Polymorphisms in the inflammatory and cellular stress related genes and risk of lung cancer

by

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Thesis submitted for the degree of

Philosophiae Doctor

UiO : Faculty of Medicine
University of Oslo

March 2011

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Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 1197

ISBN 978-82-8264-040-4

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

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To Anne and Elina

Acknowledgements

The work presented in this thesis was carried out at the National Institute of Occupational Health (NIOH), Oslo, between 2005 and 2008. I am grateful to the NIOH and The Norwegian Research Council for financially supporting this project.

I would like to express my sincere gratitude to my supervisor Dr. Shanbeh Zienolddiny for giving me the opportunity to work in his group and on this project. His enthusiasm and passion for research and his ability to encourage his co-workers is highly appreciated. Also, I would like to thank Professor Aage Haugen for being my co-supervisor and for sharing his scientific experience and knowledge in this project.

I would like to thank senior scientist David Ryberg for sharing his knowledge and enthusiasm for statistics and bioinformatics which in turn has inspired me greatly in this project. I am grateful to all my co-authors for their valuable contributions throughout various parts of this project. Professor Anne-Lise Børresen-Dale is acknowledged for being my internal supervisor at the Faculty of Medicine, University of Oslo.

I thank all present and former members of Section for Toxicology for being great colleagues during my years at NIOH. Especially, I would like to give my best wishes to Carlos Sagredo, Heidi Uppstad and Nina Landvik.

I would like to thank my family and friends for support, and also the sporty members of "STAMIs gym" for great company and sometimes hilarious conversations. I thank my colleagues at the Norwegian Asthma and Allergy Association for all their support during the completion of this thesis.

A special thank to my loving wife Anne, for all her support and for believing in me during my years at NIOH. And last but not least, I thank our lovely and fantastic daughter Elina for lots and lots of joyous moments and a much appreciated and needed distraction from life in the bubble of science.

Oslo, March 2011

Kent Hart

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List of papers

The results from this project have been published in three papers which will be referred to in this thesis by the roman numerals I – III:

Paper I

Kent Hart, Aage Haugen, Shanbeh Zienolddiny. Allele-specific induction of *IL1B* -31T/C promoter polymorphism by lung carcinogens. Mutation Research 656: 14-18, 2008.

Paper II

Nina E. Landvik, Kent Hart, Vidar Skaug, Lodve B. Stangeland, Aage Haugen, Shanbeh Zienolddiny. A specific interleukin-1B haplotype correlates with high levels of *IL1B* mRNA in the lung and increased risk of non-small cell lung cancer. Carcinogenesis 30: 1186-1192, 2009.

Paper III

Kent Hart, Nina E. Landvik, Helge Lind, Vidar Skaug, Aage Haugen, Shanbeh Zienolddiny. A combination of functional polymorphisms in the *CASP8*, *MMP1*, *IL10* and *SEPS1* genes affects risk of non-small cell lung cancer. Lung Cancer 71: 123-129, 2010.

Commonly used abbreviations and explanations

Acronym	Explanation
Allele	One of two or more variants of a gene
BaP	Benzo[a]pyrene
Carcinoma	Cancer of epithelial origin
CS/CSC	Cigarette smoke/cigarette smoke condensate
Etiology	The study of the causes of disease
ER	Endoplasmic reticulum
Haplotype	Closely linked variants inherited together as a unit
IL	Interleukin; group of cytokines such as IL1, IL6, IL8
IL1B	IL-1 beta gene
IL-1β	IL-1 beta protein
In vitro	Outside a living organism; in an artificial environment
In vivo	Within a living organism
LD	Linkage disequilibrium
Mutation	Permanent (inheritable) change in the DNA sequence
NF-κB	Nuclear factor kappa beta; a transcription factor
NSCLC	Non-small cell lung cancer
PAH	Polycyclic aromatic hydrocarbon
Polymorphism	Common variation in DNA; present in more than 1% of the population
ROS/RNS/RONS	Reactive oxygen/nitrogen species
rSNP	Regulatory SNP
SCLC	Small cell lung cancer
SNP	single nucleotide polymorphism; simple nucleotide polymorphism

Introduction

Lung cancer

Epidemiology and etiology

Lung cancer is the leading cause of cancer-related mortality worldwide. The common cause is by large tobacco smoking [1], but radon [2], asbestos [3], infectious agents [4], second hand smoking [5], and exposure to cooking fumes [6] have also been recognized as important risk factors. In addition, exposure to the various factors at working environment (occupational factors) may play an important role. It has been estimated globally that 10% of lung cancer deaths in men and 5% in women were attributable to exposure to a variety of lung carcinogens such as asbestos, arsenic, nickel, chromium, cadmium, diesel fumes, polycyclic aromatic hydrocarbons (PAHs), and silica which are often found in many working environments [7]. In Norway, lung cancer is among the most common cancer types with over 2500 diagnosed cases in 2008 and a 5-year survival of 11.0% for men and 14.3% for women [8]. Lung cancers are divided into small cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). NSCLC is further divided into three major histological subtypes; squamous cell carcinoma (SCC), adenocarcinoma (AC) and large cell carcinomas (LCC) [9]. All histological types of lung cancer are associated with smoking, although the association is strongest for SCLC and for SCC [10]. Approximately 15% of lifetime smokers develop lung cancer, and it has been estimated that 10% of male and 20% of female lung cancer cases are never smokers [11,12]. Lung cancer in never smokers is observed more frequently among females with a higher proportion of ACs and an earlier age at diagnosis [9,12]. Studies have shown that the etiology, clinical characteristics, and prognosis of lung cancer in never smokers are different from those in smokers [13]. Although the causes of lung cancer in never smokers are poorly understood, exposure to occupational and environmental factors, second-hand smoke [5], hormones, and infection may be involved [9,13].

Chronic inflammatory lung diseases such as chronic obstructive lung disease (COPD), asthma, emphysema, pneumonia and tuberculosis have to some extent been associated

with increased risk of lung cancer [14]. COPD and emphysema are co-morbid conditions often found in lung cancer patients [15]. The prospective and retrospective studies show that spirometric evidence of COPD is found in 40–60% of smokers diagnosed with lung cancer [16].

Lung carcinogenesis

Lung carcinogenesis is a multi-step process involving carcinogens, co-carcinogens and tumor promoting factors [17-19]. Upon exposure to various factors (Figure 1), a single initiated cell may acquire a mutation (initiation step) in critical genes leading to growth advantage that may produce a clone of mutated cells which forms a premalignant mass (promotion step). Some of the preneoplastic cells may acquire additional mutations and become malignant (progression).

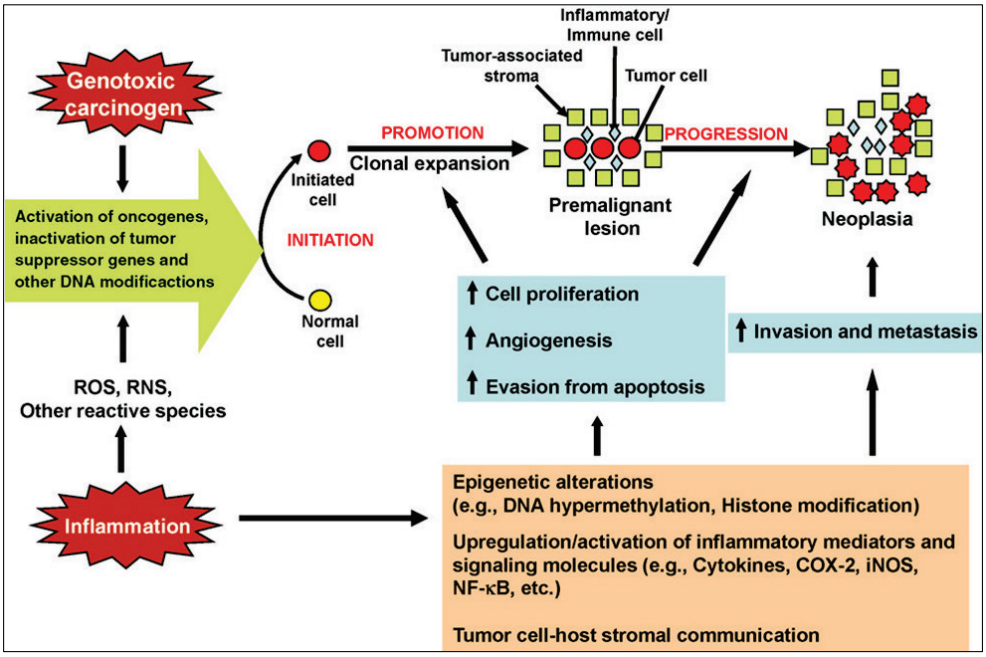

Figure 1. Multi-stage carcinogenesis. Adopted with permission from [19].

Cigarette smoke is a complex mixture containing over 4000 different compounds, with more than 60 compounds classified as carcinogens. The major carcinogens include PAH, nitrosamines, aromatic amines, aldehydes and other volatile organic compounds, metals and reactive oxygen/nitrogen species (ROS/RNS) [20,21]. Most of the chemical carcinogens require metabolic activation to form intermediates that are often more reactive than the parent compound and can form DNA adducts [1]. Benzo(a)pyrene (BaP), an abundant PAH, and an important constituent of tobacco smoke, is converted into 7,8-diol-9,10-epoxide (BPDE) which is highly reactive and capable of forming BPDE-DNA adducts, especially with guanine [22]. The nucleotide excision repair (NER) is the major DNA repair pathway responsible for repairing bulky DNA adducts such as BPDE. If unrepaired, such lesions increase the risk of somatic mutations and the induction of carcinogenesis [23]. Another constituent of tobacco smoke, NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) is converted into NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol), leading to the formation of various methyl-DNA adducts [24]. The alkyl-DNA adducts are also mutagenic and are repaired by the DNA alkyl transferases and specific DNA glycosylases [25]. Production of ROS/RNS through oxidative stress may lead to formation of 8-oxo-G, a highly mutagenic DNA adduct. This mutagenic adduct is repaired by the 8-oxo-specific DNA glycosylase, OGG1 [26]. The impact of DNA-adducts seems to be important in all stages of lung carcinogenesis particularly during the initiation step [20].

Lung carcinoma displays multiple genetic alterations, however, aberrations such as *TP53* mutations, inactivation of the pathway that controls RB1 and LOH on chromosome 3p are frequent in all histological types [27]. The tumor suppressor gene *TP53* is mutated in about 50% of NSCLC and in more than 70% of SCLC cases [28-30]. Loss of TP53 function is an early event in lung tumorigenesis and has been observed in preneoplastic lesions, such as bronchial epithelial dysplasia [31,32]. TP53 is regulated through multiple mechanisms, but the E3 ligase MDM2, Ring Finger and WD repeat domain 2 (RFWD2) and Ring finger and CHY zinc finger domain containing 1 (RCHY1) play important roles [33-35]. Alterations in *RB* are commonly found with loss of RB in SCLC types of cancer (80%) compared to NSCLC (20%), whereas $p16^{INK4A}$/CDKN2A the inhibitor of RB is more commonly altered in NSCLC. Alterations of both genes lead to disruption of cell cycle control by the RB-pathway [36]. *KRAS* and *EGFR* are the two most frequently mutated genes in AC of the lung [37]. In lung

AC, 15–25% of cases harbor a *KRAS* mutation and tumors from smokers are more likely to have KRAS mutations than tumors from non-smokers [38]. It has been shown that in lung cancer cells approximately 30% of GC to TA transversions occurs at hot spot codons 248 and 273 of the *TP53* gene and at codons 12, 13 and 61 of the *KRAS* gene (85% of cases) [39,40]. A similar mutation spectrum and pattern in the *TP53* gene has been detected *in vitro* by Smith et al. in bronchial epithelial cells [41].

The main epigenetic alterations associated with lung tumorigenesis [42] are DNA promoter hypermethylation, DNA hypomethylation [43], posttranslational modification of histones [44,45], chromatin remodeling, and microRNA silencing by DNA hypermethylation [46,47]. The CpG island methylator phenotype (CIMP) involving methylation of any one of six genes (*hOGG1, RAR-B, SEMA3B, RASSF1A, BLU*, or *FHIT*) on short arm of chromosome 3 (3p) has been linked to increased risk of NSCLC. Methylation of at least three genes was found in 43.8% of peripheral blood mononuclear cell (PBMC) specimens from NSCLC patients and only in 6.3% of normal PBMC samples [48].

About 3% of human genes encode for micro-RNAs (miRNAs) [49], and they are estimated to regulate approximately 30% of the human genome primarily through translational repression. miRNAs have the capacity to specifically inhibit translation initiation or elongation as well as induce mRNA destabilization by targeting the 3'-untranslated regions (UTR) of mRNA [49]. The miR-17-92 cluster composed of seven mature miRNAs (miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b and miR-92-1), residing in intron 3 of the C13orf25 gene at 13q31.3, is frequently overexpressed in lung cancers [50]. Findings in the literature clearly point to crucial roles for let-7 and miR-17-92 in the pathogenesis and progression of lung cancer, as they appear to affect the machinery of two key cellular functions, stemness maintenance and cell-cycle regulation. Several relevant targets for let-7 and miR-17-92 have been identified, and are suggested to play roles in cancer development [51].

Genetic polymorphisms

The most common genetic variations are single nucleotide polymorphisms (SNPs) and have been estimated to occur at an average of every 100-300 bp in the human genome [52].

The term SNP has also been used in the terms of *simple nucleotide polymorphisms* including short insertions, deletions and repeats. SNPs in protein encoding genes can influence a phenotype either by changing the function or quantity of the encoded protein [53,54]. The variants of SNPs that are located within non-coding regions of the genome can impact gene regulatory sequences such as promoters, enhancers, and silencers, and modulate sequences within the 5'UTR and 3'UTR. Especially the SNPs residing within the regulatory regions of the genome may result in altered regulation of gene transcription [53,55]. Recent research suggests that about 50% of genes have one or more common regulatory SNPs (rSNPs) [53,56-59]. Transcription factor (TF) binding sites are attractive regions to search for functional rSNPs [55]. In some cases a SNP may increase or decrease the binding, leading to allele-specific gene expression while in other cases, a SNP may generate a novel binding site (Figure 2). Consequently, the gene can no longer be regulated by the original TF. Thus, functional rSNPs in TF binding sites may lead to differences in gene expression and phenotypes, and ultimately affect susceptibility to disease [55]. Human populations show

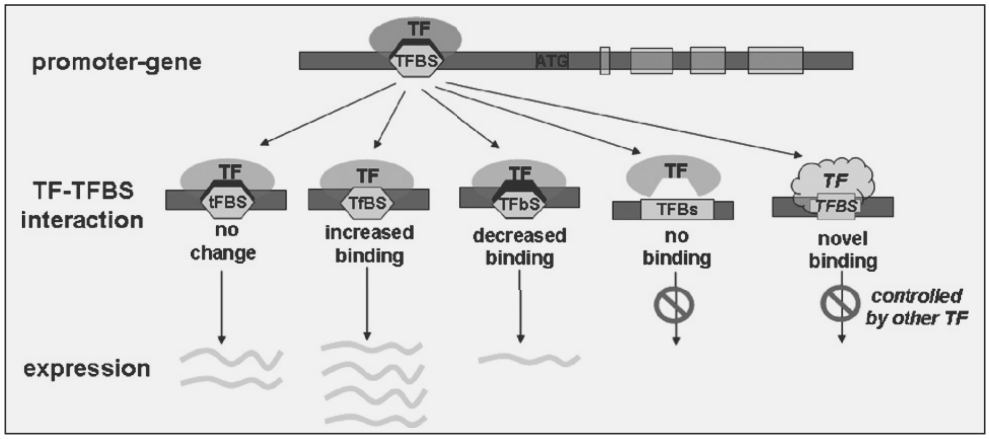

Figure 2. The impact of a SNP in a transcription factor binding site (TFBS). Adopted with permission from [55].

extensive polymorphism, both additions and deletions, and in the number of copies of chromosomal segments, known as copy number variation (CNV). CNVs are found in the germline DNA or may arise as somatic copy number alterations [60,61]. Changes in copy

number might change the expression levels of genes included in the regions of variable copy number, allowing transcription levels to be higher or lower [60]. There are some studies implicating CNVs on chromosomes 5p and 8p in lung tumors [62-64].

Susceptibility markers of lung cancer

The observation that approximately 15% of lifetime smokers develop lung cancer suggests that individuals might differ in their susceptibility to environmental risk factors [9,11]. Increased familial risk of lung cancer is an indication of a genetic contribution [65]. Bermejo and Hemminki estimated that the familial risk of lung cancer among offspring would be expected to increase by about 20% with increased familial tendency to smoke [66]. Recent studies have mapped a major susceptibility locus influencing familial lung cancer risk to chromosome 6q23-25. However, the specific susceptibility gene at this locus remains unknown [67,68]. Through a combination of genetic fine mapping and association studies the *RGS17* gene has been identified as a major candidate susceptibility gene for familial lung cancer risk [67]. However, the precise functional effect of this gene in lung cancer is unknown [65].

The risk genetic variants that might affect lung cancer risk fall into three categories: rare high-risk variants (risk of 10 or higher and prevalence of 1% or less), moderate-risk variants (risk of around 2–5 and prevalence of not more than 5%), and common low-risk variants (risk of between 1.1 – 1.5 and prevalence of more than 5%) [65]. It is believed that alleles with high frequency (typically >10%) and low penetrance contribute substantially to susceptibility to cancer [69].

Functional polymorphisms in the xenobiotic metabolism, DNA repair, inflammatory, and cell cycle pathways may alter lung cancer susceptibility. The role of individual polymorphisms has been evaluated for several genes including the CYP and glutathione S-transferase super families, the DNA repair genes, tumor suppressor or cell cycle genes [70]. Epidemiological studies have demonstrated that low-penetrance, high-prevalence polymorphic phase I and phase II enzymes of the cytochrome P450 system may alter susceptibility to lung cancer [70]. Polymorphisms in genes coding for DNA repair enzymes active in BER (*XRCC1, hOGG1*), NER (*ERCC1, ERCC6, XPD, XPA*), double-strand break repair

(*XRCC3*) and mismatch repair pathways have also been studied in relation to lung cancer risk [11,71-76]. Suboptimal DNA repair capacity is characterized by a reduced capacity for removal of DNA adducts and has been demonstrated to be associated with increased lung cancer risk, in both smokers and non smokers [72,77,78]. Some *ERCC6* polymorphisms in the NER pathway have been shown to be associated with an elevated lung cancer risk. A more extensive meta-analysis identified a slight increase in lung cancer risk for an *ERCC2* 751Gln/Gln genotype [72].

Haplotypes are a combination of alleles at different markers along the same chromosome that are inherited as a unit [79]. An understanding of haplotype structure across segments of chromosomes that contain potential disease genes is likely to increase the value of genetic association studies where haplotypes have been ascertained [79-81]. This may be especially true when genetic or epistatic interactions within a haplotype block contribute to the development of the phenotype in question [82].

Nearly 600 Genome Wide Association (GWA) studies, covering 150 distinct diseases and traits have been published [83]. These studies are carried out with high throughput genotyping techniques designed to assay hundreds of thousands of polymorphisms, and relate these variants to disease or health related traits [84]. Recently, 3 lung cancer GWA studies identified 3 loci, 15q24-25.1, 5p15.33 and 6p21 associated with lung cancer risk. – A common locus variant near rs1051730 and rs803419 on chromosome 15q contains 3 genes encoding subunits of the nicotinic acetylcholine receptor (nAChR), *CHRNA3, CHRNA5,* and *CHRNB4* [85-87]. In addition, three haplotypes (delTTC, insATC, and insTGG) were recently identified by sequence analysis in the 5' promoter region and three at the 3'-untranslated region of the *CHRNA5* locus. Luciferase reporter assays in human lung cancer cell lines also showed that the 5' region haplotypes were statistically significantly associated with changes in *CHRNA5* promoter activity, whereas the 3'-untranslated region variants were not [88]. The 5p15.33 region contains the *TERT* and the *CLPTM1L/CRR9* genes and the 6p21 region contains the *BAT3* and *MSH5* genes [89-90]. Interestingly, some of these risk SNPs were associated with a higher level of DNA-PAH adducts in normal lung tissue [90]. Li et al. published recently a GWA study on never smokers. Several SNPs were identified and this study identified the top SNP as rs2352028 at 13q31.3. A strong correlation was found

between this SNP and alteration of the expression of the *GPC5* gene in never smokers, suggesting *GPC5* as a candidate lung-cancer-susceptibility gene [91].

Recently, a novel class of functional polymorphisms in miRNAs (miRSNPs) was reported and defined as a polymorphism present at or near microRNA binding sites of functional genes that can affect gene expression by interfering with a miRNA function [92]. Hu et al. examined SNPs in four pre-miRNA sequences and found that the rs11614913 SNP was associated with survival in early stage lung cancer patients [93].

Inflammation and lung cancer

A potential link between chronic inflammation and cancer has been suspected for a long time and was initially suggested by Rudolf Virchow as early as 1863 when he postulated that the "lymphoreticular infiltrate" reflected the origin of cancer at sites of chronic inflammation [94]. Over the past two decades our understanding of the inflammatory microenvironment of malignant tissues has supported Virchow's hypothesis, and the links between cancer and inflammation are starting to have implications for prevention and treatment [94,95]. Epidemiological and clinical studies support Virchow's notion indicating that approximately 25% of all human cancers in adults result from chronic inflammation [96].

Recently, the link between cancer and inflammation has become more evident and inflammation has been proposed as the seventh hallmark of cancer [95]. Key features of cancer-related inflammation include the infiltration of white blood cells such as tumor-associated macrophages, the presence of polypeptide messengers of inflammation; cytokines and chemokines, and the occurrence of tissue remodeling and angiogenesis [95]. Two pathways have been identified: the *intrinsic* pathway where genetic events causing neoplasia initiate the expression of an inflammatory microenvironment and the *extrinsic* pathway, where inflammatory conditions facilitate cancer promotion and progression [95]. Key orchestrators at the intersection of the *intrinsic* and *extrinsic* pathway include transcription factors and pro-inflammatory cytokines [97,98].

Chronic inflammatory lung diseases may predispose to lung cancer. A recent meta-analysis of the relationship between asthma and lung cancer risk suggests that the

association might reflect a causal relationship between asthma-induced inflammation and lung cancer [99,100]. Individuals with chronic bronchitis and emphysema may also have an increased risk of lung cancer [101,102]. Joongho et al. suggests in a meta-analysis that HPV is associated with 20%–25% of NSCLC [103]. *Chlamydia pneumoniae* (*C. pneumoniae*) causing respiratory infection may lead to a high incidence of pneumonia where chronic *C. pneumoniae* inflammation correlates with increased risk of lung cancer. According to some studies, smoking assists *C. pneumoniae* to invade the lung [104]. Chronic pulmonary inflammation may develop from unresolved symptomatic acute inflammation or may evolve over time without apparent onset of clinical manifestations [105]. Several studies have recognized the role of tobacco-driven inflammation in lung carcinogenesis. Chronic inflammation is detrimental and will frequently predispose cells for an oncogenic transformation by inducing genomic instability, altering epigenetic state of the genome, increased angiogenesis, and increased cell proliferation [106].

Endotoxins are also recognized as a risk factor in lung carcinogenesis. A high level of lipopolysaccharide (LPS) endotoxin is inhaled during active smoking. Environmental tobacco smoke (ETS) also contains LPS. Larsson et al. showed that smoker's environment contains 120 times higher level of endotoxin than the smoke free indoor air [107]. Various mechanistic arguments have been advanced regarding exposure to endotoxin and carcinogenesis, focusing largely on complex interactions between the innate and adaptive immune systems [108,109].

Common inflammatory mediators including cytokines, chemokines, RNS, COX-2 and Nuclear factor-κB (NF-κB) can lead to cellular conditions favorable for tumor promotion (figure 3) [106,110]. Many effects of chronic inflammation are mediated by NF-κB, a transcription factor that controls the expression of genes such as *IL1B*, *IL6*, *IL8*, *MCP1*, *TNF-*α, *EDN1*, and *ICAM 1* [111]. These genes are involved in inflammation, immune responses, cell cycle, apoptosis, and angiogenesis in a variety of cell types, including epithelial cells, stromal cells, and macrophages [112,113]. Oxidative and nitrative species have an impact on these pathways; RNS can modulate survival signaling molecules such as c-Jun N-terminal kinase and p38 mitogen activated protein (MAP) kinase [114]. Similarly, RNS such as nitric oxide (NO) are able to cause DNA damage, leading to TP53 stabilization and engagement of apoptosis pathways [115]. Inflammatory cells also release metabolites of arachidonic acid, or

eicosanoids, including prostanoids or prostaglandins and leukotrienes [105]. *COX1* is constitutively expressed at relatively low levels, whereas *COX2* is inducible [106]. *COX2* is up regulated in NSCLC and may play a role in the promotion stage [15]. Some polymorphisms in the *COX2* gene have been associated with increased risk of lung cancer [116]. The reciprocal role between NF-κB and inflammatory cytokines makes NF-κB an important factor not only for inflammation, but also for cancer development [117].

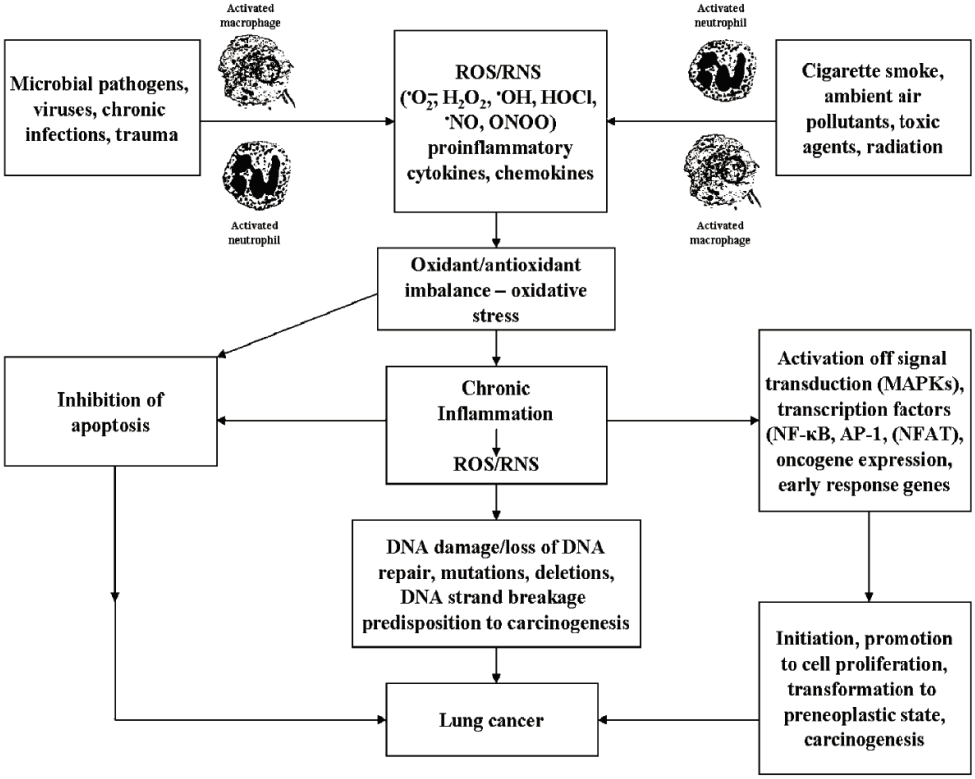

Figure 3. Schematic representation of major events leading to inflammation and lung cancer. Adopted with permission from [110].

Several different subunits of NF-κB have been identified: p65 (also called relA), p50, rel, relB, v-rel, and p52. NF-κB subunits (usually heterodimers) form complexes with IκB (IκBα and IκBβ) in the cytoplasm of unstimulated cells. More than 200 genes have been identified as NF-κB-responsive genes [118] involved in inflammation, immunity, and cell

survival [119]. NF-κB functions as an extrinsic promoter by inducing the influx of inflammatory cells, thus potentiating lung carcinoma metastasis [119].

While the majority of evidence suggests that NF-κB activity is associated with pro-tumorigenic effects, opposite results were found in other studies, indicating a complex role for NF-κB in carcinogenesis [120]. Some reports indicate a requirement for NF-κB activation in *KRAS*-driven lung cancers [121-123]. A recent study by Deng et al. has shown that a loss of the G-protein-coupled receptor C type 5a (Gprc5a) in mouse lung epithelial cells resulted in NF-κB activation accompanied by aberrant cytokine and chemokine expression *in vivo* and *in vitro* [124].

Cytokines are signaling molecules that are key mediators of inflammation or immune response [106], and represent a family of biologic response modifiers including interleukins, chemokines, interferons, growth factors, and leukocyte colony-stimulating factors [105]. The cytokines are secreted by many cell types including leukocytes, connective tissue cells, endothelial cells and also epithelial cells [105]. Chemokines consist of 8- to 10-kd proteins that stimulate leukocyte recruitment and migration as part of the host response to antigenic insults [105]. Cytokines can be generally classified as pro-inflammatory (including IL1, IL6, IL15, IL17, IL23 and TNF-α) or anti-inflammatory (including IL4, IL10, IL13, TGF-β and IFN-α) [106].

The primary sources of IL-1β are the blood monocytes, tissue macrophages, and dendritic cells (figure 4) [125]. However, many cell types including epithelial cells produce and secrete IL-1β upon activation by environmental stumuli [133]. The role of IL-1β in chemical carcinogenesis has been investigated. Krelin et al. examined the effect of 3-methylcholanthrene (3-MCA) in an IL1 transgenic mouse-model. By applying 3-MCA to wildtype and IL1 knock-out mice, *i.e*. *IL-1α*$^{-/-}$, *IL-1β*$^{-/-}$, *IL-1αβ*$^{-/-}$ (double knock out) or *IL1Ra*$^{-/-}$, they assessed the role of host-derived IL-1 molecules with respect to susceptibility to chemical carcinogens. In mice deficient in IL-1β, 3-MCA-induced tumors developed after a prolonged lag period indicating an independent role for IL-1β [134,135].

Another important pro-inflammatory property of IL-1β is its ability to increase the expression of adhesion molecules such as intercellular adhesion molecule-1 of mesenchymal cells and vascular cell adhesion molecule-1 of endothelial cells [125]. Together with the

induction of chemokines, these properties of IL-1β promote the infiltration of inflammatory and immunocompetent cells from the circulation into the extravascular space and then into tissues where tissue remodeling is the end result of chronic IL-1-induced inflammation [125]. IL-1β is also an angiogenic factor [136] and plays a role in tumor metastasis and blood vessel formation [125].

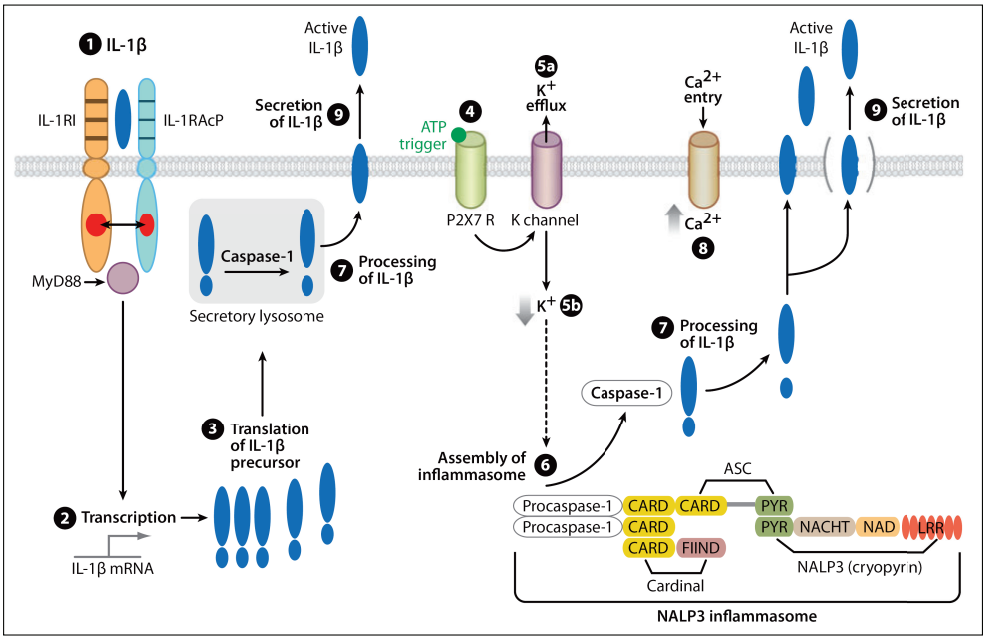

Figure 4. Generalized steps in the synthesis and secretion of IL-1β induced by IL-1β. (1) The IL-1 receptor complex heterodimer is activated by IL-1β and recruits MyD88 pathway leading to (2-3) transcription and translation of the IL-1β precursor. Activated monocytes/mactrophages (4) release ATP into extracellular space activating the P2X7 receptor with a subsequent increase in intracellular potassium levels (5a-5b). The fall in intracellular potassium levels triggers the assembly of the components of the NALP3 inflammasome (6). The assembled components of the inflammasome initiate the processing of procaspase-1, resulting in the formation of the active caspase-1. Active caspase-1 processes the IL-1β precursor (7) in the cytosol or in the secretory lysosome, resulting in the generation of the carboxy-terminal mature IL-1β. An influx of calcium into the cell (8) with an increase in intracellular calcium levels provides a mechanism by which mature IL-1β is released from the cell (9). Other pathways also exist for processed IL-1β to exit the cell. Adopted with permission from [125].

IL1B is highly inducible by proinflammatory stimuli. Its promoter contains a TATA box and the regulatory region also includes enhancer sequences distributed several thousand base pairs upstream from the transcription start site [137]. There are large interindividual

differences in *IL1B* expression, which may be explained genetically by several SNPs scattered in its long regulatory region [82]. Among the best studied SNPs are two in the promoter (C-511T and T-31C) and one in exon 5 (C+3954T) [138]. The C-511T and T-31C polymorphisms are in near-complete linkage disequilibrium and T-31C is a TATA-box polymorphism that markedly affects DNA-protein interactions *in vitro* [139,140]. These polymorphisms may affect *IL1B* expression by changing affinity for transcription factor binding or creating new binding sites for other transcription factors [82,141]. The SNPs may also interact with each other forming various haplotype structures. Several studies have shown that studying SNPs as haplotypes may better explain the interindividual differences in *IL1B* expression [82,141].

Of the anti-inflammatory cytokines, interleukin-10 (IL10) is of particular interest. The major physiological function of IL10 is to regulate macrophages activated by pathogens and their products. IL10 is required to regulate inflammation in both chronic and acute settings [142]. Human *IL10* gene spans about 4.7 kb on chromosome 1q21–32 and contains five exons that are separated by four introns [143]. So far no splice variants are known of *IL10* mRNA [144]. A number of homologs of *IL10* have been found in the genomes of Epstein-Barr virus (EBV), herpesviruses, a poxvirus, and primate cytomegaloviruses [144]. IL10 performs an irreplaceable role in negatively regulating inflammation, primarily through selectively blocking the expression of pro-inflammatory genes encoding cytokines, chemokines, cell-surface molecules and other molecules involved in the propagation of inflammation. IL10 signals via JAK1 and then STAT3 which activates genes whose products may block inflammatory signaling such as the pro-inflammatory cytokines IL-1β, IL6 and TNF-α [142,145]. The production of IL10 is predominantly from T cell subsets, and from macrophages and dendritic cells [142]. SNPs in the *IL10* promoter region have been associated with risk of NSCLC [146].

Expression of most matrix metalloproteinases (MMPs) is tightly regulated at the transcriptional level by hormones, growth factors, cytokines, cell-to-cell and cell-to-matrix interactions [147]. Activated MMPs can be inhibited by the plasma proteinase inhibitor α2-macroglobulin and by the tissue inhibitors of metalloproteinases (TIMPs), specific inhibitors that bind MMPs [147,148]. MMPs are additionally capable of modifying the function of TGF-β and IL-1β cytokines involved in the destruction of lung tissue [149,150]. Chronic cigarette smoking exposure causes increased production of MMPs by macrophages, and their

augmented release may be responsible for lung tissue destruction [151,152]. The gelatinases MMP2 and MMP9 are extensively studied in cancer, and immunohistochemical studies have demonstrated expression of MMP2 and MMP9 in patients with NSCLC [153-156]. In addition, an overexpression of MMP1 may be associated with an overall poor prognosis in lung carcinoma [157]. The MMP1 enzyme is the most expressed interstitial collagenase involved in degradation of extracellular matrix (ECM) during cancer progression [158]. An immunohistochemical study demonstrated that MMP1 was strongly expressed in AC, compared with SCC [159]. The promoter region of *MMP1* contains a guanine insertion/deletion polymorphism at position -1607 relative to the transcriptional start site, with one allele having a single guanine nucleotide (1G) and the other having two (2G) [160]. The -1607 2G/2G variant of the *MMP1* has been shown to cause elevated expression of the gene, more aggressive matrix degradation and early onset of lung cancer [158,160]. Analysis of the promoter of the gene has identified an ETS1 binding site for the 2G variant along with an increased transcriptional activity of the gene [160]. Recently, a cigarette smoke (CS) responsive region in the *MMP1* promoter has been identified, and the 2G variant reveals a higher basal and CS-responsive activity than 1G-allele [161].

The selenoprotein S (SELS/SEPS1), found in the endoplasmic reticulum (ER) membrane, is involved in the stress response within the ER [162]. SEPS1 participates in the processing and removal of misfolded proteins from the ER to the cytosol, where they are polyubiquitinated and degraded through the proteasome [163], and can induce ER-stress [164]. ER-stress is defined as an imbalance between the cellular demand for ER function and capacity of the organelle. It is characterized by a number of intracellular responses. These responses include the ER overload response (EOR), the unfolded protein response (UPR), and apoptosis [165]. A polymorphic variant of *SEPS1* G-105A has been shown to affect serum levels of secreted IL6, IL-1β and TNF-α upon ER-stress [166]. ER stress may be induced in human lung cells by several factors, such as particulate matter and by cigarette smoke [167,168]. The *SEPS1* promoter is GC-rich and contains two NF-κB binding sites in addition to a fully functional ER-stress response element (ERSE), a consensus-binding site for transcription factors regulating ER-stress responses. It has been further demonstrated that the ERSE element in the *SEPS1* promoter is functional since the ERSE element could be activated 2-3 fold by ER-stress [165,169]. The *SEPS1* G-105A single nucleotide polymorphism

is located in the putative ERSE. The A allele confers lower promoter activity than the G allele in response to stimulation with tunicamycin. Furthermore, the A allele is associated with higher cytokine levels [165].

The subset of caspases that cleaves selected substrates to produce the changes associated with apoptosis, are known as 'executioner caspases'. In mammals this subset is represented by caspase-3, caspase-6 and caspase-7. In most instances, executive apoptotic caspases are activated by 'initiator caspases' such as caspase-8 (CASP8). The mechanism of activation of this initiator caspase depends critically on the engagement and activation of recruitment platforms such as the death inducing signaling complex [170]. A novel role for CASP8 enzyme in cleaving the pro-IL-1β protein into active IL-1β in response to TLR4 stimulation has also been described in macrophages [171]. Another role for CASP8 in NF-κB activation has also been suggested where CASP8 may act as a scaffolding protein bringing the IκK complex in close proximity to its activator TAK1, a MAPKKK activated protein during IL1/TLR signaling [172]. A functional polymorphism (-652 del/ins) in the promoter of *CASP8* gene has been found to be associated with risk of several cancers including lung cancer [173]. The -652 del variant abolishes an SP1 transcription factor binding site and is associated with decreased RNA levels, lower CASP8 enzyme activity and lower apoptotic activity in T lymphocytes [173].

Aims of the study

The main goal of the study was to explore genetic variants affecting inflammatory and cellular stress related pathways and risk of lung cancer. More specifically we aimed to:

- Perform functional studies of the *IL1B* T-31C polymorphism in relation to exposure to lung carcinogens.
- Investigate the role of haplotypes of *IL1B* enhancer-promoter polymorphisms in relation to risk of lung cancer.
- Investigate the possibility of combinatory effects of several polymorphisms in genes involved in inflammation and cellular stress.

Summary of the papers

Paper I

Allele-specific induction of *IL1B* -31T/C promoter polymorphism by lung carcinogens.

Several polymorphisms in the *IL1B* gene have been identified, and some are associated with increased risk for lung cancer. Especially, the *IL1B* T-31C polymorphism has received attention. We have investigated the effect of cigarette-smoke condensate (CSC) and benzo[a]pyrene (BaP) on the promoters of the *IL1B* gene varying only at the site of the T-31C polymorphism. The promoter fragments containing either C or T were cloned in luciferase reporter vectors and transfected into human lung epithelial NCI-H2009 cells. The results show that treatment of the transfected cells with CSC or B[a]P induced the promoter significantly above the control level. Interestingly, the promoter with the wild-type allele T in position -31 showed the stronger induction when compared with the promoter with variant allele C in this position. *Jn silico* and DNA-protein analysis indicated the presence of a novel transcription-factor binding site for the YY1 transcription factor and the formation of distinct protein complexes at the C promoter.

Paper II

A specific interleukin-1B haplotype correlates with high levels of *IL1B* mRNA in the lung and increased risk of non-small cell lung cancer.

Our previous work showed that two promoter SNPs C-511T and T-31C modulated NSCLC risk. In the present study, we show that G-3893A and G-1464C located in the enhancer region of the *IL1B* gene may also affect this risk, with odds for developing NSCLC being 0.69 [95% confidence interval (CI), 0.52-0.92] for -3893 A-allele and 0.63 (95% CI, 0.47 - 0.83) for -1464 C-allele. The associations were particularly prominent in patients with *TP53* mutations

in the lung tumor. Inference of the haplotype structures showed that -3893 G, -1464 G, -511 C and -31 T formed a specific haplotype (GGCT) with near complete linkage disequilibrium in lung cancer patients but not in controls. Furthermore, the risk haplotype (GGCT) was present in 65% of cases compared with 36% of controls. Quantitative analysis of RNA in normal lung tissue of the patients showed that the risk haplotype was correlated with significantly higher *IL1B* messenger RNA (mRNA) levels compared with the non-risk haplotype (ACTC). These data suggest that a specific *IL1B* haplotype associated with increased *IL1B* gene expression increases the risk of NSCLC.

Paper III

A combination of functional polymorphisms in the *CASP8, MMP1, IL10* and *SEPS1* genes affects risk of non-small cell lung cancer.

In the present study we have investigated whether a combination of potential functional polymorphisms in genes related to inflammation may modulate risk of NSCLC. Eleven functional polymorphisms in nine genes were analyzed for association with risk of NSCLC in subjects from the Norwegian population. The results showed that individuals carrying combination of three functional polymorphisms in the *CASP8, MMP1, IL10*, and *SEPS1* genes had two-fold increased risk of NSCLC (OR 2.06 (95% CI, 1.19-3.47) whereas individuals with four risk genotypes had 4.62-fold increased risk (OR 4.62, 95% CI, 1.69-12.63). These results highlight the need to investigate the combinatory effects of multiple SNPs.

Discussion

The carcinogenic process may be influenced by both environmental factors and genetic variations in genes controlling various cellular processes. For instance, SNPs in the genes controlling metabolism and detoxification of carcinogens, DNA repair, apoptosis, cellular stress and inflammation have been found to modulate the risk of lung cancer [70,174,175]. Furthermore, the risk may also be modified by gene-gene interactions and interaction with environmental factors. Although the link between inflammation and lung cancer is documented in several reports, the role of polymorphisms in the key inflammatory genes has not been well studied.

IL-1β is a key pro-inflammatory cytokine that has been extensively studied in several diseases including cancer [125,134,135,176]. An association between *IL1B* C-511T and T-31C SNPs and risk of gastric cancer has been reported and confirmed in a recent meta-analysis [139,177]. The association between these IL1 polymorphisms and risk of lung cancer was first reported by Zienolddiny et al. [178]. Previous studies have shown that *IL1B* T-31C and C-511T are in almost complete LD. Subsequent investigations showed that the risk allele (T allele) of the T-31C polymorphism was in fact associated with an increased basal activity of the *IL1B* promoter in human lung cells [140]. We questioned whether lung carcinogens such as BaP and CSC could affect the promoter activity in human lung cells. We found that both BaP and CSC led to higher induction of the promoter with the T allele in a luciferase promoter assay system. Further molecular studies of the nuclear extracts from carcinogen-treated lung cells indicated presence of two C allele-specific DNA-protein complexes. The *in silico* search for possible transcription factors that may be bound differentially to T and C alleles suggested a novel binding site for the YY1 transcription factor to the -31 C promoter variant. One study has provided further support for the functional properties of the T-31C polymorphism. The expression of *gastrin* is modulated by the altered expression of *IL1B* due to the T-31C polymorphism. The *gastrin* promoter assays showed that IL1β inhibits *gastrin* expression at the transcriptional level and part of this inhibitory process is mediated via activation of NF-κB and involvement of histone deacetylases (HDACs) [179]. A recent study has indicated that BPDE stimulates an inflammatory response mediated through a TP53 and

JNK mediated pathway. Real time RT-PCR and ELISA revealed a time and dose-dependent-induced expression and production of *COX2*, *PGE2*, *IL1B*, *IL6* and *IL8* [180].

The *IL1B* gene expression is also regulated by enhancer elements outside of the proximal and core promoter regions. A recent study has shown that some of the polymorphisms located in the enhancer region may be functional and that haplotypes including several polymorphisms from the enhancer and proximal promoter regions may be more important than any polymorphism alone [82]. We therefore investigated the *IL1B* polymorphisms from the enhancer region and the haplotypes including the proximal promoter polymorphism and risk of lung cancer. We found that the risk alleles -3893 G and -1464 G from the enhancer region were associated with decreased risk of lung cancer and the associations were particularly prominent in patients with *TP53* mutations in the lung tumor. The mutational pattern showed a trend toward insertions or deletions that are specific types of mutations often detected in cancers with a strong inflammatory component [181,182]. Interestingly, a specific haplotype including risk alleles of the SNPs from the enhancer region and the proximal promoter was more frequent in lung cancer cases than healthy controls. Furthermore, individuals with this haplotype had higher expression of the *IL1B* mRNA in the lung (Paper II) [82,141]. The noteworthiness of the associations was examined applying False Positive Report Probability (FPRP) and Bayesian false-discovery probability (BFDP) tests, [183,184]. Associations for G-1464C and G-3893A were found noteworthy by both FPRP and BFDP, suggesting that chance alone is unlikely to explain these findings. On the basis of "noteworthiness", the G-1464C and G-3893A SNPs were included in our haplotype study. A specific haplotype, GGCT, was found in 65% of cases compared to only 36% in the control group. The LD contrast test [185] was performed on the *IL1B* SNP haplotype blocks with 200,000 permutations yielding a $P < 0.001$, indicating a robust finding. The second most common haplotype, G/ACTC, was evenly distributed between cases and controls. Data on larger *IL1B* haplotype blocks exist for control groups in Taiwan Chinese, US Caucasians and African-American populations [186,187]. This suggests that there are differences between ethnic groups that should be investigated in future studies. African-Americans carrying an *IL1B* -511T/-31C/+3954T haplotype, were more likely to be diagnosed with intestinal metaplasia or dysplasia than those carrying a common T-C-C haplotype. Carriage of *IL1B* +3954T allele was suggested to be the key factor, but this association was not significant for

Caucasians [188]. However, a recent GWA study investigating 10 potential lung cancer susceptibility variants failed to replicate this association [189], adding support to the lack of association for SNP +3954 in our data.

It is likely that polymorphisms in several genes in the inflammation and cellular stress pathways may interact to modify the risk of lung cancer. We hypothesized that SNPs that may not confer a risk individually, may increase or decrease the risk in combination with other SNPs. The results published in paper III suggest that among the genes identified with weak to moderate associations, a significant increase in the risk of NSCLC may require a combination of at least three functional polymorphisms in the *SEPS1*, *MMP1*, *CASP8* and *IL10* genes. We also found noteworthiness in FPRP and BDFP tests for this combination which further strengthens the results, however, since the number of cases or controls that carry all four risk alleles is low, the data should be interpreted with caution. Increasing the number of samples and relevant SNPs in this type of combinatory analysis may help to further strengthen the data, and may also aid in identifying potential subgroups. A recent meta-analysis of 50 studies including more than 38,000 individuals examining polymorphisms in the *MMP1* and *MMP3* genes showed that *MMP1*, -1607 2G/2G genotype carriers had an increased risk of colorectal, head and neck, and renal cancer [190]. Furthermore, a recent meta-analysis of 55 studies including more than 100,000 individuals examining polymorphisms in the *CASP8* gene showed that the minor alleles of D302H and -652 6N del were significantly associated with overall decrease in cancer risk [191]. The 6N ins/del polymorphism generates a new Sp1 binding site, resulting in differential levels of *CASP8* gene expression. In assays where tumor-infiltrating lymphocytes encounter tumor cells, the median apoptosis rate of T lymphocytes with the *CASP8* -652 6N del allele was significantly lower. Whether the 6N del variant also present in the tumor cells may be expected to reduce apoptosis and thus be associated with increased cancer risk is currently not known [173]. The SNPs *IL10* -592 and -819 are in almost complete LD [192]. The *IL10* -819 has been reported positively associated with lung cancer among never smokers [193]. The association of *IL10* -592 with lung cancer was a significant main effect from our study found noteworthy by both FPRP and BDFP tests. Data suggest that a primary effect of IL10 on lung cancer cells may be to increase metastatic potential by promoting angiogenesis and resistance to apoptosis [194]. One study reported that the *IL1B* C-511T SNP may interact with *SEPS1* G-

105A SNP affecting risk of rheumatoid arthritis [162]. We found a similar trend in our data, albeit not statistically significant, for the *IL1B* -31 T/T and *SEPS1* -105 G/G genotypes and increased expression levels of *IL1B* mRNA. The lack of significance may be due to insufficient number of samples available and should be investigated in a larger study. *SEPS1* is a gene involved in stress response in the endoplasmic reticulum and inflammation control [166]. Previous reports have provided evidence for activation of NF-κB, a major regulator of ER stress. However, recent investigation also suggested that preceding ER stress suppresses activation of NF-κB by subsequent exposure to inflammatory stimuli [195]. The molecular mechanisms are not very well understood, but potential triggers of ER stress during inflammation have been suggested to be bacteria, virus, ROS, NO and cytokines such as IL-1β, IL6, and TNF-α [195].

Concluding remarks

The results presented in this thesis provide further evidence for involvement of inflammation in lung carcinogenesis. The results from paper I provide evidence for differential induction of the *IL1B* -31 T/C variants by typical lung carcinogens found in tobacco smoke, work place and the environment. However, the results need verification in a larger panel of lung cell lines including normal bronchial epithelial cells as well as cells from other tissue types. The putative binding of YY1 to the C allele should be verified by detailed molecular studies. Results from Paper II and III suggest that a combination of SNPs in the *IL1B* and also four other genes involved in inflammation and cellular stress may lead to increased risk of lung cancer for carriers of three or more risk alleles. These results need confirmation in a larger sample size and also other ethnic groups. Furthermore, the effect of *IL1B* haplotypes should be investigated *in vitro* in human lung cells.

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