Association of a common polymorphism in the cyclooxygenase 2 gene with risk of non-small cell lung cancer

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Studies have indicated that inflammation, in conjunction with the production of reactive oxygen species, may play a key role in lung cancer development. In this study, 250 lung cancer patients and 214 controls were genotyped for polymorphisms of the inflammation-related genes prostaglandin synthase-2/cyclooxygenase-2 (COX2/PTGS2), interleukin-6 (IL6), interleukin-8 (IL8) and peroxisome proliferator-activated receptor γ (PPARγ). We found that carriers of the C allele of a polymorphism in the 3′-UTR of COX2 had a significantly increased risk of lung cancer, with odds ratios of 4.28 (95% CI, 2.44–7.49) for homozygotes and 2.12 (95% CI, 1.25–3.59) for heterozygotes. Additionally, we found that an IL8 promoter polymorphism had a protective effect for lung cancer in female subjects, whereas an IL6 promoter polymorphism was only associated with risk of squamous cell carcinoma. This is the first study implicating polymorphisms in inflammatory genes in the risk of lung cancer.

Introduction

Tobacco smoking is the most important cause of lung cancer (1). Genetic factors play a role in the etiology of lung cancer and several studies have identified predisposing, low penetrance genes. Most of the studies published so far investigated variation in genes involved in carcinogen metabolism and DNA repair (2–4), but other pathways may be relevant to lung cancer pathogenesis. Inflammation has been recognized as a contributing factor in pathogenesis of many cancers (5). Chronic inflammation, arising as a result of continuous exposure to tobacco components, may result in oxidative stress and contribute to tumor promotion and progression in the lung (6,7). Case–control studies have shown an increased risk of lung cancer in patients with inflammatory airway phenotypes such as asthma, bronchitis and emphysema (8–10).

Recent data suggest that cigarette smoke stimulates airway epithelial cells, as well as immune cells, to release pro-inflammatory cytokines such as interleukin-1β. These in turn activate the NF-κB transcription factor signal pathway, leading to up-regulation of various inflammation-related genes, including interleukin-6 (IL6), interleukin-8 (IL8) and cyclooxygenase-2 (COX2) (11–14).

Cyclooxygenases (COXs, also known as prostaglandin endoperoxide synthases or PTGSs) are the key enzymes that mediate production of prostaglandins from arachidonic acid. Prostaglandins are thought to promote tumor growth, angiogenesis and metastasis. Two COX isofoms have been identified, COX1 and COX2. In normal cells, the COX1 gene is expressed constitutively, whereas COX2 is usually undetectable but rapidly inducible (15). COX2 is overexpressed in many tumor types, including lung cancer. Up-regulation of COX2 is believed to be an early event in lung carcinogenesis, occurring in hyperplastic bronchial epithelium as well as in atypical adenomatous hyperplasia (16,17). Moreover, COX2 overexpression was reported as a poor prognostic factor in non-small cell lung cancer (NSCLC) patients (18,19). COX2 is known to be induced by cigarette smoke condensate in vitro and by the tobacco-specific carcinogen nitrosamine 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone in mice (20–22). The enzyme activity is also stimulated by various inflammatory cytokines (see below). Furthermore, several studies point to a chemopreventive effect of non-steroidal anti-inflammatory drugs (NSAIDs), of which COX2 is a major target, in lung cancer patients, particularly in smokers (23,24).

On the other hand, it is well known that NSAID metabolites which lack COX2 inhibiting activity still retain their chemopreventive potential, thus indicating that COX2 is not the only target of anti-inflammatory drugs. Several NSAIDs, for instance, can activate peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor transcription factor that regulates cell growth by inducing differentiation and apoptosis (25,26). These effects are mediated through inhibition of transcription factors, including NF-κB (27). Therefore, PPARγ activation ultimately reduces production of pro-inflammatory molecules such as IL6, IL8 and COX2. Studies conducted in lung cancer cell lines have found that this mechanism may be used to inhibit lung cancer cell growth (28).

IL6 is a major cytokine that is expressed in tumor-infiltrating cells. Lung cancer patients have been shown to have significantly higher serum levels of IL6 compared with healthy controls (29). Several studies have demonstrated that these cytokines are pivotal determinants of the angiogenic activity of NSCLC and that anti-inflammatory drugs may inhibit angiogenesis (30,31).

Studies have shown that protein levels of IL8 were significantly higher in small airway epithelial cells from smokers. In current smokers, this was positively correlated with smoking history (12,32). IL8 is believed to play an important role in the pathogenesis of various airway inflammatory diseases (33).
The aim of this study was to investigate whether germline sequence variants of the pro-inflammatory genes COX2, IL6 and IL8 and the anti-inflammatory nuclear transcription factor PPARγ act as common, low penetrance alleles for susceptibility to lung cancer. In a case–control study, the relative risk of lung cancer associated with allelic variants of these genes was determined. The single nucleotide polymorphism(s) (SNP) in each gene were selected on the basis of reported functional or biological relevance. Several SNPs in the COX2 gene have been identified, but few polymorphisms have been studied in association with diseases. COX2.926 (G→C) in the promoter region has been implicated in transcription alteration of the COX2 gene and an increase in the levels of the C-reactive protein (34). Therefore, one would expect that the C allele, which favors inflammation, could be associated with lung cancer risk. Since COX2 polymorphisms have not been extensively studied, we have also selected the other three SNPs of this gene with appreciable frequency in Caucasian populations, which cover the entire length of the gene, i.e. COX2.3050 (exon 3), COX2.5209 (intron 5) and COX2.8473 (exon 10, 3′-UTR region). Currently, there are no data on the possible function of these SNPs, therefore it is difficult to predict a priori which allelic variants would be associated with lung cancer risk. The promoter IL6 –174 (G→C) SNP has been shown to be associated with levels of IL6 protein and the C-reactive protein (35,36). The promoter IL8 –251 (T→A) SNP has also been reported to be associated with regulation of levels of IL8 chemokine (37,38). These findings are not always straightforward, and sometimes they are contrasting. It is quite difficult to predict what could be the functional outcome of these promoter polymorphisms in a specific tissue or organ, therefore, again, no a priori hypothesis was formulated on what alleles would be associated with risk. The PPARγ (Pro12 → Ala) is a coding region SNP and the Ala12 allele has been associated with a lower risk of renal carcinoma and type 2 diabetes (39,40). PPARγ has an anti-inflammatory action and the Ala12 allele of this gene has been reported to result in reduced receptor activity (41), therefore we expect that the Ala12 allele could be associated with an increased lung cancer risk.

Materials and methods

Study subjects

The study includes 464 subjects of Caucasian origin from the Norwegian population. Cases (n = 250) were newly diagnosed lung cancer patients treated by surgery at university hospitals in Oslo and Bergen between 1986 and 2001. NSCLC patients were selected consecutively whenever practically feasible. A pathologist confirmed the diagnosis of NSCLC for all cases. Histological subtyping was performed in tumors from 174 cases, which consisted of 78 adenocarcinomas (44.8%), 79 squamous cell carcinomas (45.4%) and 17 large cell carcinomas (9.8%).

Controls were recruited from 8100 individuals in the age cohort 59/60 and 75/76 participating in the Oslo Health Screening 2000–2001 (HUBRO) arranged by the National Health Screening Service. The National Health Screening Service was set up by the Norwegian state to evaluate the health status of the general population. The purpose of the HUBRO project was to perform a survey on health status of all inhabitants living in and around the city of Oslo. About 4100 healthy individuals from the general population participated in this project and contributed with blood samples and by filling out questionnaires. The controls in this study were selected from this group based on the following criteria: ≥60 years of age; smoked >5 cigarettes/day; current smokers or quit smoking for <5 years. About 950 individuals met these criteria and 214 were randomly selected.

Cases and controls were personally interviewed by trained personnel using a questionnaire to determine demographic and lifestyle characteristics. Information on smoking, age and sex was used for the present study.

Both cases and controls were informed and gave written consent to participate in the study and to allow their biological samples to be genetically analyzed. Approval for the study was given by the local ethical committees.

DNA extraction

DNA from the cases and controls was extracted from whole blood samples or normal tissue with standard proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation.

Genotyping

DNA from cases and controls was randomized and mixed on PCR plates in order to ensure that an equal number of cases and controls could be analyzed simultaneously. Genotyping was carried out using the Taqman assay (Applied Biosystems, Foster City, CA). The MGB Taqman probes were designed using the Primer Express software and synthesized by Applied Biosystems. The primer sequences, probes and their labels are reported in Table I. The reaction mix included 20 ng genomic DNA, 10 pmol each primer, 2 pmol each probe and 5 μl of 2× master mix (Applied Biosystems) in a final volume of 10 μl. The thermocycling included 40 cycles with 30 s at 95°C followed by 60 s at 60°C. PCR plates were read on an ABI PRISM 7900HT instrument (Applied Biosystems). The PCR profile and reaction conditions were tested and optimized in order to contain equal amounts of template DNA, probes and primers and to be run with unique thermal conditions. All samples that did not give a reliable result in the first round of genotyping were resubmitted to up to three additional rounds of genotyping. Data points that were still not filled after this procedure were left blank.

Statistical analysis and haplotype reconstruction

The Hardy–Weinberg equilibrium was tested for by the χ2 test in cases and controls for each polymorphism.

To test the hypothesis of association between genetic polymorphisms and lung cancer, multivariate methods based on unconditional logistic regression analyses were used.

For polymorphisms, homozgyosity for the more frequent allele among controls was set as the reference class. Tests for linear trend of odds ratios (OR) were calculated using the categorized variable as quantitative after assigning a linear score to each ordered category (i.e. the homozygote for the more frequent allele was given score 1, the heterozygote score 2 and the homozygote for the rarer allele score 3). Unconditional logistic regression was applied to calculate OR and 95% confidence intervals (CI), using the STATA (version 7) statistical package. All OR were adjusted by age, sex and smoking. COX2 haplotypes and diplotypes were reconstructed by use of the PHASE software (42).

When cases were subdivided into groups, polytomous logistic regression was used, comparing each group of cases with the whole set of controls. Cases were subdivided based on gender, amount of smoking or the location of the tumor. For stratification by smoking status, subjects were classified by smoking status (ex-smoker or current smoker of cigarettes) and by cumulative pack-years of cigarettes grouped into three logical categories (< 20, 20–35 or > 35 pack-years). Tests for interactions were performed comparing the change in deviance (−2 log likelihood) between the model with the main effects and the model that also included the interaction term (product of the main effects).

Results

The relevant characteristics of the subjects are shown by case/control status in Table II. Cases and controls were similar in terms of gender distribution and age. There were more ex-smokers among our cases than among controls, but the distribution of pack-years among current smokers was similar (Table II).

The study explored the association between COX2, IL6, IL8 and PPARγ DNA polymorphisms and NSCLC risk in smokers. The alleles at all loci were in Hardy–Weinberg equilibrium in controls, with non-significant χ2 values (data not shown). The frequencies and distributions of the genotypes and the OR for the associations of the polymorphisms are shown in Table III.

We found that, in this sample set, carriers of the C allele of COX2.8473 had a strongly increased risk of lung cancer. This effect was observed both for heterozygote individuals with an
OR of 2.12 (95% CI 1.25–3.59) and for homozygotes, with an OR of 4.28 (95% CI 2.44–7.49). The P value of the trend test was P < 0.0001, therefore our findings for this SNP will remain significant even if a very stringent correction for multiple testing, such as Bonferroni’s, is applied. Since our study subjects were all smokers or ex-smokers, our results show that this polymorphism could be an important modifier of tobacco smoking-related risk of developing NSCLC. The OR were similar for men and women when analyzed separately (data not shown). When subjects were stratified on the basis of smoking pack-years, the C/T and C/C genotypes were associated with significant odds ratios in subjects who smoked >20 pack-years. Subjects who smoked ≤20 pack-years did not show statistically significant OR for this polymorphism (data not shown).

To further elucidate the relevance of the four COX2 SNPs in lung cancer development, diplotypes (i.e. diploid pairs of haplotypes) for the four COX2 SNPs were reconstructed using PHASE software (42). Thirty-one distinct diplotypes were found, six of which were frequent enough to be meaningfully used for calculation of OR. The results showed that the haplotype effects essentially coincide with the effect of COX2.8473 alleles alone (data not shown).

No main effects were found for SNPs COX2.926, COX2.3050, COX2.5209, IL6 –174, IL8 –251 and PPARg Pro12Ala (Table III). In subgroup analyses, however, female heterozygote and homozygote subjects carrying the IL8 –251 A allele had a significantly decreased risk of lung cancer (Table IV).

When lung cancer cases were split according to histological subtypes, there was a significant over-representation of squamous cell carcinomas as compared with adenocarcinomas in cases carrying the IL6 –174 C allele (Table V).

Discussion

In a case–control study, we have explored the role of seven polymorphisms in four genes related to inflammation. Our
Table III. Distribution of SNP genotypes in healthy controls and lung cancer patients

<table>
<thead>
<tr>
<th>SNP</th>
<th>Controls</th>
<th>Cases</th>
<th>Odds ratios (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COX2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX2.926</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG/GG</td>
<td>174 (81.3)</td>
<td>202 (80.8)</td>
<td>1.00</td>
</tr>
<tr>
<td>GC/CG</td>
<td>36 (16.8)</td>
<td>45 (18.0)</td>
<td>1.03 (0.62–1.70)</td>
</tr>
<tr>
<td>CC/CC</td>
<td>4 (1.9)</td>
<td>3 (1.2)</td>
<td>0.61 (0.13–2.86)</td>
</tr>
<tr>
<td>COX2.3050</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG/GG</td>
<td>155 (72.4)</td>
<td>181 (72.4)</td>
<td>1.00</td>
</tr>
<tr>
<td>GC/CG</td>
<td>52 (24.3)</td>
<td>62 (24.8)</td>
<td>0.94 (0.60–1.48)</td>
</tr>
<tr>
<td>CC/CC</td>
<td>7 (3.3)</td>
<td>7 (2.8)</td>
<td>0.66 (0.20–2.17)</td>
</tr>
<tr>
<td><strong>COX2.5299</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT/TT</td>
<td>167 (78.0)</td>
<td>204 (81.6)</td>
<td>1.00</td>
</tr>
<tr>
<td>TG/CT</td>
<td>47 (22.0)</td>
<td>45 (18.0)</td>
<td>0.79 (0.48–1.27)</td>
</tr>
<tr>
<td>GG/GG</td>
<td>0 (0.0)</td>
<td>1 (0.4)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>COX2.8473</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT/TT</td>
<td>65 (30.4)</td>
<td>31 (12.4)</td>
<td>1.00c</td>
</tr>
<tr>
<td>CT/CT</td>
<td>99 (46.2)</td>
<td>107 (42.8)</td>
<td>2.12 (1.25–3.59)c</td>
</tr>
<tr>
<td>CC/CT</td>
<td>50 (23.4)</td>
<td>112 (44.8)</td>
<td>4.28 (2.44–7.49)c</td>
</tr>
<tr>
<td><strong>IL6 –174</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG/GG</td>
<td>55 (26.6)</td>
<td>64 (26.3)</td>
<td>1.00</td>
</tr>
<tr>
<td>GC/GC</td>
<td>105 (50.7)</td>
<td>111 (45.7)</td>
<td>0.91 (0.56–1.46)</td>
</tr>
<tr>
<td>CC/CC</td>
<td>47 (22.7)</td>
<td>68 (28.0)</td>
<td>1.24 (0.72–2.14)</td>
</tr>
<tr>
<td><strong>IL8 –251</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT/TT</td>
<td>54 (25.7)</td>
<td>71 (29.7)</td>
<td>1.00</td>
</tr>
<tr>
<td>TA/AT</td>
<td>112 (53.3)</td>
<td>119 (49.8)</td>
<td>0.83 (0.52–1.32)</td>
</tr>
<tr>
<td>AA/AA</td>
<td>44 (21.0)</td>
<td>49 (20.5)</td>
<td>0.91 (0.51–1.61)</td>
</tr>
<tr>
<td><strong>PPARα Pro12Ala</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG/GG</td>
<td>161 (76.0)</td>
<td>192 (78.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>GC/GC</td>
<td>47 (22.2)</td>
<td>52 (21.1)</td>
<td>1.06 (0.67–1.69)</td>
</tr>
<tr>
<td>CC/CC</td>
<td>4 (1.8)</td>
<td>2 (0.8)</td>
<td>0.54 (0.09–3.03)</td>
</tr>
</tbody>
</table>

*aNumber of subjects (percent). For some polymorphisms, numbers do not sum up to the totals of controls or cases due to genotyping failure. All samples that did not give a reliable result in the first round of genotyping (almost always due to low fluorescence signal intensity in the TaqMan assays) were resubmitted to up to three additional rounds of genotyping. Data points that were still not filled after this procedure were left blank.

bUnadjusted logistic regression analysis. Analysis after adjustment for sex, age and smoking (pack-years) gave essentially the same results.

cTrend test P value < 0.0001.

study showed that a polymorphism in the 3'-UTR region of the COX2 gene was associated with a significantly increased risk of lung cancer.

COXs catalyze the formation of prostaglandins from arachidonic acid. COX1 is constitutively expressed with an essentially constant level of expression, whereas COX2 is highly inducible at sites of inflammation and tumor and is induced by carcinogens, tumor promoters and various growth factors. Several studies indicate that COX2 appears to be important in chemical carcinogenesis and tumorigenesis. Overexpression of COX2 has been shown to be involved in invasion, angiogenesis and apoptosis through production of prostaglandin E2. Lung tumors express high levels of COX2 (17) and the number and size of lung metastases in Lewis lung carcinoma are inhibited by COX2 inhibitors (43).

SNPs which influence the quantity of prostaglandins produced could be associated with inflammatory diseases and tumor development (44). Elevated levels of COX2 mRNA, thus resulting in COX2 overexpression, may be due to either enhanced transcription or enhanced stabilization of the mRNA. Several consensus sequences, including NF-kB, SP1, NF-IL6 and E-box, have been identified in the COX2 promoter. Mutational analysis of this region has shown that certain base substitutions may cause differential expression of the gene in normal and tumor cells (45). Our finding that COX2.8473 polymorphism was associated with an increased risk of lung cancer points to regulation of COX2 by the alternative mechanism, namely control of mRNA stability and degradation in lung cancer cells. The COX2.8473 SNP is located downstream of the stop codon, in the 3'-UTR region. Binding of proteins to the 3'-UTR can control mRNA stability and degradation, and this may be affected by polymorphisms (46,47). This region is characterized by multiple repeats of AU-rich elements, which are also found in several other genes encoding inflammatory mediators (cytokines and protooncogenes), whose mRNA is very unstable (48). It may be possible that the T → C substitution at COX2.8473 stabilizes the mRNA of COX2, thus resulting in a larger amount of protein produced and therefore an increased pro-inflammatory and angiogenic stimulus.

Our study indicates a decreased risk of lung cancer associated with the IL8 –251 A allele in women. We are aware that subgroup analyses are of limited value, because of the smaller sample sizes, however, the effect we observed was statistically significant. We believe that it is relevant to comment on its biological meaning since differences in lung cancer risk have been demonstrated in men and women (49). At the present time, there is limited information on the actual function of the IL8 polymorphism. This SNP was found to have an effect in changing the in vitro levels of IL8 and is associated with respiratory syncytial virus bronchiolitis in children (37,38). The observation that the effect was detectable only in women could be explained by hormonal factors. It is known that hormones can have a role in the progression of tumors in many tissues. It has been suggested that sex hormones modulate the growth of malignant melanoma and, moreover, melanomas seem to metastasize more slowly in women than in men and survival after metastasis is longer in women than in men (50–52). Kanda and Watanabe, in a recent study, have suggested that this difference may be due to the fact that sex hormones reduce constitutive IL8 secretion and mRNA levels (53). The promoter region of IL8 includes an octamer motif Oct-1, which has its center at position –251 and is the binding site of the transcription factor Oct-1. In vitro experiments showed that several nuclear hormone receptors can bind to Oct-1 (54). An in silico analysis with an algorithm designed to identify transcription factor binding sites (55) (http://www.genomatix.de/software_services/software/MatInspector/matinspector.html) predicted that the sequence containing the T allele binds the octamer transcription factor Oct-1, while the sequence containing the A allele does not.

The IL6 –174 C/C genotype was associated with a risk of developing squamous cell carcinoma, but not adenocarcinoma. Squamous cell carcinoma is the predominant histological lung cancer type found in smokers. It is also located mostly in the central part of the lung, where inflammation and irritation, particularly from tobacco smoke, might exert other biological features than in the peripheral part of the lung, which more frequently hosts adenocarcinomas. It may thus be hypothesized that tobacco-induced inflammation is more frequently associated with squamous cell carcinoma and that IL6 is involved in this process. As a matter of fact, IL6 has been found to be the predominant cytokine expressed by tumor-infiltrating lymphocytes in lung cancer (56). Variations in IL6 expression may therefore have a relation to histological...
type of lung cancer. The IL6 –174 SNP is associated with altered in vivo and in vitro protein expression. However, functional studies have not fully elucidated the biological role of this polymorphism. The –174C allele was associated in vitro and in vivo with alternatively lower or higher levels of expression of IL6 (35,36,57). From all these studies it is known that the promoter harbors binding sites for multiple transcription factors and the regulation of IL6 is very complex. The polymorphism could cause different effects according to the cell type and the stimulus applied. If the –174C allele is indeed associated with reduced release of IL6, one of the consequences is to down-regulate the interleukin 1 receptor antagonist-mediated anti-inflammatory activity, thereby resulting in an increased inflammatory response at the systemic level (58). The results on SNPs in IL6 and IL8 are supported by relatively weak P values. If a stringent correction for multiple comparisons is applied, they will no longer achieve the level of significance. These results should therefore be confirmed in further studies.

In conclusion, we have investigated the role of polymorphisms in key genes related to inflammation in the context of a case–control study on NSCLC. Our results suggest that a polymorphism located in the 3′-UTR of COX2 is associated with an increased risk of developing lung cancer. Important next steps would be to determine how COX2.8473 alleles may influence the expression of COX2 or its protein function.

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