Point-mutational Mspl and Ile-Val Polymorphisms Closely Linked in the CYP1A1 Gene: Lack of Association with Susceptibility to Lung Cancer in a Finnish Study Population

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Abstract
In this study of 87 lung cancer patients, 23 patients with lung disease other than cancer, and 121 healthy controls, no association was found between the Mspl restriction fragment length polymorphism (RFLP) of the CYP1A1 gene and lung cancer risk. In the lung cancer population, histological type, smoking, and occupational histories were also examined with respect to increased lung cancer risk. No association was found between the Mspl RFLP in the CYP1A1 gene and any of these variables. This is in contrast to the results of an earlier report describing an association between the rare genotype m2m2 and susceptibility to lung cancer in a Japanese population; but another study in Norway found no such association. It is evident that, in the Nordic population, Mspl polymorphism in the CYP1A1 gene does not indicate individual susceptibility to lung cancer. We also studied a new point mutation which has recently been closely linked to the Mspl restriction site polymorphism in a Japanese study population. This mutation results in an isoleucine-valine amino acid replacement in the heme binding region of human CYP1A1. We obtained a similar linkage in our study, so the discrepancy between the Japanese and the Nordic Mspl RFLP findings cannot be based on a different degree of linkage between these two point mutations.

Introduction
Many mutagens and carcinogens are metabolically converted into their ultimate mutagenic or carcinogenic form by monooxygenases and other xenobiotic-metabolizing enzymes. The cytochrome P450 family of enzymes and their respective genes (the CYP genes) are of considerable interest, because of their ability to catalyze the transformation of a wide variety of xenobiotics including environmental carcinogens, as well as their ability to maintain body homeostasis via the metabolism of endogenous hormones and other intermediate biochemical compounds (1, 2). One member of the cytochrome P450 family, designated CYP1A1 (3), is of particular interest for its potential role in human pulmonary carcinogenesis (4, 5).

CYP1A1, responsible for AHH2 activity, is induced by polycyclic aromatic hydrocarbon components of cigarette smoke condensate (4, 5). Furthermore, a significant correlation has been reported between the high AHH inducibility phenotype and an enhanced susceptibility to pulmonary carcinoma in cigarette smokers (6–8). The original findings (6) have been both confirmed (7) and disputed (9); but the use of a more recent and substantially more reproducible assay has led to the conclusion that cigarette smokers showing high AHH inducibility are several times more prone to pulmonary carcinoma than smokers with lower inducibility (8).

RFLPs are now commonly used for diagnosis and prediction in a growing number of human clinical disorders. Knowledge of the chromosomal location and the nucleotide sequence of the gene encoding for CYP1A1 has made it possible to look for RFLPs in the CYP1A1 gene (10–13). In the early experiments, no association between RFLP patterns and AHH inducibility was observed (13); but the subsequent discovery of a polymorphic Mspl restriction site in the 3′ end of the CYP1A1 gene (14, 15) led to the detection of cosegregation of the CYP1A1 phenotype and Mspl polymorphism (16). A significant correlation between enhanced susceptibility to lung cancer and one of the detected CYP1A1 Mspl genotypes was discovered recently in a study of Japanese lung cancer patients (17). Furthermore, a subsequent Japanese study associated a much higher relative risk of lung cancer with the rare homozygous genotype, at a low level of lifetime cigarette consumption (18). The Japanese group has also found a close genetic linkage between Mspl polymorphism and another point mutation resulting in the substitution of the amino acid isoleucine for valine at residue 462 in the heme-binding region of CYP1A1 (19).

In our study, samples from 87 lung cancer patients, 23 patients with noncancerous lung disease, and 121 healthy controls have been examined for point-mutational Mspl and Ile-Val polymorphisms in the CYP1A1 gene. We also investigated the possible association be-

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2 The abbreviations used are: AHH, aryl hydrocarbon hydroxylase; RFLP, restriction fragment length polymorphism.
between increased risk of lung cancer, histological type, and extent of tobacco smoke exposure.

Subjects and Methods

Study Subjects. Three groups were included in this study: the first comprised 94 blood donors to the Finnish Red Cross Blood Transfusion Service (all males) and 27 other volunteers (12 males, 15 females) as healthy controls; the other two groups comprised 87 lung cancer patients (70 males, 17 females) and 23 patients (16 males, 7 females) with nonmalignant pulmonary disease.

Both of the latter groups consisted of individuals who came to Helsinki University Central Hospital within a 3-year study period, for surgical pulmonectomy or lobectomy for suspected, operable lung carcinoma. The blood samples were obtained the day before surgery. None of the patients had received chemotherapy or radiotherapy.

All the patients were interviewed to obtain their occupational and smoking histories as described in detail elsewhere. No such detailed information was available for the blood donors or the volunteers. Based on urinary cotinine measurements in a subgroup of the patients the information given in the interview about the tobacco smoke exposure appeared to be very reliable. For the smokers, pack-years of cigarette smoking were calculated from daily cigarette consumption and the number of years of smoking.

The mean age of the lung cancer patients was 63 years (SD 9.5); most were either current cigarette smokers (n = 60) or ex-smokers who had stopped smoking 3 or more years prior to surgery (n = 19). According to the interview data seven patients had never smoked. The mean age of the patients with nonmalignant lung disease was 52 years (SD 13.9). Half were current cigarette smokers (n = 12); the rest either were ex-smokers (n = 5) or had never smoked (n = 6).

DNA Isolation and Southern Blot Analysis. DNA was isolated from heparinized peripheral blood samples (10-20 ml). Total lymphocyte DNA (5 μg) was digested completely with restriction endonuclease Mspl (25 units) for 4-5 h at 37 °C under conditions recommended by the supplier (Boehringer Mannheim, Germany). The products were subjected to electrophoresis in 0.9% Sepharose agarose gelatin; 0.2 mM each deoxynucleotide triphosphate; 1.0 mM magnesium chloride; 10 μM Tris-hydrchloric acid, pH 7.8; 50 mM potassium chloride; 0.1% (w/v) gelatin; 0.2 mM each deoxynucleotide triphosphate; 1 μM each primer; 400-600 ng genomic DNA as template; and 1.5 units Taq polymerase. After an initial denaturation at 94 °C for 90 s, 25 cycles of 60 s at 94 °C and 90 °C at 70 °C were performed. Final samples (10 μl) were electrophoresed on parallel lanes in a 1.8% agarose gel.

The oligonucleotides used in the PCR reactions were complementary to the following stretches of the CYP1A1 sequence, numbered according to the method of Jaiswal et al. (13): primer 1 (5'-GAAAGCTGGTGCACACCTCTC-3') 4912-4932; primer 2a (5'-AAGACCTCAGGCACAGGCAAT-3') 5159-5179; and primer 2g (5'-AAGACCCTCAGGCACCAAC-3') 5215-5234.

Statistical Analyses. The χ² (contingency tables) test with Yates' correction for continuity was used to test the associations between the different genotypes and cancer incidence, as well as other parameters studies. χ² tests were performed by comparing the genotype homozygous for the m1 allele to the genotypes homozygous or heterozygous for the m2 allele, or vice versa. The same procedure was used to compare the different ile-Val genotypes.

Results

The genetic restriction site polymorphism in the CYP1A1 gene was detected by the presence or absence of the Mspl site located at the 3' end of the gene (Fig. 1). The presence of the polymorphic Mspl restriction site in both alleles, arising from a T→C base change 264 base pairs downstream from the polyA signal, gave a 1.9-kilobase band in a Southern autoradiogram, which characterized the homozygous genotype m2m2 (14, 15). Likewise, the homozygous genotype m1m1 was defined by a 2.5-kilobase band on the Southern autoradiogram, whereas the appearance of both bands indicated the heterozygote m1m2 genotype (Fig. 2A).

The point mutation resulting in an amino acid change at residue 462 in CYP1A1 was studied using PCR as described in “Subjects and Methods.” In the allele-specific amplification, the primer pair amplified a 322-
and primer pair 1/2g was used for the mutation-specific amplification. No differences were seen between the lung cancer and groups. The point mutations appeared to be very closely linked.

and mim2, mlm2,

The allelic frequencies were calculated to be in very good correlation (86%) was also detected between the Mspl genotype groups. Furthermore, a total lack of occurrence of mlm2 genotype with the m2m2 genotype was observed (Table 2). Similar results were observed (data not shown).

Finally, the genotypes of patients having had work-related exposure to asbestos were distributed normally (Fig. 2).

table 1

Table 1 shows the frequencies of Mspl RFLP and Ile-Val alleles of the CYP1A1 gene in the three study groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of alleles (frequency)</th>
</tr>
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<tbody>
<tr>
<td>Healthy controls (n = 242)</td>
<td>m1</td>
</tr>
<tr>
<td></td>
<td>214</td>
</tr>
<tr>
<td>Lung cancer patients (n = 174)</td>
<td>(0.88)</td>
</tr>
<tr>
<td>Patients with other lung diseases (n = 46)</td>
<td>(0.87)</td>
</tr>
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</table>

Table 2 shows the observed frequencies of Ile-Ile, Ile-Val, and Val-Val in the observed Mspl genotype groups m1m1, m1m2, and m2m2 for the lung cancer patients. The point mutations appeared to be very closely linked. The Ile-Val and Val-Val genotypes were always associated with m1m2 and m2m2 genotypes, respectively, and a very good correlation (86%) was also detected between the Ile-Ile and m1m1 genotypes. Furthermore, a total lack of occurrence of Ile-Ile genotype with the m2m2 genotype was observed (Table 2). Similar results were obtained for the controls and for the patients with nonmalignant lung disease (data not shown).

Table 3 shows the distribution of Mspl RFLP genotypes in the three groups. No significant differences could be observed between the study populations. The results also failed to show any significant variation in the genotypic frequencies for the various cell types of lung carcinoma. The distribution of genotypes for the most cases associated with smoking, squamous cell carcinoma (n = 44), did not differ from that seen for adenocarcinoma (n = 32). Neither could we find any skewed distribution of Mspl RFLP genotypes in the two patient populations, in relation to smoking habits (Table 4).

Discussion

We investigated the potential association of two CYP1A1 gene polymorphisms as indicators of a modified host-related susceptibility to lung cancer. The results indicate a close linkage between the two polymorphisms studied, i.e., the Mspl and Ile-Val polymorphisms in the CYP1A1 gene, in a Finnish study population. These observations agree with a report describing a similar close linkage in a Japanese study population (19). We could not, however, demonstrate any association between the Mspl polymorphism and an increased risk of lung cancer. This is in contrast to the results of Kawajiri et al. (17), who showed a statistically significant association between the homozygosity for the rare m2 allele and certain types of lung cancer. According to them, the frequencies of Mspl genotypes in lung cancer patients were significantly different from those observed in healthy control subjects. They estimated the increased risk of lung cancer for patients with the rare m2m2 genotype to be 2.6 or 3.1 (odds ratio), compared to the two more frequently occurring genotypes, m1m1 and m1m2. Of the histological types, squamous cell carcinoma gave the most noticeable deviation in the distribution of genotypes, compared to the distribution for healthy controls. Kawajiri and co-workers (17) calculated that patients with the m2m2 genotype were 5 times more likely to develop squamous cell carcinoma than patients with the m1m1 genotype.

More recently, the same group has also reported that smokers with the susceptible genotype m2m2 were at remarkably high risk, at an odds ratio of 7.31 (95% confidence interval, 2.13 to 25.12) at a low level of cigarette smoking and that the difference in susceptibility between genotypes was reduced at high levels of smoking (18).

In our study, m1 and m2 alleles were very similarly distributed between the different populations. However, the frequencies observed (0.88 and 0.12 for the controls, 0.87 and 0.13 for the lung cancer patients) clearly differed.
from the Japanese findings (0.69 and 0.31 for the controls, 0.56 and 0.44 for the lung cancer patients) (17). The most striking difference was in the proportion of the rare genotype m2m2, which was almost absent in the Finnish study populations. Furthermore, no statistically significant differences in genotypic distribution were observed between the histological types or the various smoking groups.

The present study design did not allow for a fully matched case-referent comparison, and the power calculations point to the need for larger study populations in order to demonstrate a significant difference in the rate of the rare genotype. Although the background frequency of the rare CYP1A1 Mspl genotype m2m2 was low in our study group (0.0165), the populations in the Finnish and Japanese studies were approximately the same size. Furthermore, we were able to use as an additional control group patients with lung diseases other than cancer, who entered the hospital the same time as the lung cancer cases.

Our results are consistent with a recent Norwegian report which found no association between the Mspl polymorphism in the CYP1A1 gene and lung cancer (22). The Norwegian control population (n = 212) exhibited frequencies of 0.88 and 0.12, and the lung cancer population (n = 221) frequencies of 0.89 and 0.11, for the m1 and m2 alleles, respectively. These frequencies were very close to those we observed, and, as we found, the incidence of the rare genotype homoygous for m2 was almost negligible in the Norwegian population.

The two Nordic studies have very similar findings, which differ clearly from the Japanese results. Several explanations for these divergences can be made, but the large discrepancy in genotypic frequencies points to a population-based genetic difference. In this study, we found a close linkage between Mspl and Ille-Val polymorphisms in the CYP1A1 gene; but we failed to show any association between the Mspl RFLP and susceptibility to lung cancer. In the Japanese population the Mspl polymorphism may therefore be linked to other unknown point mutations important for CYP1A1 expression or to other genes involved in tumorigenesis. This would explain the divergence between the findings for the Japanese and Nordic populations.

In an attempt to explain further the observed differences between our study and the Japanese one, we compared lung cancer rates and the fraction of cases attributable to tobacco smoking in the Finnish and the Japanese populations. The data from Cancer Incidence in Five Continents show that the incidence of lung cancer in Finnish men is more than double that in Japanese men; in women, the rates are similar (23). The annual consumption of cigarettes per capita was higher in Finland until around 1950; thereafter, the Japanese consumption figures have been higher. In fact, around 1975, the average annual number of cigarettes consumed per person in Japan reached the world's highest level (24, 25). The chronological relationship between cancer pattern (especially lung cancer mortality), cigarette consumption, and the prevalence of smoking suggests that cigarette smoking is the main cause of lung cancer, in both Finland and Japan. Given that more than 80% of Finnish and Japanese lung cancer cases are attributable to smoking, numerous explanations occur for smoking to appear more detrimental in terms of lung cancer to Finnish men than to Japanese men. These range from differences in genetic susceptibility to differences in environmental factors including life style.

To summarize, our study used a molecular epidemiological approach to assess whether genetic variations in the CYP1A1 gene can be used to predict individual variations in responses to exposure to carcinogens. In addition to the CYP genes, genetic variations in genes controlling the enzymes involved in detoxification reactions, such as one of the glutathione S-transferases, have been suggested as determinants of susceptibility to lung cancer.
cancer (26). Further studies are needed to examine the role of such variations in determining susceptibility to lung cancer in humans.

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