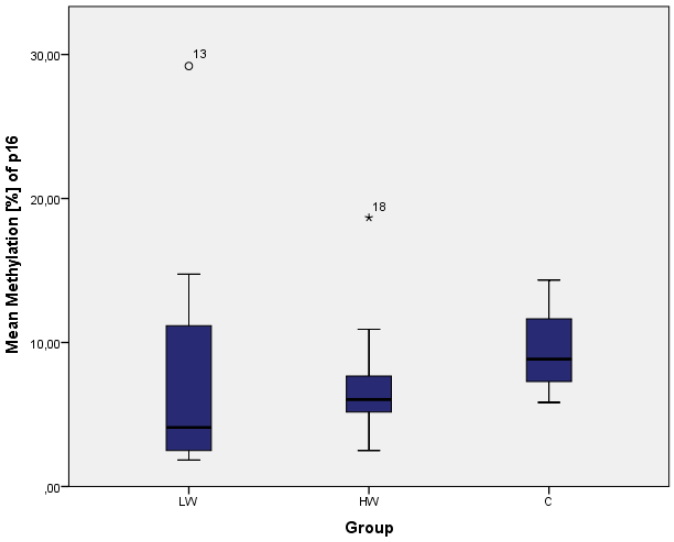
Table 8: p16: Methylation of each CpG and Mean Methylation of all CpGs of all 3 groups

Group	**CpG 1**	**CpG 2**	**CpG 3**	**CpG 4**	**CpG 5**	**CpG 6**	**Mean of all 6 CpGs**
LW	6.94	9.54	6.49	6.56	7.76	6.01	7.50
	±4.58	±14.79	±5.72	±7.65	±8.42	±11.00	±7.50
HW	12.25	8.75	9.85	5.95	4.65	2.60	7.34
	±11.82	±10.32	±9.02	±5.93	±4.26	±1.35	±4.60
C	8.46	5.91	9.05	7.88	9.54	4.83	9.47
	±4.50	±2.83	±0.07	±4.91	±11.18	±3.12	±3.54
Significance							
LW – C	0.940	0.864	0.882	0.938	0.912	0.964	0.842
HW – C	0.704	0.920	0.988	0.880	0.534	0.886	0.832
LW – HW	0.261	0.987	0.497	0.975	0.594	0.569	0.998

Table 9: p16: Methylation of each CpG and Mean Methylation of all CpGs of all welders in total and controls

Group	**CpG 1**	**CpG 2**	**CpG 3**	**CpG 4**	**CpG 5**	**CpG 6**	**Mean of all 6 CpGs**
LW+HW	9.15	9.20	7.89	6.30	6.47	4.59	7.43
	±8.58	±12.77	±7.29	±6.81	±7.05	±8.49	±6.33
C	8.46	5.92	9.05	7.88	9.54	4.83	9.47
	±4.50	±2.83	±0.07	±4.91	±11.18	±3.11	±3.54
Significance							
LW+HW – C	0.308	0.256	0.100	0.617	0.261	0.722	0.397

Figure 21: p-16: Mean Methylation of all CpGs of all groups



10 Discussion

Recently there is growing interest in the potential that the environment may influence not only the genome by mutations but also the epigenome by epimutations. The topic of how epigenetic processes can significantly modulate cellular behavior and potentially complex diseases risk, including cancer, especially in response to environmental chemicals is still in its infancy. As there is a widespread use and dispersion in the environment of these environmental agents, including nano-sized materials and PM, at present and predictable in the near future more attention should be paid to this issue [Stoccoro et al, 2012]. Pulmonary dysfunction including asthma and an increased lung cancer risk with an odds ratio of 1.4 are common diseases of welders [Wultsch et al, 2013]. In this study we investigated, if toxic fumes from welding processes increase the risk of cancer due to alteration in the methylation in LINE-1, IL-6 and p16. We compared two different groups of welders with a control group. It was one of the first studies using buccal cells to determine the alteration of methylation. LINE-1, p16 and IL-6 are three genes which play a role in cancer. The aim of the study was to investigate if the level of methylation in these three genes can be used as a biomarker for cancer growth.

There is a great potential of using DNA methylation as a nucleic acid based biomarker for clinical implementation in cancer medicine and a number of recent studies highlighted the prospective utility of this biomarker in lung cancer prognosis, predicting response to therapy. DNA is the most stable biological macromolecule and the methyl groups on the cytosines are part of the covalent structure of the DNA which enables a relatively easily transformation from a research laboratory setting into routine diagnostic. Biomarkers with sufficient sensitivity and specification are needed but so far no single gene has been found that is always methylated in a certain type of cancer. Therefore most likely panels of DNA methylation based biomarkers will be used in future [How Kit et al, 2012; Liloglou et al, 2014].

Around half of the human genome consists of repetitive elements. LINE-1 is the most prevalent repetitive sequence and is dispersed throughout approximately 17 % of the entire gene [Kitkumthorn et al, 2012]. Wherefore LINE-1 methylation quantification has relevance as a marker of global DNA methylation. LINE-1 hypomethylation is

correlated with genetic changes during carcinogenesis and consequently the methylation of this gene can be used as a potentially useful tumour marker for the detection of cancer DNA [Turcot et al, 2012; Pobsook et al, 2011; An et al, 2002]. Furthermore different studies have observed an association between the exposure to air pollution and decreased repetitive element methylation [Guo et al., 2014].

The result of this study showed no significant alteration in methylation within the three groups in the three determined CpGs. This supports the justification of using LINE-1 as a marker for global DNA methylation.

IL-6 is one of the best-characterized pro-tumourigenic cytokines and plays critical roles in a wide range of biological activities, such as infection, cell proliferation and differentiation and tumour growth. IL-6 is elevated in many cancers such as lung, esophageal and liver cancer [Taniguchi et al, 2014; Tang et al, 2011]. Gasche et al. (2011) demonstrated significant IL-6 induced global LINE-1 hypometylation in an in-vitro model of OSCC cell lines. Furthermore, IL-6 induced CpG promoter methylation changes in several important tumour suppressor genes were observed [Gasche et al., 2011]. Tang et al. came to the result that the activity of IL-6 decreased with increased methylation compared to an unmethylated promoter. Hence it can be assumed that DNA methylation alteration might be involved in the activation of IL-6 [Taniguchi et al, 2014; Tang et al, 2011]. Furthermore a correlation between the methylation of distinct CpG-sites within the IL-6 promoter region and mRNA expression could be observed in several studies [Dandrea et al., 2009].

The observed CpG-sites of IL-6 were described in present studies to play important roles in the regulation of IL-6 expression. In general we report light decreases of methylation in promoters of welders in all three measured CpGs and in mean values of methylation assuming increased inflammatory activity.

Nevertheless it should be considered that the methylation levels at the investigated CpG-sites are rather low (1-10 %) wherefore the variation within a group might be higher than the differences between the means of the study groups.

p16 is a tumour suppressor gene and plays a key role in the regulation of the cell cycle by inducing the cell cycle arrest in G1. Due to inhibiting the cyclin dependent kinases

CDK4 and CDK6 by p16 the retinoblastoma protein can be inactivated. Hypermethylation of the promoter region of p16 is an important mechanism of gene silencing and could be a potential biomarker for early diagnosis, for as it occurs as an early event in cancer [Endo et al, 2011; Zainuddin et al, 2011]. DNA hypermethylation of promoter of gene p53 and p16 was reported in arsenic-exposed people with and without malignancy [Chanda et al, 2006]. We analysed six CpGs of the promoter region of p16 and report higher methylation in CpG 1 and 2 in welders. The percentage of methylation at each site was between 2.6 % and 12.3 % whereby the highest and the lowest value was both measured in the high exposed group. Bihl et al., (2012) reported from data of p16 methylation using pyrosequencing ranging from 4.2 % to 11 % in normal tissue. Tumour tissue was almost twice that high, with maximum value of 20.9 % [Bihl et al, 2012].

The benefit working with buccal cells is the simple and fast sample taking. Certainly a disadvantage of this method is the low DNA yields compared to extraction from DNA from blood. The standard deviation is quite high in some CpGs and indicates that the percentage of methylation diversifies within the group. This could be seen as a consequence of the low DNA concentration because it could lead to more fluctuations in the measurement.

Furthermore, it is worth mentioning that different CpG sites within the promoter region and perhaps different CpG sites even within the same promoter region may respond differently to environmental stimuli [Kile et al, 2013]. This might be a handicap, comparing different studies, when not analysing exactly the same CpG sites.

DNA methylation might have some advantages, making it a more robust biomarker comparing to mRNA or protein expression. There are several stages within gene expression and each stage is regulated through a multitude of fine-tuning processes. Thereby a number of effects can influence the gene expression, making an association with the target parameter complex.

11 Conclusion

In summary epigenetic changes due to genotoxic carcinogens may play a quite important role in tumourigenic process. For better understanding and using this information as a biomarker for early cancer detection further investigations, especially in buccal cells are necessary to examine bigger cohorts. Furthermore, it would be interesting if you can deflect these results for a general indication of the hazard of nanoparticles and PM for the human body in reference to epigenetic alteration.

12 Paper

Epigenetic changes of p16, IL-6 and LINE-1 in buccal cells after exposure to toxic fumes produced in welding, a pilot study

Summary

Environmental toxins such as from traffic pollution have been shown to change epigenetic methylation in the promoter region of genes and to induce nuclear anomalies. For fumes from welding, known to contain particulate matter, chromosomal alterations were reported. The study investigated effects from welding fumes on epigenetic methylation in promoter CpGs of IL-6, p16 and LINE-1 in DNA of buccal cells of welders using DNA bisulfite conversion and pyrosequencing. For IL-6 a methylation rate between 1 and 10 % was measured, this range was already observed in other studies. Hereby a general decrease of methylation could be shown for welders in the IL-6 promoter. Comparing welders with the control group a significant decrease was observed in one of the CpGs. This may correlate with increased inflammatory responses in buccal cells. Average methylation for p16 was about 10 %. The CpG 1 and 2 of welders showed an increased methylation as already before observed in other studies. As p16 plays a key role in the regulation of the cell cycle, hypermethylation of this gene in the promoter region is an important mechanism of gene silencing and therefore associated with tumour growth. LINE-1 values were in the same range in all groups. These results add evidence that damaging of DNA induced by environmental toxins should be further investigated and associated diseases can be early detected.

Key words

Welders, Buccal cells, Epigenetic

Introduction

Humans are exposed to a number of environmental agents like metals, tobacco smoke and airborne mixture of particles which influence the risk of various developing chronic diseases, such as cancer [1]. Some studies have already investigated the toxic effects of certain nano-sized compounds and highlighted the ability of alteration of global DNA methylation, as well as changes of gene specific methylation in tumour suppressor

genes, inflammation genes and DNA repair genes which are all potentially involved in cancer development [3].

Metals are found throughout the environment whereat certain metals are indispensable for living others are toxic with mutagenic and genotoxic effects. However most metals and the majority of environmental factors do not interact with DNA and do not promote genetic mutations but have the capacity to induce epigenetic changes which account for their carcinogenic activity [5, 3]. The outcome of in vitro, animal and human studies have identified several classes of environmental chemicals which are associated to modify epigenetic marks, including metals (cadmium, arsenic, chromium, nickel and methylmercury) peroxisome proliferators (trichloroethylene, dichloroacetic acid and trichloroacetic acid), air pollutants (particulate matter, black carbon and benzene) and endocrine-disrupting/reproductive toxicants (diethylstilbestrol, bisphenol A, persistent organic pollutants and dioxin) [3]. Most studies connected to this topic investigated changes in DNA methylation and have reported changes in global and gene specific DNA methylation [1]. Only little research has been done on the influence of environmental chemicals relating to histone modifications and non–coding RNA [3].

Epigenetic changes due to exposure to nano-sized materials

Epigenetic can be literally interpreted as "above genetics" and describes heritable changes in gene expression that do not involve alterations in DNA sequence [2, 3]. Epigenetic can be grouped into DNA methylation, microRNAs (miRNAs), covalent histone modification and nucleosome remodeling [4]. Many factors from the environment , such as toxins, food ingredients or many forms of stress have been shown to effect on multiple epigenetic mechanisms even in a transgenerational way. Particles, from the environment or occupational origin which may both effect, genetic and epigenetic regulation of gene expression, such as nanoparticles, are of special interest.

Nanoparticles are basically defined around a threshold dimension of 100 nm [6]. The welding process in conjunction with extreme heat produces a complex mixture of gases, aerosols and particulate matter. The product of this molten mixture condenses into ultrafine and fine particulate matter (PM) with an aerodynamic diameter $\leq$ 2.5 µm ($PM_{2.5}$) which can be easily inhaled. Epidemiological studies investigated on the effects

of chronic exposure of welding fumes and accompany that these fumes are associated with respiratory health effects, such as asthma, bronchitis, lung function changes and an increased risk of lung cancer. Epigenetic changes especially altered DNA methylation could be shown in previous studies in combination with different metals from welding fumes. Kile et al. (2013) hypothesized that exposure to particulate matter generated from welding activities can alter DNA methylation [7]. More and more studies investigated the cytotoxic effects of nanoparticles and it could be shown that the intracellular uptake of unmodified nanoparticles is size and shape dependent. Nanoparticles can penetrate the cell membrane which can lead to cell damaging and altered enzymatic, metabolic and genomic activities. The main mechanism for the uptake of nanoparticles into the human cells is via endocytosis. Hence nanoparticles are able to reach the nucleus through different barriers and can bind to DNA which could lead to long-term or chronic mutations [8].

Wultsch et al. (2013) investigated the effects of toxic fumes produced by welding activity in context with chromosomal alteration and acute cytotoxicity in epithelial cells from the respiratory tract of welders and unexposed controls. Additional biochemical parameters were monitored for reflecting the redox status and the concentration of different metals in body fluids. The outcome of the nasal cells showed significant alterations in welders which are indicative for chromosomal alteration and was therefore the most important finding of this study. Elevated rates of nuclear anomalies reflecting cytotoxic effects were detected in cells from nose and buccal cells and the levels of certain metals were significantly higher in the body fluids of the welders compared to the control group. The result of this study indicated that epithelial cells from the respiratory tract are suitable for the detection of DNA damaging and cytotoxic effects. This observation could be used to asses health risk associated with genomic instability [9].

The aim of the present study was to find out if there are changes in DNA-methylation due to higher exposure of toxic fumes in the promoter region of LINE-1, p16 and IL-6. These three genes play an important role in carcinogenesis and the level of methylation could be used as a biomarker for early detection of cancer.

Materials and Methods

For the study three different groups of men between the ages of 30 to 63 years were examined. Two of the three groups included welders. Demographic data, such as age, height, weight and smokers were measured and collected with questionnaires and are listed in Table 1. Due to the type of welding process and thereby the degree of exposition two groups differed: low exposed (LW) and high exposed welders (HW). The difference between these two groups is the type of welding process. One group works as so called factory mechanics and operates with manual metal arc welding only with steel, which has no carcinogenic effect, and labeled with LW. The other group of welders was involved mainly in two types of welding processes, tungsten inert gas (TIG) welding and Gas metal arc welding process, more precise Metal active gas (MAG) welding, labeled with HW. This group worked with high-grade alloy steel and different metals. The HW had to work in confined space with some more breaks but nevertheless with an exposure of eight hours a day whereby the LW had a daily exposure to the toxic fumes of only five to six hours. The third group was a control group which was matched by gender, age and body mass index (BMI), labeled with C.

The study included a total of 61 men, namely 20 participants in the LW and the control group and 21 in the HW group. Buccal cells were collected of each participant with cotton buds. DNA was extracted with QuickExtract™ DNA Extraction Solution (Epicenter, Chicago, USA). Bisulfite conversion of all samples was done with EpiTect® Bisulfite Kit (Qiagen, Hilden, Germany). Bisulfite modified DNA was amplified with gene-specific primers in the promoter region.

Table 1: Demographic data of the study participants

Parameters	**Low exposed Welders (LW)**	**High exposed Welders (HW)**	**Control (C)**
Age	44.3 ± 8,7	44.8 ± 9,8	43.0 ± 9.1
BMI	26.0 ± 3,2	28.2 ± 3,9	23.6 ± 2.4
Smoker	6 current smokers (30%) 3 occassionally smokers (15%)	21 smokers (100%)	1 current smoker (5%) 2 former smokers (10%)

Hight and body weight to calculate the BMI were measured before sampling, other characteristics were collected by questionnaires.

Table 2: Primers for PCR and Pyrosequencing

Name of primer	Sequence 5'-3'	Size	Annealing Temperature
LINE-1 (F)	TTT TGA GTT AGG TGT GGG ATA TA	23	50 °C
LINE-1 (R)	Biotin-AAA ATC AAA AAA TTC CCT TTC	21	50 °C
LINE-1 (S)	AGT TAG GTG TGG GAT ATA GT	20	
IL-6 (F)	AAA TGT GGG ATT TTT TTA TGA	21	50 °C
IL-6 (R)	Biotin-AAT TCC AAA ACT AAA AAT TTC CT	23	50 °C
IL-6 (S)	ATG TTT GAG GTT TAT TTT GTT	21	
p16 (F)	TGG GGA GAT TTT AGG GGT GTT A	22	58.5 °C
p16 (R)	Biotin-AAC CAA CCC CTC CTC TTT	18	58.5 °C
p16 (S)	GGG AGA TTT TAG GGG TGT TAT AT	23	

PCR conditions were 95 °C for 15 minutes, 45 cycles of 94 °C for 30 seconds, primer specific cf. Table 2 for 45 seconds and 72 °C for 45 seconds, and a final elongation at 72 °C for 10 minutes.

Methylation of three CpGs in the promoter region of LINE-1 and IL-6 and six CpGs in the promoter region of p16 was evaluated. Analysis of methylation was done by pyrosequencing using the PyroMark® Q24 System (Qiagen, Hilden, Germany).

Statistical analysis

IBM® SPSS® Statistics Version 20 (IBM, Armonk, NY) was used for quantitative methylation analysis of LINE-1, IL-6 and p16. Normal distribution of data was tested with the Kolmogorov-Smirnov test. Statistical significance was defined as a p-value <0.05 and was tested with the student's two tailed paired t-test and one-way analysis of variance (ANOVA) with the Tukey post hoc correction. All data shown are mean ± standard deviation (SD).

Results

We analysed the percent of methylation in the promoter region in three different genes, namely LINE-1, p16 and IL-6 in a total of 62 participants. Afterwards, a comparison

between the groups, respectively, a comparison between the welders in total and the control group was carried out.

The methylation of LINE-1 indicated very consistent methylation between the three groups (Fig.1). This supports the assumption of LINE-1 as an indicator of the overall methylation [10].

In IL-6 three CpGs were analysed and the highest value was measured in CpG 3 in the control group with 4.2 % and was significantly increased (p= 0.018) when compared with all welders (Fig.2). Both groups of welders show a tendency of decreased values of methylation in the different CpGs as well as in the mean value.

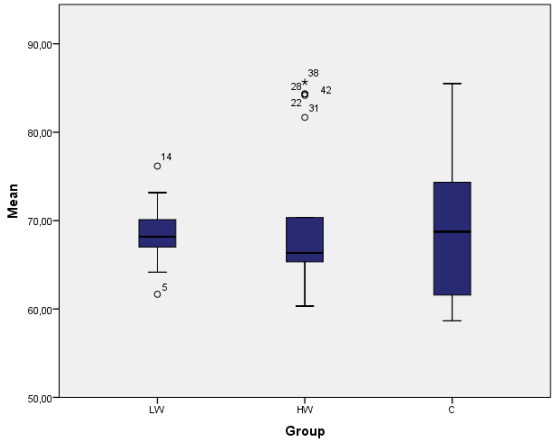


Figure 1: LINE-1 Mean Methylation [%] of all three groups LW (low exposed welders), HW (high exposed welders) and C (control group)

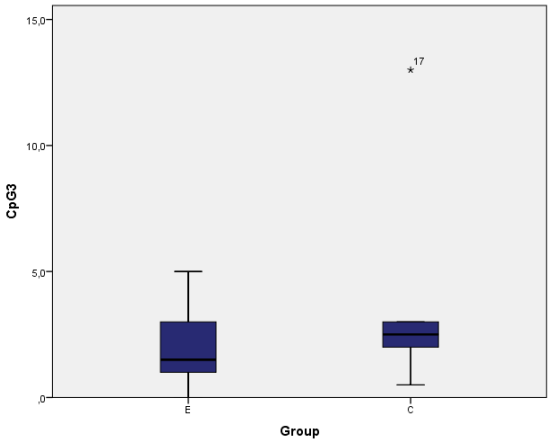


Figure 2: IL-6 Methylation [%] on CpG 3 Comparison between E (all welders in total) and C (control group)

In p16 six CpGs of the promoter region were analysed. CpG 1 and 2 showed increased methylation in both exposed groups when compared with the control group. The methylation in CpG 2 was 64.3 % higher in welders as in the control group but the difference did not reach statistical significance. In all groups at each site average percentage of methylation was between 2.6 % and 12.3 %.

Discussion

Recently there is growing interest in the potential that the environment may influence not only the genome by mutations but also the epigenome by epimutations. The topic of how epigenetic processes can significantly modulate cellular behavior and potentially complex diseases risk, including cancer, especially in response to environmental chemicals is still in its infancy. As there is a widespread use and dispersion in the environment of these environmental agents, including nano-sized materials and PM, at present and predictable in the near future more attention should be paid to this issue [3]. Pulmonary dysfunction including asthma and an increased lung cancer risk with an odds ratio of 1.4 are common diseases of welders [9]. In this study we investigated, if toxic fumes from welding processes increase the risk of cancer due to alteration in the methylation in LINE-1, IL-6 and p16. We compared two different groups of welders with a control group. It was one of the first studies using buccal cells to determine the alteration of methylation. LINE-1, p16 and IL-6 are three genes which play a role in cancer. The aim of the study was to investigate if the level of methylation in these three genes can be used as a biomarker for cancer growth.

There is a great potential of using DNA methylation as a nucleic acid based biomarker for clinical implementation in cancer medicine and a number of recent studies highlighted the prospective utility of this biomarker in lung cancer prognosis, predicting response to therapy. DNA is the most stable biological macromolecule and the methyl groups on the cytosines are part of the covalent structure of the DNA which enables a relatively easily transformation from a research laboratory setting into routine diagnostic. Biomarkers with sufficient sensitivity and specification are needed but so far no single gene has been found that is always methylated in a certain type of cancer. Therefore most likely panels of DNA methylation based biomarkers will be used in future [11, 4].

Around half of the human genome consists of repetitive elements. LINE-1 is the most prevalent repetitive sequence and is dispersed throughout approximately 17 % of the entire gene [12]. Wherefore LINE-1 methylation quantification has relevance as a marker of global DNA methylation. LINE-1 hypomethylation is correlated with genetic changes during carcinogenesis and consequently the methylation of this gene can be

used as a potentially useful tumour marker for the detection of cancer DNA [10, 13, 14]. Furthermore different studies have observed an association between the exposure to air pollution and decreased repetitive element methylation [15].

The result of this study showed no significant alteration in methylation within the three groups in the three determined CpGs. This supports the justification of using LINE-1 as a marker for global DNA methylation.

IL-6 is one of the best-characterized pro-tumourigenic cytokines and plays critical roles in a wide range of biological activities, such as infection, cell proliferation and differentiation and tumour growth. IL-6 is elevated in many cancers such as lung, esophageal and liver cancer [16, 17]. Gasche et al. (2011) demonstrated significant IL-6 induced global LINE-1 hypometylation in an in-vitro model of OSCC cell lines. Furthermore, IL-6 induced CpG promoter methylation changes in several important tumour suppressor genes were observed [18]. Tang et al. came to the result that the activity of IL-6 decreased with increased methylation compared to an unmethylated promoter. Hence it can be assumed that DNA methylation alteration might be involved in the activation of IL-6 [16, 17]. Furthermore a correlation between the methylation of distinct CpG-sites within the IL-6 promoter region and mRNA expression could be observed in several studies [19].

The observed CpG-sites of IL-6 were described in present studies to play important roles in the regulation of IL-6 expression. In general we report light decreases of methylation in promoters of welders in all three measured CpGs and in mean values of methylation assuming increased inflammatory activity.

Nevertheless it should be considered that the methylation levels at the investigated CpG-sites are rather low (1-10 %) wherefore the variation within a group might be higher than the differences between the means of the study groups.

p16 is a tumour suppressor gene and plays a key role in the regulation of the cell cycle by inducing the cell cycle arrest in G1. Due to inhibiting the cyclin dependent kinases CDK4 and CDK6 by p16 the retinoblastoma protein can be inactivated. Hypermethylation of the promoter region of p16 is an important mechanism of gene silencing and could be a potential biomarker for early diagnosis, for as it occurs as an

early event in cancer [20, 21]. DNA hypermethylation of promoter of gene p53 and p16 was reported in arsenic-exposed people with and without malignancy [22]. We analysed six CpGs of the promoter region of p16 and report higher methylation in CpG 1 and 2 in welders. The percentage of methylation at each site was between 2.6 % and 12.3 % whereby the highest and the lowest value was both measured in the high exposed group. Bihl et al., (2012) reported from data of p16 methylation using pyrosequencing ranging from 4.2 % to 11 % in normal tissue. Tumour tissue was almost twice that high, with maximum value of 20.9 % [23].

The benefit working with buccal cells is the simple and fast sample taking. Certainly a disadvantage of this method is the low DNA yields compared to extraction from DNA from blood. The standard deviation is quite high in some CpGs and indicates that the percentage of methylation diversifies within the group. This could be seen as a consequence of the low DNA concentration because it could lead to more fluctuations in the measurement.

Furthermore, it is worth mentioning that different CpG sites within the promoter region and perhaps different CpG sites even within the same promoter region may respond differently to environmental stimuli [7]. This might be a handicap, comparing different studies, when not analysing exactly the same CpG sites.

Conclusion

In summary epigenetic changes due to genotoxic carcinogens may play a quite important role in tumourigenic process. For better understanding and using this information as a biomarker for early cancer detection further investigations, especially in buccal cells are necessary to examine bigger cohorts. Furthermore, it would be interesting if you can deflect these results for a general indication of the hazard of nanoparticles for the human body in reference to epigenetic alteration.

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13 Appendix

13.1 Rapid DNA Extraction Protocol

1. Label the appropriate number of tubes containing QuickExtract DNA Extraction Solution 1.0.

2. Thoroughly rinse out the subject's mouth twice with water. We recommend that subjects abstain from drinking coffee before tissue collection. Alternatively, instruct subjects to gently brush the inside surface of both cheeks with a toothbrush (without toothpaste) followed by a thorough rinsing of the mouth with water.

3. Collect tissue by rolling the Catch-All sample collection swab firmly on the inside of the cheek, approximately 20 times on each side, making certain to move the brush over the entire cheek. If storing or transporting the sample, air dry the swab for 10-15 minutes at room temperature. Store the dry swab in the original packaging at 22-37 °C for up to one week before extracting the DNA. For longer storage, place the dry swabs in the original packaging at –20 °C for up to 6 months. Yield is directly correlated with the starting amount of buccal cells. If yield is not a concern, use only one swab; if yield must be maximized, use a separate swab for each cheek surface, and if necessary, use a third swab, collecting tissue from both cheeks.

4. Place the swab end of the Catch-All sample collection swab into a tube containing QuickExtract DNA extraction solution and rotate the brush a minimum of five times. Press the brush against the side of the tube and rotate the brush while removing it from the tube to ensure most of the liquid remains in the tube.

5. Screw the cap on the tube tightly and vortex mix for 10 seconds. Incubate the tube at 65 °C for 1 minute.

6. Vortex mix for 15 seconds.

7. Transfer the tube to 98 °C and incubate for 2 minutes.

8. Vortex mix for 15 seconds.

9. Measure the amount of DNA using the Pico100 (Picodrop Limited, Hinxton, UK).

10. Store the DNA at –20 °C, or at –70 °C for long term storage.

The yield of DNA is usually between 2-14 ng/µl. The QuickExtract DNA Extraction Solution 1.0 contains the MasterAmp PCR Enhancer (with betaine). The presence of this reagent may change the annealing temperature of a given primer pair. We recommend using 5 µl of extracted DNA in a 50 µl PCR amplification reaction. For target sequences containing high G+C content or secondary structure, we recommend using 5-15 µl of the extracted sample.

13.2 Complete bisulfite conversion and cleanup of DNA for methylation analysis

For bisulfite conversion EpiTect® Bisulfite kit was used (Qiagen, Hilden, Germany). DNA amounts of 1 ng-2 µg in a volume of up to 20 µl can be processed using this standard protocol.

The methylation status of a DNA sequence can be determined using sodium bisulfite

One of the best ways to determine the methylation status of a DNA sequence is by the use of sodium bisulfite. The incubation of the target DNA with sodium bisulfite results in the conversion of unmethylated cytosin residues into uracil, while methylated cytosines remain unchanged. The most critical step is the complete conversion of unmethylated cytosines, which is achieved by incubating the DNA in high bisulfite salt concentrations at high temperature and low pH. These harsh conditions often results in a high degree of DNA fragmentation and subsequent loss of DNA during purification. Purification is necesarry to remove bisulfite salts and chemicals used in the conversion process that inhibit sequencing procedures.

The EpiTect Bisulfite Kit provides a fast 6-hour procedure for efficient conversion and purification of as little as 1ng of DNA. DNA Protect Buffer prevents DNA fragmentation during the bisulfite conversion and enables confirmation of the correct pH for cytosine conversion. A high cytosine conversion rate of over 99 % can be obtained with the bisulfite thermal cycling program and optimized series of incubation. Carrier RNA is provided when using less than 100 ng genomic DNA template to enhance the binding of small quantities of DNA to the EpiTect spin column membrane.

The final step in chemical conversion of cytosines is the desulfination and is achieved by a convenient on-column step included in the purification procedure.

Important points before starting

Each aliquot of Bisulfite Mix is sufficient for 8 conversion reactions. If converting fewer than 8 DNA samples, dissolved Bisulfite Mix can be stored at –20 °C for up to 4 weeks without any loss of performance.

DNA Protect Buffer should turn from green to blue after addition to DNA–Bisulfite Mix (step 2), indicating sufficient mixing and correct pH for the bisulfite conversion reaction.

Perform all centrifugation steps at room temperature (15–25 °C).

Things to do before starting

- Add 30 ml ethanol (96–100 %) to Buffer BW and store at room temperature (15–25 °C). Invert the bottle several times before starting the procedure.
- Add 27 ml ethanol (96–100 %) to Buffer BD and store at 2–8 °C. Invert the bottle several times before starting the procedure and make sure to close the bottle immediately after use. White precipitates may form in the Buffer BD–ethanol mix after some storage time. These precipitates will not affect the performance of Buffer BD. However, avoid transferring precipitates to the EpiTect spin column.
- Add 310 µl RNase-free water to the lyophilized carrier RNA (310 µg) to obtain a 1 µg/µl solution. Dissolve the carrier RNA thoroughly by vortexing.
- When processing 48 samples at once, add the complete volume of dissolved carrier RNA to the bottle of Buffer BL, and check the box on the bottle lid label. If processing fewer samples, split the dissolved carrier RNA into conveniently sized aliquots (e.g., 50 µl) and store at –20 °C. Aliquots can be stored for up to 1 year. If fewer than 48 conversions will be performed in a 2-week period, then only make up enough Buffer BL–carrier RNA solution as required.

Table 10: Carrier RNA and Buffer BL volumes

Number of samples	1	4	8	16	24	48
Volume of Buffer BL*	620 µl	2.5 ml	5 ml	10 ml	15 ml	31 ml
Volume of carrier RNA solution†	6.2 µl	25 µl	50 µl	100 µl	150 µl	310 µl

* The volumes given contain a 10% surplus for pipetting inaccuracies.

† Resulting in a final concentration of 10 µg/ml carrier RNA in Buffer BL.

Carrier RNA enhances binding of DNA to the EpiTect spin-column membrane, especially if there are very few target molecules in the sample. Carrier RNA is not necessary if >100 ng DNA is used. Add dissolved carrier RNA to Buffer BL. Calculate the volume of Buffer BL and dissolved carrier RNA required for the number of samples to be processed (see Table 10 for example volumes). If Buffer BL contains precipitates, dissolve by heating (maximum 70 °C) with gentle agitation.

- Equilibrate samples and buffers to room temperature.
- Optional: Set a thermomixer, heating block, or heated orbital incubator to 60 °C for use in step 1.

Procedure

Bisulfite DNA conversion

1. Thaw DNA to be used in the bisulfite reactions. Dissolve the required number of aliquots of Bisulfite Mix by adding 800 µl RNase-free water to each aliquot. Vortex until the Bisulfite Mix is completely dissolved. This can take up to 5 min. If necessary, heat the Bisulfite Mix–RNase-free water solution to 60 °C and vortex again. Do not place dissolved Bisulfite Mix on ice.

2. Prepare the bisulfite reactions in 200 µl PCR tubes according to Table 11. Add each component in the order listed. The combined volume of DNA solution and RNase-free water must total 20 µl

Table 11: Bisulfite reaction components

Component	Volume per reaction (µl)
DNA solution (1–500 ng)	Variable* (maximum 40 µl)
RNase-free water	Variable*
Bisulfite Mix (dissolved), see step 1	85
DNA Protect Buffer	15
Total volume	**140**

* The combined volume of DNA solution and RNase-free water must total 40 µl.

3. Close the PCR tubes and mix the bisulfite reactions thoroughly. Store the tubes at room temperature (15–25 °C). DNA Protect Buffer should turn from green to blue after addition to DNA–Bisulfite Mix, indicating sufficient mixing and correct pH for the bisulfite conversion reaction.

4. Perform the bisulfite DNA conversion using a thermal cycler. Program the thermal cycler according to Table 12. The complete cycle should take approximately 5 h.

Table 12: Bisulfite conversion thermal cycler conditions

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	25 min	60°C
Denaturation	5 min	95°C
Incubation	85 min (1 h 25 min)	60°C
Denaturation	5 min	95°C
Incubation	175 min (2 h 55 min)	60°C
Hold	Indefinite*	20°C

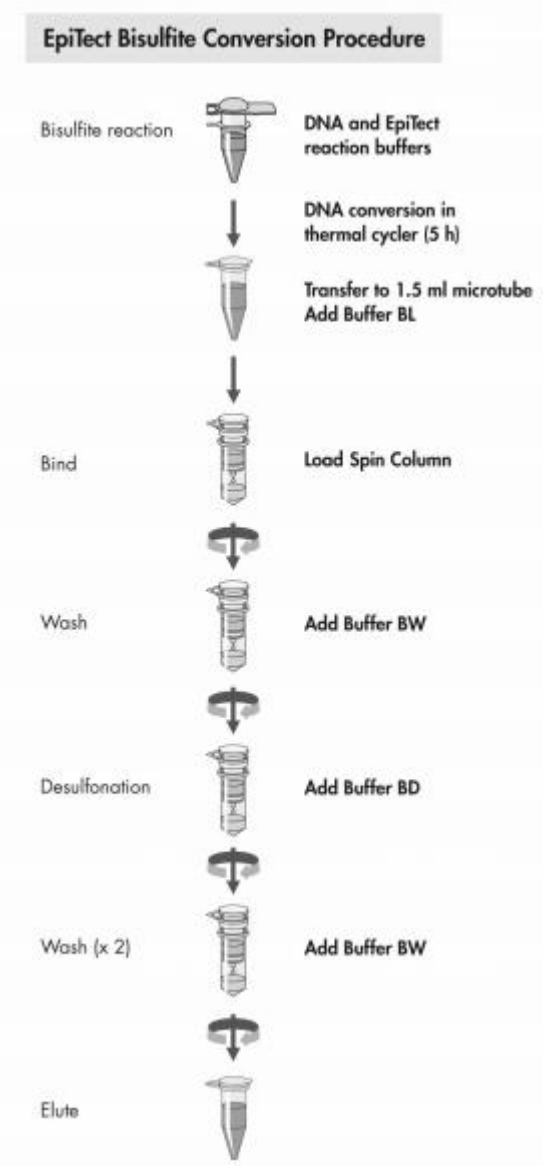
* Converted DNA can be left in the thermal cycler overnight without any loss of performance.

5. Place the PCR tubes containing the bisulfite reactions into the thermal cycler. Start the thermal cycling incubation. Converted DNA can be left in the thermal cycler overnight without any loss of performance.

Cleanup of bisulfite converted DNA

6. Once the bisulfite conversion is complete, briefly centrifuge the PCR tubes containing the bisulfite reactions, and then transfer the complete bisulfite reactions to clean 1.5 ml microcentrifuge tubes. Transfer of precipitates in the solution will not affect the performance or yield of the reaction.

Figure 22: Scheme of bisulfite conversion (Qiagen, Hilden, Germany)



7. Add 560 µl freshly prepared Buffer BL containing 10 µg/ml carrier RNA. Mix the solutions by vortexing and then centrifuge briefly.

8. Place the necessary number of EpiTect spin columns and collection tubes in a suitable rack. Transfer the entire mixture from each tube in step 7 into the corresponding EpiTect spin column.

9. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.

10. Add 500 µl Buffer BW to each spin column, and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.

11. Add 500 µl Buffer BD to each spin column, and incubate for 15 min at room temperature (15–25 °C). If there are precipitates in Buffer BD, avoid transferring them to the spin columns.

    The bottle containing Buffer BD should be closed immediately after use to avoid acidification from carbon dioxide in the air. It is important to close the lids of the spin columns before incubation.

12. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.

13. Add 500 µl Buffer BW to each spin column and centrifuge at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.

14. Repeat step 13 once.

15. Place the spin columns into new 2 ml collection tubes, and centrifuge the spin columns at maximum speed for 1 min to remove any residual liquid.

16. Recommended: Place the spin columns with open lids into clean 1.5 ml microcentrifuge tubes (not provided) and incubate the spin columns for 5 min at 56 °C in a heating block. This step enables evaporation of any remaining liquid.

17. Place the spin columns into clean 1.5 ml microcentrifuge tubes (not provided). Dispense 20 µl Buffer EB onto the center of each membrane. Elute the purified DNA by centrifugation for 1 min at approximately 15.000 x g (12.000 rpm). To increase the yield of DNA in the eluate, dispense an additional 20 µl Buffer EB to the center of each membrane, and centrifuge for 1 min at maximum speed. If the purified DNA is to be stored for up to 24 h, we recommend storage at 2–8 °C. For storage longer than 24 h, we recommend storage at –20 °C. At –20 °C, DNA converted and purified using the EpiTect Bisulfite Kit can be stored for at least 3 years without decrease of quality or conversion.

13.3 PCR amplification of template DNA optimized for Pyrosequencing® analysis

PCR amplification of template DNA was done using PyroMark PCR kit (Qiagen, Hilden, Germany).

Several points have to be checked before starting. One primer must be biotinylated at its 5' end in order to prepare a single-stranded PCR product for use in the subsequent Pyrosequencing procedure. For primer design using PyroMark Assay Design Software 2.0 is recommended. The optimal PCR amplicon length for Pyrosequencing is between 80 and 200 bp, although products up to 500 bp might work well. HotStarTaq DNA Polymerase requires an activation step of 15 min at 95 °C (see step 6 of this protocol). All reaction mixtures should be set up in an area separate from that used for DNA preparation or PCR product analysis. The use of disposable tips containing hydrophobic filters is recommended to minimize cross-contamination.

Procedure:

1. Thaw the PyroMark PCR Master Mix, CoralLoad Concentrate, primer solutions, and 25 mM MgCl2 (if required) at room temperature or on ice. It is important to mix the solutions before use in order to avoid localized concentrations of salt.

2. Set up the reaction according to Table 13. It is not necessary to keep reaction vessels on ice since HotStarTaq DNA Polymerase is inactive at room temperature.

Table 13: Reaction composition using PyroMark PCR Master Mix

Component	Volume/reaction	Final concentration
Reaction mix		
PyroMark PCR Master Mix, 2x	12.5 µl	Contains HotStarTaq DNA Polymerase, 1x PyroMark PCR Buffer,* and dNTPs
CoralLoad Concentrate, 10x	2.5 µl	1x
25 mM $MgCl_2$ (optional)	Variable, see Table 2	See Table 2
Primer A	Variable	0.2 µM†
Primer B	Variable	0.2 µM†
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	≤ 500 ng/reaction‡ or 10–20 ng bisulfite converted DNA
Total volume	25 µl	

*contains 1.5 mM MgCl2, **final primer concentration in PCR reaction of 0.2µM is normally optimal

3. Gently pipet the master mix up and down for thorough mixing and dispense appropriate volumes into PCR tubes.

4. Add template DNA (≤ 500 ng/reaction) to the individual PCR tubes. We recommend 10 ng human genomic DNA or 10–20 ng bisulfite converted DNA.

5. When using a thermal cycler with a heated lid, do not use mineral oil. Proceed directly to step 6. Otherwise, overlay with approximately 100 µl mineral oil.

6. Program the thermal cycler according to the manufacturer's instructions. Each PCR program must start with an initial heat activation step at 95 °C for 15 min.

Table 14: Optimized cycling protocol when using PyroMark PCR Master Mix

			Additional comments
Initial PCR activation step	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step
3-step cycling:			
Denaturation	**30 s**	**94°C**	
Annealing*	**30 s**	**60°C**	For genomic DNA
		56°C	For bisulfite converted DNA
Extension	**30 s**	**72°C**	
Number of cycles	**45**		
Final extension	**10 min**	**72°C**	

*An annealing temperature that gives the highest specificity for the desired PCR product should be used.

7. Place the PCR tubes in the thermal cycler and start the cycling program. After amplification, samples can be stored overnight at 2–8 °C or at –20 °C for longer storage.

8. Use 5–20 µl of a 25 µl PCR for subsequent Pyrosequencing analysis.

Recommended step:

Check your PCR product prior to Pyrosequencing analysis, e.g. by agarose gel analysis. PCR products can be directly loaded onto an agarose gel without prior addition of a PCR loading buffer and gel tracking dyes when using CoralLoad Concentrate. CoralLoad Concentrate contains a gel loading reagent and gel tracking dyes.

13.4 Reagents

DNA Extraction

- QuickExtract™ DNA Extraction Solution 1.0 (Epicenter, Chicago, USA)

EpiTect Bisulfite Kit (48): (Qiagen, Hilden, Germany)

- Bisulfite Mix (Qiagen, Hilden, Germany)
- DNA Protect Buffer (Qiagen, Hilden, Germany)
- RNase-free water (Qiagen, Hilden, Germany)
- Buffer BL (Qiagen, Hilden, Germany)
- Buffer BW (Qiagen, Hilden, Germany)
- Buffer BD (Qiagen, Hilden, Germany)
- Buffer EB (Qiagen, Hilden, Germany)
- Ethanol 96%

PCR: (Qiagen, Hilden, Germany)

- PyroMark PCR Master Mix, 2x (Qiagen, Hilden, Germany)
- CoralLoad® Concentrate, 10x (Qiagen, Hilden, Germany)
- RNase-Free Water (Qiagen, Hilden, Germany)

Pyromark: (Qiagen, Hilden, Germany)

- Streptavidin-coated Sepharose® beads (GE Healthcare, Vienna, Austria)
- Pyromark binding buffer (Qiagen, Hilden, Germany)
- PyroMark Annealing Buffer (Qiagen, Hilden, Germany)
- 70% Ethanol
- Denaturation solution (Qiagen, Hilden, Germany)
- Wash Buffer (Qiagen, Hilden, Germany)
- PyroMark Gold Q24 Reagents (Qiagen, Hilden, Germany)

Gelelectrophoresis:

- TAE buffer (50x) (genXpress Service & Vertrieb GmbH)
- Biozym LE Agarose (Biozym, Wien, Austria)

Primer:

p16: designed with primer design software (Qiagen, Hilden, Germany)

- p16 (fw) (©Biomers.net GmbH)
- p16 (R*) (©Biomers.net GmbH)
- p16 (S) (©Biomers.net GmbH)

IL-6: designed with primer design software (Qiagen, Hilden, Germany)

- IL-6 (fw) (©Biomers.net GmbH)
- IL-6 (R*) (©Biomers.net GmbH)
- IL-6 (S) (©Biomers.net GmbH)

LINE-1: was chosen from a paper of Bollati et al. (2007) [Bollati et al. 2007]

- LINE-1 (fw) (©Biomers.net GmbH)
- LINE-1 (R*) (©Biomers.net GmbH)
- LINE-1 (S) (©Biomers.net GmbH)

*biotinylated

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