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

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ABSTRACT

Exposure to tobacco smoke as well as environmental and occupational factors is the major cause of lung cancer. Non-small cell lung cancer (NSCLC) is the major histological type. Genes in pathways affecting inflammation, cellular stress and apoptosis are important, and the extent of inflammation in the lung could be affected by polymorphisms modifying these responses. 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 882 subjects from the Norwegian population. The results showed that individuals carrying combination of three functional polymorphisms in the caspase-8, matrix metalloproteinase-1, seleno-protein S1, and interleukin-10 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 in the carcinogenesis of the lung.



## 1. Introduction

Exposure to tobacco smoke as well as environmental and occupational factors is major cause of lung cancer [1]. Enhanced inflammation due to environmental and intrinsic factors is an important co-factor in promotion and progression of lung cancer. Major genes modulating the level of inflammation in chemical carcinogenesis include interleukins 1 (IL-1) and 6 (IL-6), tumor necrosis factor alpha (TNF-α), and the transcription factor nuclear factor kappa B (NF-κB). Cigarette smoke also induces an endoplasmic reticulum (ER) stress response in the lung [2]. Several genes including Selenoprotein S (*SEPS1*) have been implicated in regulation of the ER-stress response.

Studies suggest that inflammatory reactions in the lung could be affected by polymorphisms in the genes regulating these responses. For example, polymorphisms in the *TNF-α* (G−308A, G+488A) and *NF-κB* (Ins-94Del) genes have been shown to modulate risk of several cancers [3–6]. We have recently demonstrated that regulatory polymorphisms in the *IL1B* gene were correlated with increased risk of NSCLC and higher expression of IL1B mRNA in the lung [7]. *In vitro* studies using human lung epithelial cells showed that the functional polymorphism in the *IL1B* gene promoter (*IL1B*-31 T/C) had increased expressional activity after exposure to the lung carcinogen benzo(a)pyrene [8]. Among others, SEPS1 has been identified as an ER protein participating in processing and removal of misfolded proteins from the ER to the cytosol where they are destroyed by the proteasome in an ubiquitin-dependent manner [9]. Recently, the *SEPS1* G-105A promoter SNP was identified and shown to influence the efficiency of removal of the misfolded proteins from the ER, resulting in increased ER-stress and elevated circulatory levels of the pro-inflammatory cytokines, particularly IL1β [9]. An interaction between the *SEPS1* -105 SNP and the *IL1B* promoter SNPs towards an increased risk of rheumatid arthritis has been suggested as evidence for epistasis between the *IL1B* and *SEPS1* genes [10].

The cysteine-dependent aspartate-specific protease-8 (CASP-8) is involved in apoptotic signaling as well as inflammation [11]. Apoptotic signals can lead to activation and cleavage of pro-caspase 8 into active caspase 8, thereby inducing apoptosis [12]. Caspase-8 may also trigger inflammation [13]. A functional six nucleotide insertion–deletion polymorphism (-652 ins/del) in the *CASP8* gene promoter was recently described and reported to be associated with susceptibility to multiple cancers [14]. Yet another caspase protein, Caspase-1 (CASP-1) may mediate cytokine production caused by tobacco smoke. CASP-1 enzyme is essential for the cleavage of pro-IL1β protein into its active and mature form [15]. A polymorphism in the *CASP1* gene (G+5455A) may affect the enzymatic activity of the CASP1 enzyme towards pro-IL1β and hence formation of the active IL1β protein [16].

The inflammation-related molecules such as the matrix metalloproteinase 1 (MMP-1) are associated with degradation of the

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**Table 1**
Characteristics of lung cancer cases and healthy controls.

| Parameter | Cases | Controls |
|---|---|---|
| | *N* = 442 | *N* = 440 |
| Median age (min–max) | 66 (25–85) | 60 (50–83) |
| Sex (male/female) | 323/115 | 335/105 |
| Number of cigarettes pr day | | |
| Mean ± SD | 15.52 ± 8.21 | 14.59 ± 6.29 |
| Median (min–max) | 14 (2–60) | 15 (3–40) |
| Total smoking years | | |
| Mean ± SD | 41.04 ± 12.03 | 42.30 ± 8.45 |
| Median (min–max) | 42 (2–69) | 41 (15–65) |
| Total pack-years | | |
| Mean ± SD | 31.41 ± 17.58 | 31.78 ± 15.07 |
| Median (min–max) | 28.5 (1–112.5) | 28.5 (5–84) |
| TP53 status in tumors | *n* = 272 (100%) | |
| Mutated | 151 (55.5%) | |
| Wild-type | 121 (44.5%) | |
| Histological details | | |
| Adenocarcinoma | 159 | |
| Squamous cell carcinoma | 192 | |
| Large cell carcinoma | 71 | |
| NSCLC (not otherwise classified) | 20 | |

SD, standard deviation.

extracellular matrix (ECM), tissue remodeling and alteration of cellular signals in multiple cancer types [17]. MMP-1 may also be involved in the migration of immune cells from the bloodstream to sites of inflammation. A functional insertion/deletion polymorphism in the -1607 promoter region (-1607 2G/1G) of the *MMP1* gene has been shown to affect expression levels of the gene [18].

Interleukin-10 (IL-10) is an important immune regulatory cytokine, mostly with anti-inflammatory functions [19,20]. The implication of IL-10 in lung cancer has previously been studied, but its significance is still not clear [21,22]. Three promoter SNPs at positions -1082, -819 and -592, forming a specific haplotype, have been associated with decreased expression of *IL10* gene [23].

Studies indicate that Toll-like receptor 4 (TLR-4) signaling contributes to environmentally induced airway inflammation [24]. The A-2026G polymorphism in the regulatory region of the *TLR4* gene has been shown to be functional and has been associated with several diseases [25–27]. Another gene involved in the *TLR4* signaling is the *CD14* which is a receptor for bacterial wall components such as LPS and is a susceptibility locus for asthma and may be a critical factor in lung inflammation as well [28]). The functional C-260T polymorphism in the *CD14* gene has been shown to affect expression of the gene [29].

We hypothesized that functional polymorphisms that are individually associated with a weak to moderate risk of lung cancer, may have additive effects leading to a much higher lung cancer risk. We examined the association between NSCLC and 11 functionally verified polymorphisms in genes related to lung inflammation, cellular stress and apoptosis. Our results suggest that a significant increase in the risk of NSCLC may require a combination of at least three functional polymorphisms. Specifically, we found that a combination of functional polymorphisms in the *CASP8, MMP1, IL10* and *SEPS1* genes increased risk of NSCLC to more than four-fold. The odds ratios were tested and further confirmed using statistical tools False-Positive Report Probability (FPRP) and Bayesian False Discovery Probability (BFDP) tests [30,31] developed to correct for multiple testing errors in association studies.

In view of the significance of the *TP53* gene in lung carcinogenesis, we also examined the genotypes in relation to *TP53* mutations in the lung tumors available from a subset of lung cancer patients.

## 2. Materials and methods

### 2.1. Study population

The characteristics of lung cancer patients and healthy controls included in the present study are summarized in Table 1. The details of the study population are recently published by Landvik et al. [7]. Briefly, 455 lung cancer patients were admitted for surgery at the university hospitals in Oslo or Bergen between 1986 and 2001. Diagnosis of lung cancer was ascertained by reviewing histological slides from tumor tissue and 442 non-small cell lung cancer (NSCLC) cases were enrolled in the study consecutively whenever practically feasible. Controls were recruited from a general health survey conducted by the National Health Surveys in the Oslo area (HUBRO) of the general population (http://www.fhi.no/hubro). A total of 440 smokers without any known history of cancer were randomly selected and frequency matched with the cases on age, smoking dose (pack-years) and sex. Cases and controls were interviewed by trained health personnel using questionnaires containing comparable information on demographic and lifestyle details. All subjects gave written consent to participate in the study which was approved by the Regional Ethical Committee.

### 2.2. DNA extraction, polymorphism selection and genotyping

DNA was extracted from whole blood or normal lung tissue with standard proteinase K digestion and phenol/chloroform extraction. DNA from 442 cases and 440 controls were randomized and mixed on PCR plates in order to ensure that an equal number of cases and controls could be analyzed simultaneously. Eleven polymorphisms in nine genes reported in the literature to be functional were selected (Table 2). The insertion/deletion polymorphisms in the *CASP8* (6 bp -652 ins/del), the *MMP1* (1 bp -1607 ins/del), and the *NF-κB1* (4 bp -94 ins/del) were genotyped by capillary fragment length analysis as described elsewhere [32]. Genotyping of the remaining eight SNPs were carried out using pre-designed or custom TaqMan genotyping assays as instructed by the supplier (Applied Biosystems, Foster City, CA, USA). Approximately 10 ng genomic DNA was amplified in 5 μl reaction mixture in a 384 well plate containing 1× universal TaqMan master mix and 1× assay mix containing a pre-mix of the respective primers and MGB-probes labeled either with FAM or VIC. After initial denaturation and enzyme activation at 95 °C for 10 min the reaction mixture was subjected to 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The reactions were performed on an ABI 7900HT sequence detection system from Applied Biosystems (Applied Biosystems, Foster City, CA, USA). Equal numbers of cases and controls were run simultaneously, and negative controls containing water instead of DNA were included in every run. Genotypes were determined in the SDS 2.2 software (Applied Biosystems, Foster City, CA, USA). There were more than 92% genotyping success rate in both cases and controls and no deviation from Hardy–Weinberg equilibrium was observed ($P > 0.05$). A 10% blinded replicate samples were re-genotyped with a concordance rate of 100%.

### 2.3. Determination of mRNA levels by quantitative real-time RT-PCR

Total RNA was isolated from normal lung tissue of patients with SEPS1G/G genotype ($n = 34$) and SEPS1 G/A+A/A genotypes ($n = 12$) and the IL1B mRNA levels were determined by quantitative real-time RT-PCR as described elsewhere [7]. Total RNA was extracted from frozen, crushed normal lung tissue by the TRIzol reagent (Invitrogen, Paisley, UK) and reverse transcribed using ran-

**Table 2**
Genes, polymorphism, type, location and frequencies in cases and controls.

| Gene name | Genotype | Cases | Controls | Location | Polymorphism | Type | [a]Rs number |
|---|---|---|---|---|---|---|---|
| Caspase 8 | | | | -652 | -/CTTACT | Deletion/insertion | rs3834129 |
| | ins/ins | 125 | 106 | | | | |
| | ins/del | 210 | 209 | | | | |
| | del/del | 101 | 118 | | | | |
| Matrix metalloproteinase 1 | | | | -1607 | 2G/1G | Insertion/deletion | rs1799750 |
| | 2G/2G | 114 | 104 | | | | |
| | 2G/1G | 207 | 198 | | | | |
| | 1G/1G | 115 | 132 | | | | |
| Selenoprotein S1 | | | | -105 | G/A | Base exchange | rs28665122 |
| | G/G | 322 | 335 | | | | |
| | G/A | 110 | 95 | | | | |
| | A/A | 5 | 4 | | | | |
| Interleukin 10 | | | | -592 | C/A | Base exchange | rs1800872 |
| | C/C | 264 | 243 | | | | |
| | C/A | 144 | 175 | | | | |
| | A/A | 26 | 15 | | | | |
| | | | | -1082 | A/G | Base exchange | rs100896 |
| | A/A | 120 | 104 | | | | |
| | A/G | 207 | 226 | | | | |
| | G/G | 109 | 105 | | | | |
| Tumor necrosis factor alpha | | | | -308 | G/A | Base exchange | rs1800629 |
| | G/G | 287 | 270 | | | | |
| | G/A | 130 | 140 | | | | |
| | A/A | 18 | 25 | | | | |
| | | | | 488 | G/A | Base exchange | rs1800610 |
| | G/G | 383 | 372 | | | | |
| | G/A | 55 | 62 | | | | |
| | A/A | 0 | 1 | | | | |
| Caspase 1 | | | | 5455 | G/A | Base exchange | rs501192 |
| | G/G | 286 | 282 | | | | |
| | G/A | 134 | 141 | | | | |
| | A/A | 16 | 12 | | | | |
| Nuclear factor kappa-B, subunit 1 | | | | -94 | -/ATTG | Deletion/insertion | rs28362491 |
| | del/del | 141 | 151 | | | | |
| | del/ins | 207 | 187 | | | | |
| | ins/ins | 71 | 73 | | | | |
| Toll-like receptor 4 | | | | -2026 | A/G | Base exchange | rs1927914 |
| | A/A | 196 | 196 | | | | |
| | A/G | 197 | 183 | | | | |
| | G/G | 42 | 52 | | | | |
| CD14 antigen | | | | -260 | C/T | Base exchange | rs2569190 |
| | C/C | 134 | 121 | | | | |
| | C/T | 213 | 228 | | | | |
| | T/T | 88 | 85 | | | | |

[a] Rs numbers are from PubMed SNP database.

dom primers (Roche Molecular Biochemicals, Basel, Switzerland). B-actin (*ACTB*) was used as a reference gene and the PCR amplicons were designed over introns to avoid the amplification of possible traces of genomic DNA contamination (Primer Express 2.0 software, Applied Biosystems, Foster city, CA, USA). The sequences of the primers used for *ACTB* have been published previously [33]. *IL1B* primers were pre-designed (Qiagen, Hilden, Germany) and used according to the manufacturers' recommendations. Analysis was performed on an ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA) with SYBR Green I. After initial denaturation at 95 °C for 10 min the reaction mixtures were subjected to 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by one cycle of 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s for melting curve analyzes. The internal standard, a known amount of PCR-product for *IL1B* and *ACTB*, was used in dilution series as template in the real-time PCR and served as a control of successful PCR and for estimating target cDNA quantity. Specific gene expression levels were normalized to the expression of ACTB mRNA and calculated by the standard curve method.

### 2.4. Bioinformatics analysis of the region flanking the polymorphisms

Short sequences, varying only at the location of the SNPs, were evaluated for putative binding sites of known transcription factors. To assess the putative binding sites for transcription factors we used the program PROMO and set the cut-off for the dissimilarity matrix at 15%. To give a measure of the reliability of the different predictions, the expectation of finding each of the matches, in a random sequence of 1000 nucleotides, is calculated, considering both a model with equiprobability of the four nucleotides and a model with the same nucleotide frequency as the query sequence [34,35].

### 2.5. Statistical analysis

Differences in demographic variables, smoking, grouped genotypic frequencies between cases and controls were evaluated by $X^2$-test and reported $P$ values are two-sided with $P < 0.05$ considered as significant, odds ratios (ORs) and 95% confidence intervals

**Table 3**
Risk of lung cancer associated with combination of SNPs in *CASP8, MMP1, SEPS1* and *IL10* genes.

| Number of risk genotypes | Cases | Controls | *P* | OR | 95% CI | BFDP | | *Prior probability* | Score | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Z | ABF | | FPRP | BFDP |
| "0–1" | 32 | 45 | | Ref. | | | | | | |
| 3 | 206 | 168 | 0.009 | 2.063 | (1.193–3.475) | 2.607 | 0.363 | 0.100 | 0.385 | 0.766 |
| "3–4" | 272 | 230 | 0.003 | 4.616 | (1.688–12.627) | 2.517 | 0.733 | 0.100 | 0.646 | 0.868 |
| "0–2" | 158 | 200 | | Ref. | | | | | | |
| 3 | 206 | 168 | 0.000 | 1.716 | (1.266–2.325) | 3.485 | 0.034 | 0.025 | 0.091 | 0.571 |
| "3–4" | 272 | 230 | 0.001 | 1.625 | (1.222–2.161) | 3.338 | 0.042 | 0.025 | 0.102 | 0.620 |

OR, odds ratio; CI, confidence interval; BFDP, Bayesian false discovery probability; FPRP, false-positive report probability; ABF, approximate Bayes factor; Ref, referent. ORs were obtained from unconditional logistic regression analysis adjusted for age, sex and smoking and *P* values were obtained from the same logistic regression analysis.

(CIs) were estimated from unconditional regression analyses using SPSS (version 15.0) with age, sex and pack-years as covariates unless stated otherwise.

To evaluate the chance of obtaining a false-positive association in our dataset, a FPRP was calculated as described by Wacholder et al. [30] as well as for the Bayesian false discovery probability (BFDP)-test described by Wakefield [31]. Both methods rely on the decision of a *prior probability* level to indicate whether a test is considered noteworthy or not. We, have previously suggested three levels of *prior probability* for SNP analysis: (i) *prior probability* 0.1 when a strong biological plausibility consistent with epidemiological evidence exist, (ii) *prior probability* 0.01 when either biological knowledge or epidemiological data is missing and (iii) *prior probability* 0.001 when no biological or epidemiological data are present or adequate [36]. In this report we chose to include an intermediate level at 0.025 with the exclusion of the 0.001 level as strong evidence for the selected SNPs existed. Data were presented at the *prior probability* 0.1 level unless scoring for more stringent levels emerged. The *a priori* odds ratio was set at 1.5, as well as the selected levels of noteworthiness for FPRP was 0.2 and 0.8 for BFDP as recommended in the original papers [30,31]. In Table S4 the statistical relationship between individuals carrying 0–1 through 4 risk genotypes were evaluated by $R \times C$ contingency tables using $\chi^2$ test statistics (Pearson Chi-Square, two-sided) and a linear-by-linear association test for trend to evaluate linear trend using SPSS (version 16.0). To evaluate the relationship between *TP53* status and polymorphisms, the cases were dichotomized by the status of the *TP53* gene in tumor (wild-type or mutated *TP53*) and genotypes of the subjects as shown in Table S3 and *P* values were obtained using two-sided $\chi^2$ test statistics with Yates correction. Wherever there were 5 or fewer subjects, a two-sided Fisher's exact test was used.

### 2.6. Analysis of the TP53 status in tumors

The mutations were screened as described recently [50]. Briefly, tumor tissues were obtained from 272 lung cancer patients and kept frozen at −80 °C and DNA was extracted from the tissue using standard proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation. Tumor DNA was screened either by SSCP or by denaturating gradient capillary electrophoresis covering the exons 4–9 of the *TP53* gene. Mutated exons were sequenced either by direct sequencing using BigDye chemistry (Applied Biosystems) or by the use of the arrayed primer extension platform (APEX). Primers and conditions used in PCR reactions and sequencing are available on request.

### 2.7. Determination of IL6 levels

Frozen serum or plasma samples from 71 lung cancer patients were available. The IL6 cytokine levels were determined by ELISA using the commercially available Human IL-6 CytoSet ELISA kit (Cat. No: CHC1263, Lot No: 092603) purchased from Invitrogen (Biosource, CA, USA) as instructed by the manufacturer.

## 3. Results

The characteristics of NSCLC patients and healthy controls are shown in Table 1. Eleven polymorphisms in nine genes (Table 2), involved in inflammation, cellular stress and apoptosis were analyzed for association with risk of NSCLC. The polymorphisms were selected on the basis of confirmed functionality [3,9,14,16,18,23,25,29,37,38]. Genotypes were obtained for >92% of cases and controls for all eleven polymorphisms as shown in online supplementary Tables 1 and 2 (Table S1-S2). There was complete concordance between duplicate samples; hence there was no evidence of any systematic bias in genotyping. The frequency of alleles of each SNP in controls in our study was similar to previously published data from European populations (http://www.hapmap.org and http://www.ncbi.nlm.nih.gov/projects/SNP/). Furthermore, there was no evidence of population stratification as the genotype distribution in controls for each of the eleven polymorphisms satisfied Hardy–Weinberg equilibrium ($P > 0.05$).

The results for the main effects of the polymorphisms on NSCLC risk are shown in Table S1. The *IL10* -592 SNP showed a significant association with risk of NSCLC with an odds ratio (OR) of 1.35 (95% CI, 1.01–1.81, $P = 0.019$). The 1G/1G variant of *MMP1* was associated with an increased risk in younger subjects (≤59 years) with an OR of 2.284 (95% CI, 1.14–4.57, $P = 0.020$). The *del/del* variant of *CASP8* was associated with an increased risk in subjects older than 59 years with OR of 1.72 (95% CI, 1.06–2.79, $P = 0.029$) and the risk was particularly associated with male gender OR 1.61 (95% CI, 1.02–2.53, $P = 0.039$). For *TNF-α* +488 SNP there was increased risk associated with the inheritance of the A allele (OR 2.02; 95% CI, 1.196–3.413, $P = 0.009$).

The polymorphisms were also analyzed in relation to accumulated smoking dose (pack-years). For light smokers (≤20 pack-years) we found a reduced risk for *IL10* -592 A/A genotype with an OR of 0.22 (95% CI, 0.06–0.08, $P = 0.022$). For moderate smokers (>20–≤40 pack-years) an increased risk for the *CASP8* -652 del/del carriers, OR 1.85 (95% CI, 1.03–3.32, $P = 0.038$) was observed. When subjects were grouped according to smoking intensity (number of cigarettes smoked per day) we found that presence of the *CASP8 del* allele [OR 1.77 (95% CI, 1.03–3.02, $P = 0.039$)] and the *SEPS1* G-allele [OR 0.58 (95% CI, 0.37–0.92, $P = 0.022$)] were associated with lower smoking intensity (≤14 cigarettes/day).

Analysis of the main effects showed that only *IL10* -592, *CASP8* -652 ins/del, *MMP1* 1G/2G, *SEPS1 G*-105A and *TNF-α*+488 polymorphisms were associated with risk of NSCLC. The combinatory effects of these SNPs were further investigated, excluding *TNF-α* +488 SNP due to the low frequency of the rare genotype. Cases and controls were grouped into four groups based on carrying 0, 1, 2, 3 or 4 risk genotypes (Table 3). Compared to subjects having 0 or 1

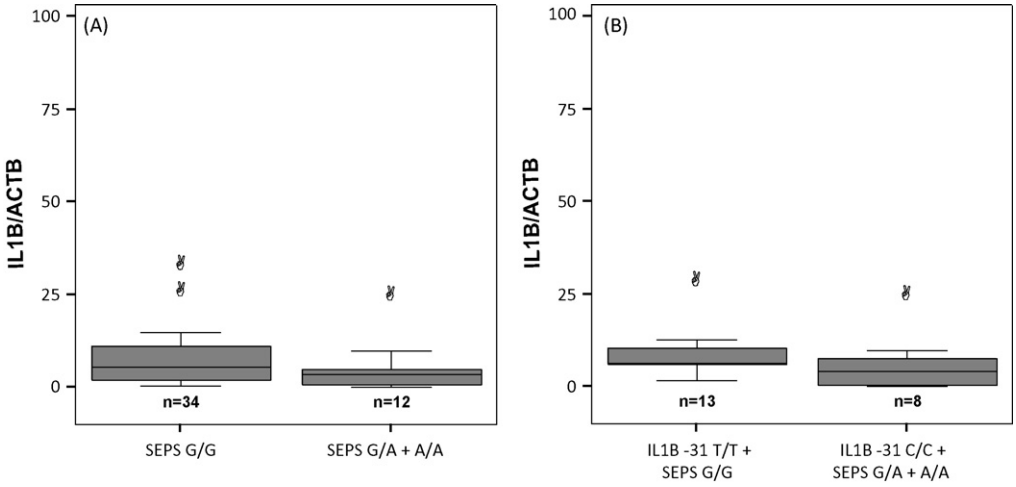


**Fig. 1.** *SEPS1* and *IL1B* promoter polymorphisms and mRNA levels of *IL1B* gene. Total RNA was isolated from normal lung tissue of patients with *SEPS* 1G/G genotype ($n = 34$) and *SEPS1* G/A+A/A genotypes ($n = 12$) and the IL1B mRNA levels were determined by qPCR. (A) Levels of IL1B mRNA normalized to Actin from individuals with *SEPS1* G/G genotype was compared to individuals with G/A+A/A genotype using a non-parametric test. (B) Levels of IL1B mRNA normalized to Actin from individuals with *IL1B* T/T and *SEPS1* G/G genotype was compared to individuals with *IL1B* C/C and *SEPS* G/A+A/A genotype by non-parametric testing.

genotypes (reference), subjects carrying 3 risk genotypes showed a two-fold increase in risk (OR 2.04 95% CI, 1.19–3.47, $P = 0.009$), whereas the risk in subjects carrying 3 or 4 risk genotypes was more than four-fold (OR 4.62, 95% CI, 1.69–12.63, $P = 0.003$). The effects were "noteworthy" even after applying multiple correction FPRP and BDFP-tests when 0–2 risk genotypes was used as reference and compared to the group carrying three risk genotypes (OR 1.72, 95% CI, 1.27–2.32, $P < 0.001$) or 3–4 (OR 1.62, 95% CI, 1.22–2.16, $P = 0.001$) risk genotypes. The combinatory effect of polymorphisms was evident as there were significant trends towards increased risk of lung cancer with increasing number of risk genotypes ($P_{trend} = 0.013$ for 0–1 versus 2, 3 or 4 risk genotypes; $P_{trend} = 0.007$ for 0–2 versus 3 or 4 genotypes). The number of cases and controls are shown in Table S4.

The *SEPS1* -105 G/A SNP has previously been reported to affect serum levels of the IL1β protein after ER-stress [9]. We investigated whether expression of the IL1B mRNA in the lung was correlated to SEPS1 -105G/A genotypes. The results showed that subjects with *SEPS1* G/G genotypes produced more IL1B ($P = 0.076$) mRNA (Fig. 1A). The results were also analyzed in relation to the biologically functional *IL1B* -31T/C SNP genotypes which have been shown to affect IL1B mRNA levels [7]. The results showed that subjects with *SEPS1* G/G and *IL1B* -31T/T genotypes ($n = 14$) had higher mRNA levels (Fig. 1B) compared to subjects with *SEPS1* G/A+A/A and *IL1B* C/C genotypes ($n = 8$) although being not statistically significant ($P = 0.111$).

The *TP53* mutational data for tumors from 272 lung cancer patients were available. The genotypes of patients which had mutations in the *TP53* were compared with genotypes of patients without mutations in the tumor tissue (Table S3). For the SEPS1 gene, 27 of 151 (17.9%) of the cases had the -105A allele and a mutation in the tumor tissue compared with thirty-six of one hundred and thirteen (31.8%) of the cases that had the 105A allele but with a wild-type *TP53* in the tumor ($P = 0.008$, $X^2$-test). The patients with a T-allele of the CD14 polymorphism were more likely to have a mutation in the tumor tissue ($X^2$-test, $P = 0.031$).

The level of the inflammatory IL6 protein was measured in 71 serum/plasma samples available from lung cancer patients and the protein levels were compared in subjects with different genotypes. There were no significant correlations between serum IL6 levels and any of the polymorphisms investigated (data not shown).

## 4. Discussion

In this study we have examined the risk of NSCLC associated with eleven functional polymorphisms in nine genes. We investigated further the interaction between four of the polymorphisms in *CASP8, MMP1, SEPS1* and *IL10* genes. The results showed a step-wise increase in risk of NSCLC with increasing number of risk genotypes.

IL-10 is an important immunoregulatory cytokine with anti-inflammatory properties and capacity to inhibit pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α [39] and is dysregulated in human cancer [22]. IL-10 production has also been implicated in the tumorigenesis of various types of cancer, and may also protect tumors by inhibiting cytotoxic T lymphocyte-mediated tumor specific cell lysis [22]. A group of tissue inhibitors of metalloproteinases (TIMPs), capable of inhibiting almost every member of the MMP-family, is up-regulated by IL-10 signaling [40]. Transcriptional regulation of the *IL10* gene by NF-κB transcription factor is not clear, however, homodimers of NF-κB/p50 subunits have been demonstrated to be transcriptional activators of the *IL10* gene [41].

The MMP-1 enzyme is the most expressed interstitial collagenase involved in degradation of extracellular matrix (ECM) during cancer progression [42]. 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 and progression of cervical cancer [18,42]. 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 [18]. Recently, a cigarette smoke (CS) responsive region in the *MMP1* promoter was also identified, and the 2G variant revealed higher basal and CS-responsive activities than 1G-allele [43]. The pro-inflammatory IL-1β cytokine has been shown to have the potential for inducing MMP1 mRNA expression [44]. The induction of MMP1 mRNA levels by IL-1β may be reduced by the inheritance of the *MMP1* 1G-allele enough to make the cells more vulnerable to DNA damage as the ECM is broken down.

The CASP-8 has been extensively studied as the initiator protein in the caspase-dependent apoptosis pathway which is triggered by death receptors of the TNF-receptor 1. The *del* variant of the *CASP8* gene abolishes an SP1 transcription factor binding site and is associated with decreased RNA levels, lower CASP-8 enzyme activity and lower apoptotic activity in T lymphocytes [14]. A role for CASP-8 in NF-κB activation in innate immunity has been suggested where

CASP-8 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 [12]. A novel role for CASP-8 enzyme in cleaving the pro-IL-1β protein into active IL-1β in response to TLR4 stimulation has been described in macrophages, further supporting the immune regulative functions of CASP-8 [13].

For the *SEPS1* -105 G/A SNP, a previous study reported increased circulatory levels of the inflammatory cytokines IL-1β, IL-6 and TNF after conditions inducing ER-stress due to protein overload [9]. We, therefore, investigated transcriptional activity from the *IL1B* promoter, as we hypothesized that cases with the *SEPS1* G-allele together with the *IL1B* functional T-allele may produce more IL1B mRNA. Even though the data did not reach statistical significance ($P = 0.111$), a trend was present. Accumulation of proteins in the ER has been shown to activate the transcription factor NF-κB [45]. Within the *SEPS1* promoter, an ER-stress responsive element (ERSE) with two NF-κB binding sites has been identified which is located in close proximity to the -105 G/A polymorphism [46]. The effect of the -105 G/A polymorphism is suggested to be caused by disruption of the ERSE [47]. The mechanisms leading to ER-stress are not fully known, but NF-κB and/or TLR/IL1R dependent pathways may be involved [48,49]. The expression levels of pro-inflammatory cytokines, such as IL-1β, are suggested to be elevated as an effect of impaired coping with ER-stress by the *SEPS1* G/G variant [9]. The *SEPS1* G/G genotype, on the other hand, may also increase inflammatory stress through generating elevated levels of IL-1β cytokine.

The results showed multiplication of risk of lung cancer associated with a combination of specific functional SNPs in inflammation-related genes. The *CASP8, SEPS1, MMP1* and *IL10* SNPs contain potential candidates of functional SNPs that can affect the expression levels of the mRNA produced by these genes. The combinatory effect of these SNPs suggests that the combination of SNPs may predict risk of lung cancer with a greater specificity when three or more risk genotypes are present. There are several links between these genes and carcinogenesis as discussed above. However, from the analysis it is not possible to predict exactly which gene(s) and SNP(s) are the most important ones, but modulation of the central transcription factor NF-κB may provide a link between these SNPs.

We found that lung cancer patients with a -105A allele of the SEPS1 polymorphism were more likely to have a wild-type *TP53* gene in the tumor tissue. Mutations in the *TP53* gene are the most common genetic alteration in lung tumors. Studies indicate an increased *TP53* mutation load in a number of inflammatory diseases [51]. We have also found that tumors from lung cancer patients carrying the pro-inflammatory IL1B-31T/T genotype were more likely to harbor *TP53* mutations [52]. A higher expression of the IL1B mRNA in lungs of the patients carrying the SEPS1-105G/G-IL1B-31T/T genotype was also observed (Fig. 1). This is an interesting observation since inflammatory cytokines such as IL1β have been shown to induce DNA damage and inhibit DNA repair *in vitro* [53]. Furthermore, the IL1β has been shown to reduce apoptosis by changing the ratio of BCL-2/BAX proteins. In this context, it is biologically plausible to hypothesize that impairment of the SEPS1 protein and increased levels of the IL1β both may lead to an increased *TP53* mutation load due to increased levels of reactive oxygen/nitrogen species and also increased ER-stress. The significance of the CD14 polymorphism on *TP53* mutation load is not clear and needs further investigation.

The sequences flanking the SNP sites from the *CASP8, MMP1, SEPS1* and *IL10*-592 SNPs were analyzed by the Alggen PROMO bioinformatics tool for alterations in putative transcription factor binding sites. Interestingly, the variant alleles of three of the polymorphisms could bind to glucocorticoid receptor alpha (GR-alpha) which is a common transcription factor (data not shown).

The results of this study highlight the need to investigate the combinatory effects of multiple SNPs in lung cancer. However, due to the limited sample size the findings should be interpreted with caution and larger studies are needed to replicate and verify the results obtained in this moderate-sized case-control study.

## Conflict of interest

The authors declare no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lungcan.2010.04.016.

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