

Cytochrome P450E1 Genetic Polymorphisms and Risk of Nasopharyngeal Carcinoma: Results from a Case-Control Study Conducted in Taiwan¹

Allan Hildesheim,² Chien-Jen Chen, Neil E. Caporaso, Yu-Juen Cheng, Robert N. Hoover, Mow-Ming Hsu, Paul H. Levine, I-How Chen, Jen-Yang Chen, Czau-Siung Yang, Ann K. Daly, and Jeffrey R. Idle

Epidemiology and Biostatistics Program [A. H., R. N. H.], Genetic Epidemiology Branch [N. E. C.], Viral Epidemiology Branch [P. H. L.], National Cancer Institute, Bethesda, Maryland; Institute of Epidemiology, College of Public Health, National Taiwan University, Taipei, Taiwan [C.-J. C., Y.-J. C.]; Department of Otolaryngology, National Taiwan University Hospital, Taipei, Taiwan [M.-M. H.]; Department of Otolaryngology, MacKay Hospital, Taipei, Taiwan [I.-H. C.]; Institute of Microbiology, College of Medicine, National Taiwan University, Taipei, Taiwan [J.-Y. C., C.-S. Y.]; GenoType Limited, Newcastle Upon Tyne, United Kingdom [A. K. D., J. R. I.]

Abstract

CYP2E1 is responsible for the metabolic activation of nitrosamines believed to be involved in the pathogenesis of various tumors. Nasopharyngeal carcinoma (NPC) is a tumor thought to be linked to nitrosamine exposure. To investigate the possible role of CYP2E1 genetic polymorphisms in the etiology of this tumor, we investigated 50 histologically confirmed NPC cases from Taiwan and 50 controls matched to cases on age, sex, and residence. Samples were examined for RFLPs in the CYP2E1 gene by PCR amplification followed by digestion with *DraI* and *RsaI*. Among healthy controls, the allelic frequency of wild-type and variant forms of CYP2E1 were 79 and 21%, respectively, using *DraI* enzyme digestion and 82 and 18%, respectively, using *RsaI* enzyme digestion. As compared with individuals who were homozygous for the wild-type CYP2E1 gene, those found to be homozygous for the variant form of the gene by *DraI* digestion were at a 5-fold excess risk of disease (95% confidence interval = 0.95-16). Similarly, subjects homozygous for the variant form of the CYP2E1 gene by *RsaI* digestion were at 7.7-fold excess risk of developing NPC (95% confidence interval = 0.87-68). Individuals found to be heterozygous for the gene were at similar risk of disease compared to those homozygous for the wild-type gene. A strong association was observed between the RFLPs detected by *DraI* and *RsaI* digestion of CYP2E1; a correlation coefficient of 0.86 for controls and 0.91 for cases was observed. Interestingly, all

individuals with the variant form of CYP2E1 detected by *RsaI* enzyme digestion also exhibited the variant form of the gene using *DraI* enzyme digestion, although the reverse was not always true. Our results confirm previous findings suggesting that the distribution of CYP genotypes among Oriental populations varies from that observed among Caucasians and demonstrate for the first time a possible association between CYP2E1 genetic polymorphisms and the risk of developing NPC.

Introduction

The CYP³ family of enzymes is responsible for the metabolism of numerous xenobiotics (1). In addition, some of the CYP enzymes are involved in the activation of procarcinogens into reactive intermediates capable of forming adducts and damaging DNA, a step believed to be essential in chemical carcinogenesis (1-3). Nitrosamines are among the substrates of CYP enzymes believed, once activated, to be linked to the development of numerous cancers (4). Of the over 250 CYP enzymes cloned to date, CYP2E1 is believed to play an important role in the activation of these potentially carcinogenic nitrosamines (5, 6).

NPC has been associated with nitrosamine exposure (7). Evidence from previous epidemiological studies suggests that salted fish (7, 8) and cigarette smoke (7, 9-12), both of which contain nitrosamines and nitrosamine precursors (13-15), are associated with NPC risk. Animal studies have demonstrated that rats fed nitrosamine-containing salted fish develop nasal cavity tumors (16, 17).

Furthermore, a recent study conducted in two regions of China having different rates of NPC showed that individuals residing in the community with high NPC rates had an increased ability to endogenously form nitrosamines compared to individuals residing in the community with low NPC rates (18). Because the carcinogenic action of nitrosamines requires that they first be activated and because CYP enzymes have been shown to be involved in the activation process and to be expressed in the nasal epithelium of numerous animal species and humans (7, 19-23), we hypothesized that genotypic and/or phenotypic differences in CYP enzymes responsible for the activation of nitrosamines might partially account for differences in risk of developing NPC. In the present report, we investigate the possible role of CYP2E1 genotypes in NPC pathogenesis among 50 NPC cases and 50 matched healthy controls from Taiwan. PCR and RFLP methods were used to identify polymorphisms in the gene of interest. *DraI* and *RsaI* restriction enzyme digestions were utilized to detect genotypic

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² To whom requests for reprints should be addressed, at Environmental Epidemiology Branch, National Cancer Institute, Executive Plaza North Room 443, Bethesda, MD 20892-7374.

³ The abbreviations used are: CYP, cytochrome P-450; NPC, nasopharyngeal carcinoma; CI, confidence interval.

polymorphisms. The use of these restriction enzymes was dictated by previous findings suggesting that the variant detected by *DraI* digestion might be associated with lung cancer risk and that the two forms of the gene detected by *RsaI* digestion exhibit widely different levels of expression (24–27).

Materials and Methods

As part of a larger epidemiological investigation of NPC currently underway in Taipei, Taiwan, 50 incident cases of NPC were selected for the present study. These subjects were randomly chosen from among the participants in the larger investigation for whom adequate amounts of lymphocytes were available. Cases were diagnosed in one of two large referral hospitals in Taipei. All cases were histologically confirmed as having NPC by an ear, nose, and throat specialist (M-M. H.). Subjects were required to be <75 years old, to have no prior diagnosis or treatment for NPC, and to have resided in Taipei city or county for at least 6 months.

For each eligible NPC case, an individually matched healthy control was selected for study. Controls were matched to the NPC cases on age (5-year groupings), sex, and residence. In addition, controls were required to have no history of NPC before identification for the present study.

All subjects were asked to donate 30 ml of blood for study. Samples were collected and kept at room temperature until processing began. Peripheral blood lymphocytes obtained by Ficoll-Hypaque separation were diluted in a freezing medium containing RPMI 1640 (70%), DMSO (15%), and FCS (15%) and stored in a liquid nitrogen tank. Shipments to the United States were made in dry ice on a monthly basis. Once frozen, samples were never thawed until testing.

DNA extraction was performed by using standard methods (28). All PCR reactions were carried out in a final volume of 50 μ l. The reaction buffer was 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂, 50 mM KCl, 0.1% (v/v) Triton X-100 with each primer at 0.25 μ M, nucleotides at 0.25 mM, and Tbr polymerase (1 unit/50 μ l reaction; NBL Gene Sciences, Cramlington, Northumberland, United Kingdom). The assays for *CYP2E1* (*RsaI*) also contained 3% DMSO.

The method of Hayashi *et al.* (26) was used to identify genotypes of *CYP2E1* using *RsaI* digestion. In brief, two primers used were: (a) CCAGTCGAGTCTACATTGTCA; and (b) TTCATTCTGTCTTCTAACTGG. The PCR conditions were 35 cycles of 1 min at 95°C, 2 min at 45°C and 2 min at 70°C. Twenty μ l of each sample was digested with 10 units *RsaI* at 37°C for 3 h and the products analyzed by electrophoresis on a 4% Nusieve 3:1 agarose gel. Bands were visualized on a UV transilluminator (Fig. 1A). A sample of DNA from a subject known to be heterozygous was included in each set of 10 samples. All samples were analyzed twice, and concordant results were obtained on both occasions.

The method described previously by Kim *et al.* (29) was used to determine *CYP2E1* genotypes using *DraI* digestion. The primers were: (a) TCGTCAGTTCCTGAAAGCAGG; and (b) GAGCTCTGATGCAAGTATCGCA. The PCR conditions were 35 cycles of 1 min at 95°C, 2 min at 55°C, and 2 min at 70°C. Twenty μ l of each sample was digested with 10 units *DraI* at 37°C for 3 h and the products analyzed by electrophoresis on a 1.8% agarose gel. Bands were visualized on a UV transilluminator (Fig. 1B). Samples of DNA from subjects known to be homozygous wild type, heterozygous, and homozygous mutant were included in each set of 20 samples. All samples were analyzed on two separate occasions and yielded concordant results.

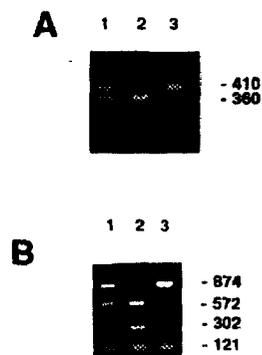


Fig. 1. Detection of the *RsaI* and *DraI* polymorphisms. A, analysis with *RsaI*. Lane 1, heterozygous; Lane 2, homozygous wild type; Lane 3, homozygous variant. B, analysis with *DraI*. Lane 1, heterozygous; Lane 2, homozygous wild type; Lane 3, homozygous variant. The fragment sizes are indicated.

Table 1 Demographic characteristics of study subjects

	Cases	Controls
Sample size	50	50
Age		
Mean (yrs)	45.1	45.0
Range (yrs)	17–63	19–64
Sex		
% Male	82%	82%
% Female	18%	18%

A 10% random sample ($n = 10$) of subjects were blindly tested twice for each of the assays described above. The results were concordant for all 10 blind duplicate sets on all assays.

Allele and genotype frequencies were computed. The relative risk, as estimated by the odds ratio, was the estimate used to determine the association between CYP genotype and disease (30). 95% CIs were calculated to determine the statistical significance of findings (30). Correlation coefficients were computed by using the method proposed by Pearson (31).

Results

The age and sex distributions of the 50 cases and 50 controls selected for study are presented in Table 1. Eighty two % of cases were male and the median age of cases was 45.1 years (range = 17–63 years). The similar age and sex distribution observed among controls, relative to the cases, was dictated by the matching criteria utilized in selecting controls for the present study.

CYP2E1 RFLP data were available on 48 NPC cases and 50 healthy controls; 2 cases had insufficient DNA available for analysis. The distribution of alleles among our healthy controls is presented in Table 2. As shown in the table, the wild-type and variant forms of *CYP2E1* detected by *DraI* digestion were present in 79 and 21% of control subjects, respectively. Similarly, the wild-type and variant forms of *CYP2E1* detected by *RsaI* digestion were present in 82 and 18% of control subjects, respectively.

Table 3 presents results of our analysis that investigated the association of *CYP2E1* genotypes and NPC risk. Subjects who were homozygous for the variant form of *CYP2E1* by *DraI* digestion were at a 5-fold excess risk of disease compared to

Table 2 Allele frequencies of CYP2E1 genotype among healthy control subjects

	Wild type	Variant
CYP2E1- <i>DraI</i>	79.0%	21.0%
CYP2E1- <i>RsaI</i>	82.0%	18.0%

Table 3 Frequency distribution among cases and controls and relative risks associated with genotypic variants of CYP2E1

CYP2E1	Frequency		RR ^a	95% CI
	Cases	Controls		
<i>DraI</i>				
Homozygous wild type	25	31	1.0	
Heterozygous	15	17	1.1	0.45-2.7
Homozygous variant	8	2	5.0	0.95-16
<i>RsaI</i>				
Homozygous wild type	30	33	1.0	
Heterozygous	11	16	0.76	0.30-1.9
Homozygous variant	7	1	7.7	0.87-68

^aRR, relative risk.

Table 4 Correlation and joint distribution of the *DraI* and *RsaI* genotypes

<i>DraI/RsaI</i>	Homozygous wild type	Heterozygous	Homozygous variant
Among NPC cases			
Homozygous wild type	25	0	0
Heterozygous	4	11	0
Homozygous variant	1	0	7
Correlation coefficient	0.91 ($P < 0.001$)		
Among healthy controls			
Homozygous wild type	31	0	0
Heterozygous	2	15	0
Homozygous variant	0	1	1
Correlation coefficient	0.86 ($P < 0.001$)		

subjects who were homozygous for the wild-type gene (95% CI = 0.95-16). Similarly, subjects who were homozygous for the variant form of *CYP2E1* by *RsaI* digestion were at a 7.7-fold excess risk of disease compared to subjects who were homozygous for the wild-type gene (95% CI = 0.87-68). No excess in risk was observed for individuals heterozygous for the wild-type and variant forms of the gene using either *DraI* or *RsaI* digestions, relative to those who were homozygous for the wild-type gene.

Previous studies have reported a high degree of association between RFLPs observed after *DraI* and *RsaI* digestion. We examined this issue among our Taiwanese study population (Table 4). Our results suggest a strong association between the RFLPs observed by *DraI* and *RsaI* digestion. The two RFLPs were associated in 47 (94%) of 50 healthy controls and in 43 (86%) of 48 NPC cases examined. Interestingly, all individuals with the variant form of *CYP2E1* detected using *RsaI* enzyme digestion also exhibited the variant form of the gene using *DraI* enzyme digestion, although the reverse was not always true.

Discussion

Results from the present study suggest that genotypic variants of the *CYP2E1* gene might be associated with risk of NPC. Subjects found to be homozygous for the variant form of

CYP2E1 using *DraI* digestion were at a 5-fold excess risk of disease and those homozygous for the variant form of the gene using *RsaI* digestion were at a near 8-fold excess risk of disease. These results were of marginal statistical significance due to the relative small size of the present study. Given the strong association observed between the variants of *CYP2E1* detected by *DraI* and *RsaI* digestion, we were unable to disentangle the independent effects of these polymorphisms on disease risk. However, it is interesting to note that a previous study reported a 10-fold increase in gene expression of the variant form of the *CYP2E1* gene (detected by *RsaI* digestion) relative to the wild-type form of the gene, when a chloramphenicol acetyltransferase-containing construct was transfected into HepG2 cells and analyzed for chloramphenicol acetyltransferase activity (26). The polymorphism detected by *DraI* digestion of *CYP2E1* is located in intron 6, and no functional significance of this polymorphism is currently known to exist.

NPC is a tumor thought to occur, in part, as a result of exposure to nitrosamines (7). Studies have consistently observed that individuals who consume salted fish and other processed/preserved foods known to contain nitrosamines and nitrosamine precursors are at excess risk of developing NPC (13, 14). Risk of disease appears to be particularly accentuated when exposure occurs at young ages (8). Also, several studies to date have reported that cigarette smoking is related to risk of NPC (7, 9-12), although some studies have failed to observe this association (7, 32, 33). As with salted fish, the nitrosamines present in cigarettes are believed to be the chemical carcinogens linked to NPC development.

Because nitrosamines require activation to impart their carcinogenicity and *CYP2E1* is known to be important in the activation of numerous nitrosamines, the association between *CYP2E1* genotype and NPC risk observed in this study is biologically plausible (5, 6). Furthermore, evidence suggesting that CYP enzymes are expressed in the nasal cavity of numerous animal species and humans further strengthens the biological plausibility of the observed association (19-21, 23). The fact that an increased risk of disease was apparent only among individuals who were homozygous for the variant form of the gene suggests that *CYP2E1* could function in a recessive manner.

Results from our study are consistent with those from previous investigations suggesting that the allelic frequency distribution of the different forms of *CYP2E1* varies between Caucasian and Oriental populations (27). The allelic frequency distribution of the variant form of the *CYP2E1* gene observed in two studies conducted among Japanese individuals was very similar to that noted in this report (~20%; Refs. 24, 27). In contrast, the allelic frequency distribution of the variant form of the *CYP2E1* gene is reported to be much lower among Caucasians ($\leq 10\%$; Refs. 25, 34).

In summary, results from the present study suggest an association between *CYP2E1* genotype and risk of developing NPC. Larger studies that are capable of simultaneously assessing *CYP2E1* polymorphisms and nitrosamine exposure are needed to confirm and extend the present findings.

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