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Original Article

Restriction fragment length polymorphism of the human *CYP2E1* (cytochrome P450IIE1) gene and susceptibility to lung cancer: possible relevance to low smoking exposure

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Polymorphic metabolism of certain chemical carcinogens may result in differences in susceptibility to cancers. Human CYP2E1 (cytochrome P450IIE1) is an enzyme involved in the metabolic activation of precarcinogens such as nitrosamines. We detected a restriction fragment length polymorphism (RFLP) of the human *CYP2E1* gene for the restriction endonuclease *Dra* I. The distribution of this polymorphism was examined among lung cancer patients ($n = 91$), patients with cancer of the digestive tract ($n = 45$) and controls ($n = 76$). A significant difference in the distribution was observed between lung cancer patients and controls ($\chi^2 = 11.4$ with 2 df; $p < 0.005$). On the other hand, there was no significant difference between patients between cancer of the digestive tract and controls ($\chi^2 = 4.87$ with 2 *df*; NS). This finding suggests that the *Dra* I polymorphism of the *CYP2E1* gene is associated with susceptibility to lung cancer. In addition, an association was found between the amount of lifelong smoking exposure and the distribution of the genotypes of the RFLP among lung cancer patients. The distribution pattern seemed deviated from that of controls especially in the population of low smoking exposure. Our Northern blot analysis data using RNA from human liver autopsy samples suggest that the *Dra* I polymorphism might be associated with the gene expression of *CYP2E1* at mRNA level.

Introduction

Cytochrome P450 (CYP or P450) enzymes constitute the microsomal multisubstrate monooxygenase system responsible for the oxidative metabolism of both endogenous and exogenous compounds (Gonzalez, 1988). Reactions mediated by P450s often result in the production of substances involved in tumourigenesis. Human CYP2E1 (P450IIE1) is involved in the oxidation of potential pretoxicants or precarcinogens such as *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, benzene, styrene, carbon tetrachloride, and trichloroethylene (Bartsch & Montesano, 1984; Hong & Yang, 1985; Wrighton *et al.*, 1986, 1987; Yoo *et al.*, 1988; Guengerich & Shimada, 1991; Guengerich *et al.*, 1991). Therefore, differences in the genotypes or phenotypes of *CYP2E1* may be responsible for inter-individual variations in susceptibility to cancers caused by these chemicals (Nebert, 1988). The CYP2E1 enzyme is constitutively expressed in liver and can also be induced by substances such as ethanol and acetone (Gonzalez, 1988). Protein levels and catalytic activities of CYP2E1 have been shown to vary among individuals (Wrighton *et al.*, 1986; Yoo *et al.*, 1988). However, the genetic basis of such differences has not been precisely elucidated.

Restriction fragment length polymorphisms (RFLPs) of the human *CYP2E1* gene have been detected for the restriction endonucleases *Taq* I (McBride *et al.*, 1987), *Dra* I (Uematsu *et al.*, 1991a, b), *Rsa* I (Uematsu *et al.*, 1991a), and *Msp* I (Uematsu *et al.*, 1991c). In addition, polymorphisms in the upstream region of the human *CYP2E1* gene have been observed for *Pst* I and *Rsa* I (Watanabe *et al.*, 1990; Hayashi *et al.*, 1991). For the last two RFLPs, racial differences in allelic

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frequency have been shown (Kato *et al.*, 1992). We previously reported an association between the *Dra* I polymorphism of the *CYP2E1* gene and susceptibility to lung cancer (Uematsu *et al.*, 1991b, 1992) and cancer of the digestive tract (Uematsu *et al.*, 1992). This time, we devised a genotyping method employing the polymerase chain reaction (PCR). In this study, the distribution of the polymorphism was examined among lung cancer patients, patients with cancer of the digestive tract and controls using PCR analysis. The relation of the polymorphism with the expression of the gene at mRNA level in human liver autopsy samples was also investigated by Northern blot analysis.

Subjects and methods

Population samples

The study population consisted of 136 cancer patients and 76 controls ($\bar{x} = 64.6$ y, SD = 13.9 y, 57 males and 19 females). Controls were chosen from the population without known history of cancer until their blood samples were collected. Cancer patients were divided into group A (91 lung cancer patients; $\bar{x} = 66.0$ y, SD = 9.2 y, 79 males and 12 females) and group B (45 patients with cancer of the digestive system; $\bar{x} = 61.3$ y, SD = 14.6 y, 35 males and 10 females). Group B included 14 cases of gastric cancer, 10 cases of colon and rectum cancer, 10 cases of esophageal cancer. All the patients had a definite diagnosis proven by histology or cytology from clinically obtained specimens. Southern blot analysis was performed as previously described (Uematsu *et al.*, 1991b) using the cDNA probe λhPD4 (Komori *et al.*, 1989).

Human liver samples ($n = 17$) were obtained at autopsy and provided from Department of Pathology, Tohoku University Hospital, Sendai, Japan.

PCR analysis

PCR was performed in a final volume of 100 µl containing 2 µg genomic DNA, 100 pmol of each oligonucleotide primer (5′-TGTCGTTCCTTCCACAGGGC-3′ and 5′-TCTGTTGTCAGGCTAGAGTG-3′), 200 µM each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 2.5 U AmpliTaq (Perkin-Elmer Cetus Corp., Norwalk, CT, USA) and 1 U Perfect Match polymerase enhancer (Stratagene, La Jolla, CA, USA). Thirty cycles at 94° C for 1 min, 60° C for 2 min and 72° C for 3 min were performed with the final extension step lengthened to 10 min. One tenth of the PCR products (10 µl) were digested with 12 U *Dra* I (Takara Shuzo, Kyoto, Japan) overnight at 37° C and separated by electrophoresis on 2.0% agarose gels. Fragment sizes were determined by co-electrophoresis with *Hind* III-digested λDNA. The PCR-amplified fragment was also cloned into the pUC19 vector and the DNA sequence including the polymorphic site was

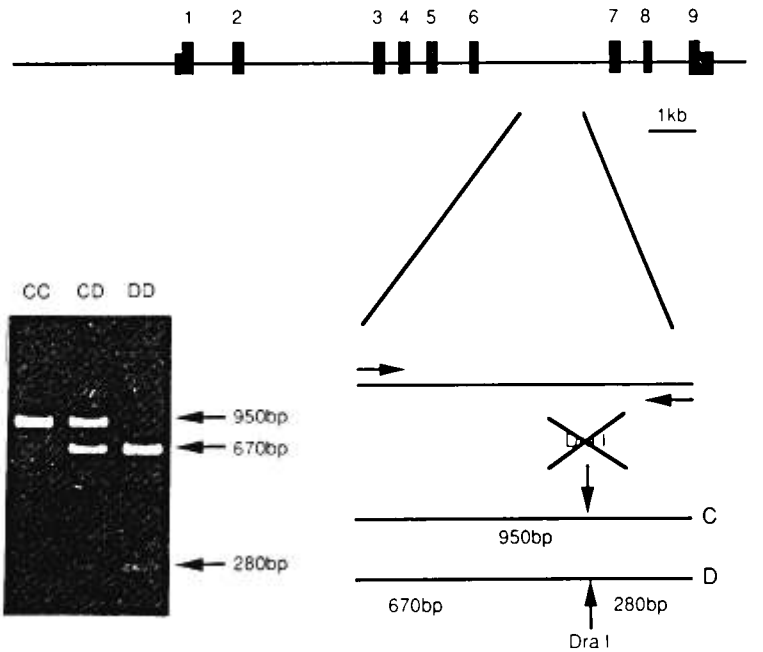

Fig. 1. *Dra* I polymorphism of the *CYP2E1* gene detected by polymerase chain reaction. The amplified fragment is a 950 bp sequence containing the polymorphic site in intron 6. By digestion with *Dra* I, individuals are divided into three genotypes, namely, CC, CD, and DD.

determined by the dideoxy-mediated chain termination (Sanger) method using Sequenase Version 2.0 (United States Biochemical Corp., Cleveland, OH, USA).

Northern blot analysis

Total RNA was extracted from the human liver samples obtained at autopsy by the method employing buffer-saturated phenol and proteinase K (Frazier *et al.*, 1983). The RNA was subjected to electrophoresis on 1.0% agarose gels containing 0.66 M formaldehyde in 1 × MOPS (3-[*N*-morpholino]propane sulfonic acid) buffer and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The blotted RNA was then hybridized with the cDNA probe λhPD4 labelled with ^{32}P-dCTP (Amersham, Amersham, UK) using random oligonucleotide primers (Boehringer Mannheim GmbH, Mannheim, Germany). Washing and autoradiography were then performed (Sambrook *et al.*, 1989). The levels of *CYP2E1* mRNA were assayed by densitometry and normalized to the levels of β-actin mRNA.

Results

PCR analysis

Dra I detected a two-allele polymorphism with bands at 950 bp or 670 bp and 280 bp (Fig. 1). The polymorphic site was located in intron 6 of the *CYP2E1* gene on the basis of the published genomic sequence data (Umeno *et al.*, 1988). The sequence analysis revealed that the *Dra* I polymorphism was caused by a T → A base transversion which is responsible for the presence or absence of the *Dra* I site (Fig. 2).

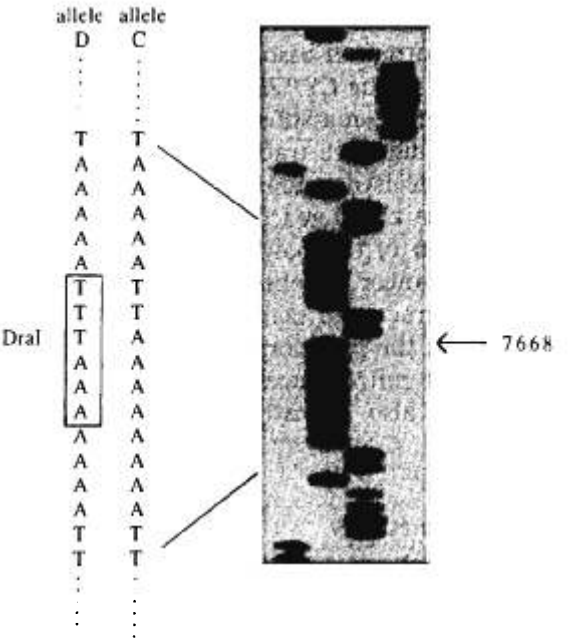

Fig. 2. The DNA sequence ladder in a part of intron 6 of the *CYP2E1* gene (allele C). The *Dra* I polymorphism is caused by a T → A bp change.

Table 1 shows the distribution of the three genotypes of the *Dra* I polymorphism of the *CYP2E1* gene in each group. In the control group, the distribution did not strictly follow the Hardy-Weinberg equilibrium, gene frequencies being 0.29 for allele C and 0.71 for allele D. But the gene distribution in lung cancer patients was in Hardy-Weinberg equilibrium. The distribution among lung cancer patients was different from that among controls ($\chi^2 = 11.4$ with

Table 1. Distribution of the different genotypes of the *Dra* I polymorphism of the *CYP2E1* gene in the various populations

	Genotypes			
	CC (%)	*CD (%)*	*DD (%)*	*Total*
Control	11 (14.5)	22 (28.9)	43 (56.6)	76 (100)
Cancers of the digestive tract	5 (11.1)	22 (48.9)	18 (40.0)	45 (100)
Lung cancer	2(2.2)	42 (46.2)	47 (51.6)	91 (100)
Squamous cell carcinoma	0	10	14	24
Small cell carcinoma	0	10	11	21
Large cell carcinoma	0	5	5	10
Adenocarcinoma	2	17	17	36
Odds ratio based on the comparison between lung cancer patients and controls (95% confidence interval)	0.13 (0.04–0.51)	2.1* (1.11–3.99)	0.82 (0.54–1.51)	

*$p < 0.05$.

Table 2. Distribution of the genotypes of the *CYP2E1* gene in relation to smoking

	Genotype			
	CC (%)	*CD* (%)	*DD* (%)	*Total*
⩽20 pack years[a]	1 (4)	16 (64)	8 (32)	25
>20 pack years	1 (2)	21 (39)	32 (59)	54
				$\chi^2 = 5.08$[b] $p < 0.05$

[a]1 pack year (PY) = 20 cigarettes per day for 1 year.
[b]χ^2 is based on the comparison between the presence and absence of the C allele.

2 *df*; $p < 0.005$). On the other hand, there was no difference between the distribution among patients with cancer of the digestive system and that among controls ($\chi^2 = 4.87$ with 2 *df*; NS). The odds ratio based on the comparison between lung cancer patients and controls is 2.1 in the case of CD genotype, suggesting a statistically significant difference between the two groups. These results indicate that the *Dra* I polymorphism of the *CYP2E1* gene seems to be associated with susceptibility to lung cancer. There was, however, no association between the RFLP and histological type of lung cancer cells.

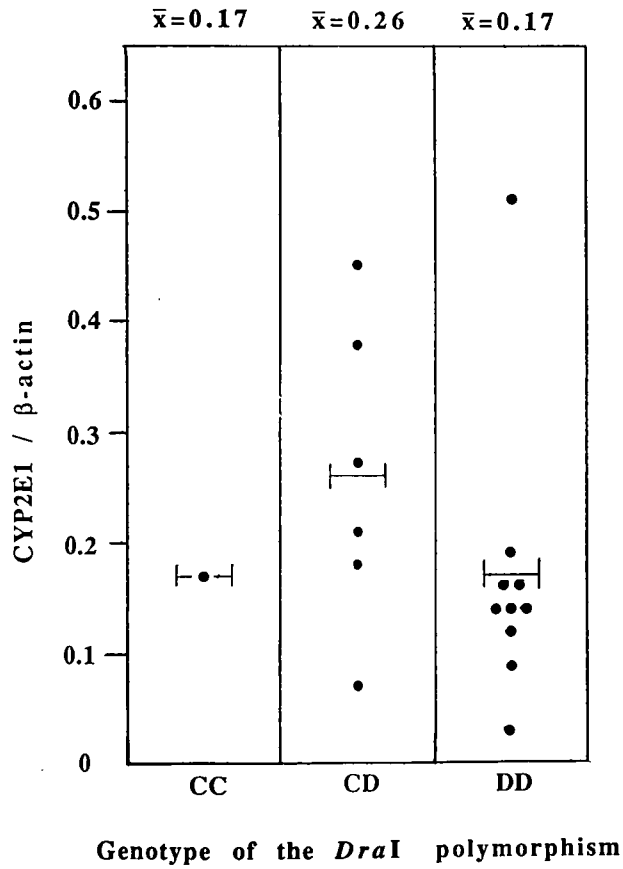

Fig. 3. mRNA levels of *CYP2E1* in human liver autopsy samples. Each value is normalized to mRNA amount hybridizing with the β-actin probe.

Smoking history was confirmed for 79 lung cancer patients. The distribution of the different genotypes among these lung cancer patients with regard to lifelong smoking exposure is shown in Table 2. There was no statistically significant difference in the distribution of three genotypes between those with smoking exposure of ⩽20 PY (packyears) and >20 PY ($\chi^2 = 5.15$ with 2 *df*, NS). However, when comparison was made between the presence and absence of the C allele, such as CC and CD versus DD genotypes, a statistically significant difference in the distribution was detected between those with smoking exposure of ⩽20 PY and those with >20 PY ($\chi^2 = 5.08$ with 1 *df*, $p < 0.05$).

Northern blot analysis

The expression of the gene at mRNA level was determined by Northern blot densitometry (Fig. 3). The mean value of the mRNA level was not significantly different between the CD genotype and the DD genotype by Mann-Whitney *U* test ($n_A = 6$, $n_B = 10$, $U = 66$; NS). Then the mean value of the mRNA level was compared between the presence and absence of the C allele. Likewise, there was no significant difference ($n_A = 7$, $n_B = 10$, $U = 81$; NS). The distribution of the values in the DD genotype showed a normal distribution, so the outlier among the DD genotype (0.51) may be rejected ($T = 2.7$; $p < 0.01$), and therefore, under this condition, the mean value was different for the presence and absence of the C allele ($n_A = 7$, $n_B = 9$, $U = 81$, $p < 0.05$).

Discussion

In our results, using PCR analysis, the frequency of the heterozygote (CD) in lung cancer patients was higher than that in the control group, while the frequency of the homozygotes (CC and DD) was lower (Table 1). Hence it would be difficult to conclude that either of the two alleles is associated with a high susceptibility to cancer. The odds ratios for lung cancer risk were 0.13 for CC (95% CI = 0.04–0.51; $\chi = -2.9$, NS), 2.1 for CD (95% CI = 1.11–3.99; $\chi = 2.3$, $p < 0.05$), 0.82 for DD (95% CI = 0.54–1.51, $\chi = -0.63$, NS). These findings were similar to those in our previous report using Southern blot hybridization for RFLP analysis (Uematsu *et al.*, 1991b).

The data shown in Table 2, however, might give a key to this problem. As stated above, there was an

association in the lung cancer patients between the high frequency of the C allele and the low level exposure to smoking. This relationship further supports the association between the *Dra* I polymorphism and lung cancer susceptibility. Interestingly, among lung cancer patients with high smoking exposure the distribution of the genotypes seems to be similar to that among controls, whereas among those with low smoking exposure the distribution does not. This suggests that the *CYP2E1* polymorphism could be closely associated with susceptibility to lung cancer especially among populations with low smoking exposure. From our data reactions mediated by CYP2E1 may be important in pulmonary carcinogenesis in those with low smoking exposure, while reactions unrelated to CYP2E1 may play a major role in the pathogenesis of lung cancer in populations with high smoking exposure. It is also possible to speculate that environmental carcinogens from sources other than tobacco smoke, such as air pollutants and diet, may be modified by CYP2E1 and contribute to human pulmonary carcinogenesis.

The *Dra* I polymorphism is caused by a base pair change in intron 6 of the *CYP2E1* gene. Therefore, this is unlikely to be the site directly affecting the variability of the gene expression. It could, however, be a marker in linkage disequilibrium with such a site. Our data of Northern blot analysis is interesting in that mRNA levels of human liver *CYP2E1* tend to be high in the presence of the C allele as compared with the absence of it as stated above. This may be compatible with our data in which the frequency of the DD genotype was low among lung cancer patients.

There are some reports that a genetic factor is important in the etiology of lung cancer. The familial influence was more evident among nonsmokers than smokers (Tokuhata & Lilienfeld, 1963; Ooi *et al.*, 1986). Sellers *et al.* (1990) recently proposed a model of a Mendelian codominant inheritance of an autosomal gene causing onset of lung cancer at earlier age. It is possible that *CYP2E1* itself may act as a gene controlling inheritance in susceptibility to lung cancer. Another possibility is that the polymorphic site of the *CYP2E1* gene is in linkage disequilibrium with other tumour-related genes, such as oncogenes, tumour suppressor genes, or genes related to the structure or expression of other P450s. Indeed, the genes for P450IIC (*CYP2C*) (Okino *et al.*, 1987), P450XVII (*CYP17*) (Matteson *et al.*, 1986) and multiple endocrine neoplasia type2A (*MEN2A*) (Mathew *et al.*, 1987; Simpson *et al.*, 1987) have been located on the same chromosome as *CYP2E1* — chromosome 10 (Umeno *et al.*, 1988).

The number of the subjects analysed here may be too small to allow any definite conclusion on which genotypes are actually related to the susceptibility to lung cancer. However, from our data it seems likely that the C allele is relevant to a high risk for lung cancer in populations with low smoking exposure. These results may support the hypothesis that susceptibility to lung cancer is under genetic control. In this study, susceptibility to gastrointestinal tract cancer was not associated with the *Dra* I polymorphism, although an association was found in our previous study (Uematsu *et al.*, 1992). However, our study samples contained various sites of cancer in the gastrointestinal tract and, therefore, there may be a great degree of heterogeneity. The association between the polymorphism of the *CYP2E1* gene and susceptibility to other types of cancer except lung cancer should be further explored. Characterization of the molecular basis of the above findings would explain the relation between the *Dra* I polymorphism and gene expression. This might lead to the elucidation that *CYP2E1* plays a role in human carcinogenesis and also to the confirmation of a novel risk factor.

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