

# Genetic Susceptibility to Squamous Cell Carcinoma of the Lung in Relation to Cigarette Smoking Dose<sup>1</sup>

Kei Nakachi,<sup>2</sup> Kazue Imai, Shin-ichi Hayashi, Junko Watanabe, and Kaname Kawajiri

Departments of Epidemiology [K. N., K. I.] and Biochemistry [S-i. H., J. W., K. K.], Saitama Cancer Center Research Institute, 818 Komuro, Ina-machi, Saitama 362, Japan

## ABSTRACT

Cytochrome P450IA1 is responsible for the metabolic activation of benzo(a)pyrene in cigarette smoke; an association of lung cancer with DNA polymorphisms of P450IA1 gene was shown in our previous study.

In this paper, we investigated the interindividual difference of genetically determined susceptibility to squamous cell carcinoma of the lung in relation to cigarette smoking dose. We first compared the total amounts of cigarettes consumed over the lifetime of patients and showed that the patients with a "susceptible" P450IA1 gene genotype contracted carcinoma after fewer cigarettes [mean  $\pm$  SD,  $31.3 \pm 12.8 \times 10^4$  cigarettes ( $n = 12$ )] than those with other genotypes [ $42.5 \pm 18.2 \times 10^4$  ( $n = 33$ )], with a statistical significance of  $P < 0.05$ . Next, we carried out a case-control study to estimate the odds ratios of susceptible to nonsusceptible individuals in relation to the cumulated cigarette dose. We thus showed that the individuals with the susceptible genotype were at remarkably high risk with an odds ratio of 7.31 (95% confidence interval, 2.13 to 25.12) at a low dose level of cigarette smoking and that the difference in susceptibility between genotypes was reduced at high dose levels.

## INTRODUCTION

An individual difference in susceptibility to chemical carcinogens is one of the most important factors in the estimate of risks to human cancer. Many different types of host factors may be involved in the mechanism of difference in susceptibility, including possible polymorphic changes of oncogenes or related genes in germ line, the activation and detoxification of carcinogens, and predispositions due to impaired antibody production or cellular immunity. Genetic differences in the metabolism of chemical carcinogens have been suggested to be associated with the different predispositions to cancers (1-8).

The cytochrome P450-dependent monooxygenases, involving a variety of P450 isozymes (9, 10), play a prominent role in the metabolism of various chemical carcinogens from the environment. Genetic variability in the oxidative activation of chemical carcinogens may well explain the interindividual difference in response to carcinogens. In our previous paper (11), we studied the association of lung cancer with DNA polymorphisms of the P450IA1 gene, because cytochrome P450IA1 is responsible for the metabolic activation of benzo(a)pyrene and other aromatic procarcinogens in cigarette smoke. Namely, we reported that: (a) three polymorphisms of P450IA1 gene originated from the presence or absence of one *MspI* site in the 3' flanking region; (b) the segregation profile of these polymorphisms in family pedigree analysis showed mendelian inheritance; (c) three genotypes were classified in the predominant homozygote (genotype *A*) characterized by 2.7-, 2.3-, and 0.8-kilobase fragments

in RFLPs,<sup>3</sup> the heterozygote (genotype *B*) by 2.7, 2.3, 1.9, and 0.8 kilobases and the homozygous rare allele (genotype *C*) by 2.7, 1.9, and 0.8 kilobases; and (d) the frequency of genotype *C* among lung cancer patients was more than 2-fold higher than that among healthy controls, showing statistical significance. The patients with squamous cell carcinoma of the lung especially showed a remarkable deviation of frequency from controls. In addition, we recently identified the origin of these polymorphisms to be substitution of one base at the 264th base downstream from the additional poly(A) signal, forming an *MspI* site in genotype *C* (12).

The individual differences in susceptibility to lung cancer may be investigated by two different approaches to the aspect of gene-environment interactions. The first one entails the comparison of genotype frequencies between patients and a healthy population, and the difference indicates genetic variability in susceptibility due to different phenotypic expressions of the gene. Then, it may be plausible to infer that the individuals with differing susceptibilities to cancer must be also different in response to the dose of carcinogens. In this paper we provide the results of both approaches and clarify that the individuals at genetically high risk for squamous cell carcinoma of the lung, which is most closely associated with cigarette smoking, can contract the cancer with a smaller dose of cigarette smoke than other people.

## MATERIALS AND METHODS

**Experimental.** Blood samples (5 to 15 ml) were obtained from both patients and controls, and DNAs were isolated from peripheral lymphocytes. The identification of the P450IA1 gene genotypes ascribed to the *MspI* site at the 264th base from the additional poly(A) signal in the 3' flanking region was carried out with two methods: Method 1, RFLPs of the P450IA1 gene by a genomic Southern blotting, digesting the sample DNAs with *MspI*, and hybridizing with a probe of the *XbaI-EcoRI* fragment of the cloned P450IA1 gene (11, 13); and Method 2, DNAs amplified by the use of a PCR, where two synthetic oligonucleotide primers of 21 bases (from the 130th to 150th and from the 449th to 469th bases, counting from the additional poly(A) signal) were used (12). PCR was carried out by 25 cycles under the following conditions: 1 min at 95°C for denaturation; 1 min at 68°C; and 1 min at 72°C for primer annealing and primer extension. Other conditions used were as described previously (14). The amplified fragments including the *MspI* site were digested with *MspI* for 2 h at 37°C, and the products were subjected to electrophoresis in 1.8% agarose gel. Genotype *A* was characterized by a 0.34-kilobase fragment; genotype *B* by 0.14, 0.20, and 0.34 kilobases; and genotype *C* by 0.14 and 0.20 kilobases.

These two methods were in complete accordance with each other in the identification of the genotypes. Method 2 was used to identify the genotypes of the controls selected for the present case-control study.

**Epidemiological.** All possible inpatients with all sites of cancer, excluding recurrence or cases with a history of cancer, have been interviewed in Saitama Cancer Center Hospital since 1984 by the staff of the epidemiology department using a standardized questionnaire on

<sup>3</sup> The abbreviations used are: RFLP, restriction fragment length polymorphism; poly(A), polyadenylate; PCR, polymerase chain reaction; CI, confidence interval.

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<sup>2</sup> To whom requests for reprints should be addressed.

Table 1 Distribution of the three genotypes of the P450IA1 gene among lung cancer patients compared with distribution among healthy controls

Histological types	P450IA1 genotypes			Total
	A	B	C	
<b>Kreyberg type I</b>				
Squamous cell carcinoma	19 (33.3) <sup>a</sup>	23 (40.4)	15 (26.3)	57 (100.0)
Small cell carcinoma	10 (41.8)	7 (29.1)	7 (29.1)	24 (100.0)
Large cell carcinoma	2 (20.0)	6 (60.0)	2 (20.0)	10 (100.0)
Total	31 (34.0)	36 (39.6)	24 (26.4)	91 (100.0)
<b>Kreyberg type II</b>				
Adenocarcinoma	30 (50.3)	22 (36.7)	8 (13.3)	60 (100.0)
Healthy controls	166 (44.3)	169 (45.1)	40 (10.6)	375 (100.0)

<sup>a</sup> Numbers in parentheses, percentage.

their occupational career, dietary habits, cigarette smoking, and alcohol drinking history. This interview was not focused on a particular cancer and was carried out without any knowledge of their cancer sites. Our study focused on squamous cell carcinoma of the lung, and epidemiological information of the patients was collected from these interview data. Among the 57 patients with squamous cell carcinoma of the lung in Table 1, a total of 45 patients were interviewed; 12 patients could not be interviewed because of their serious condition or combination of other diseases, such as brain stroke and dementia. The 0.79 ratio (45 of 57) of interviewed patients was almost the same over the three genotypes. In this study we summarized the smoking history of the patients as the total amount of cigarettes consumed over their lifetime until interview. Included in the calculation of the total amount of cigarettes used were age smoking was begun, average consumption of cigarettes per day below 39 years of age, average consumption between 40 and 59 years of age, average consumption at 60 years of age and over, and abstinence durations longer than 1 year. Drastic changes of consumption within those age brackets were taken into account to estimate each average consumption. To compare two means of the cigarette consumption among the patients with different genotypes, the *t* distribution was calculated to test statistical significance.

We then designed a case-control study to estimate the susceptibility of the genotypes in relation to cigarette dose. Controls were selected from the subjects of our prospective cohort study, which aims to investigate host-environment interaction in carcinogenesis in the general population. A total of 8553 individuals were surveyed by a self-administered questionnaire, comprising 95% of the total residents over 40 years of age in a town near Saitama Cancer Center Hospital. Of these individuals, peripheral blood samples of 3625 have been collected thus far at the time of yearly health screening and subjected to a variety of biochemical and immunological assays designed for the cohort study. We also collected DNA samples of 2486 individuals from those surveyed since 1989. For this case-control study, we randomly selected 1700 individuals from those with DNA samples and interviewed them to estimate the total cigarette consumption. Controls used in this study were chosen from them and were individually matched to the patients with respect to sex and age (in 1-year age units). Three controls were randomly selected for each of the patients within the matching conditions. Responses to cigarette dose in the genotypes were then estimated from the distribution of patients and controls with respect to their genotypes and cigarette dose levels in terms of odds ratio.

**RESULTS**

Since first reporting the association of the polymorphic P450IA1 gene with the predisposition to lung cancer (11, 15), we greatly increased the numbers of subjects for study in both patients and healthy controls. Table 1 shows our present results. The frequency distribution of genotypes among the patients with Kreyberg type I lung cancer (16), which is closely associated with cigarette smoking (17), was clearly different from that among controls ( $P < 0.001$ ;  $\chi^2 = 15.462$ , d.f. = 2). It is noted that the frequency of genotype C was more than 2-fold higher

than that among controls ( $P < 0.001$ ;  $\chi^2 = 15.249$ ), although the frequencies of other genotypes did not show statistical significance. On the other hand, the frequency distribution of genotypes among patients with adenocarcinoma, which has little association with cigarette smoking (18), was almost identical to that among controls. The frequency distributions among the patients with cancer of the stomach, colon, and breast were also examined (15) and were identical to those among controls; their genotype C frequencies were 8.7% (9 of 104), 11.5% (9 of 78), and 9.7% (3 of 31), respectively.

Our first purpose in this paper entails the comparison of cigarette dose among patients with different P450IA1 gene genotypes. We focused on the squamous cell carcinoma, which is most closely associated with smoking in Kreyberg type I lung cancer (17). The total amounts of cigarettes consumed over a lifetime were compared among the patients with genotypes A, B, and C (Fig. 1). We found that the distribution of amounts in genotype C shifted to the lesser dose from that of the other two genotypes. Since there was no difference in the distributions between genotypes A and B (see Table 2 for mean  $\pm$  SD), we combined these two genotypes as A+B and compared the mean cigarette consumption with that of genotype C. The mean value of  $31.3 \times 10^4$  cigarettes ( $n = 12$ , SD =  $12.8 \times 10^4$ ) was lower than the mean of  $42.5 \times 10^4$  ( $n = 33$ , SD =  $18.2 \times 10^4$ ) of genotypes A+B with a statistical significance of  $P < 0.05$  [ $t_0 = 2.117$  for d.f. = 43, noting that the variance of the two samples estimated the same parametric variance

$$F = s_{A+B}^2/s_C^2 = 2.010 < F_{0.1(32,11)} = 2.076.$$

We next estimated the relative risk of the genotypes to squamous cell carcinoma of the lung by means of a case-control study. The characteristics of the patients and controls are shown

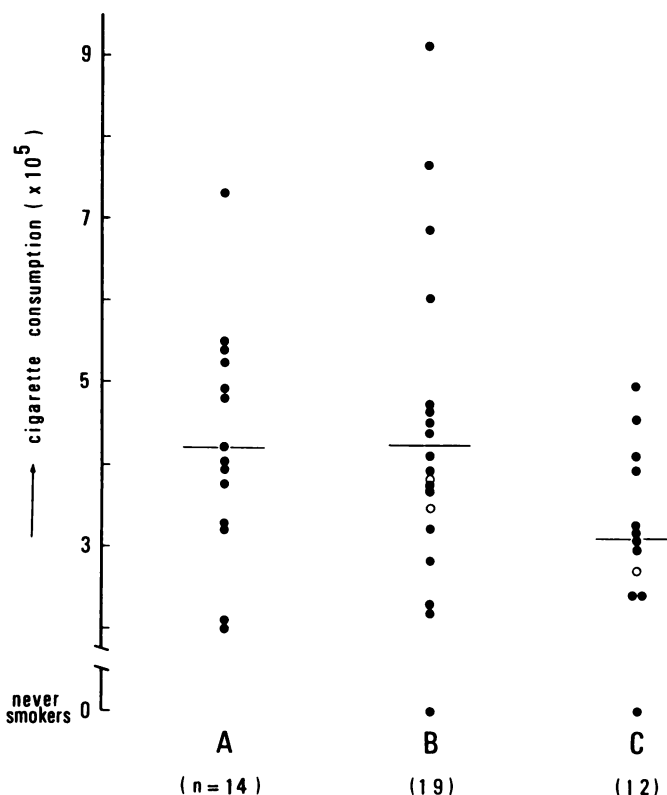


Fig. 1. Cigarette consumption of the patients with squamous cell carcinoma of the lung in genotypes (●, male; ○, female).

Table 2 Characteristics of patients and controls matched for sex and age

Genotypes	No. of subjects	Age (yr)	Cigarette consumption (×10 <sup>4</sup> cigarettes)
<b>Patients</b>			
A	14 (0.311) <sup>a</sup>	66.1 ± 11.8 <sup>b</sup>	42.2 ± 14.9 <sup>b</sup>
B	19 (0.422)	66.9 ± 6.2	42.7 ± 20.6
C	12 (0.267)	67.5 ± 8.2	31.3 ± 12.8
Total	45 (1.000)	66.8 ± 8.6	39.5 ± 17.5
<b>Controls</b>			
A	61 (0.452)	66.3 ± 9.3	22.3 ± 18.1
B	58 (0.429)	66.2 ± 7.5	23.4 ± 20.6
C	16 (0.119)	67.3 ± 9.4	23.9 ± 20.7
Total	135 (1.000)	66.8 ± 8.5	23.0 ± 19.4

<sup>a</sup> Numbers in parentheses, frequency.

<sup>b</sup> Mean ± SD.

in Table 2, where mean ages and lifetime mean cigarette consumption were calculated in each of the genotypes together with frequencies of the genotypes. Among the controls, mean cigarette consumptions among the genotypes were identical to each other in contrast with those among patients. This means that there were no interactions between the genotypes and the exposure to cigarette smoking among controls. The mean ages of the genotypes showed no differences among patients or among controls.

The distributions of patients and controls by genotypes and cigarette dose levels are shown in Table 3, where genotypes A and B are combined as A+B. The frequency of genotype C among controls was almost constant in cigarette dose levels, while that among patients clearly depended on cigarette dose levels; *i.e.*, the frequency reduced with increased dose levels. This indicated that the susceptibility of genotype C to the carcinoma varied as a function of cigarette dose level, and we must estimate the odds ratios in different dose levels. The odds ratios were then calculated in the same table for the combinations of genotypes and cigarette dose, taking the risk of the first category with genotype A+B at the lowest dose level of less than 3 × 10<sup>5</sup> cigarettes to be at a baseline of 1.0. These odds ratios were designated in the table as “odds ratios for genotypes and dose.” We could not take the baseline of exposure to be never-smokers, since only two never-smokers were observed among the patients. Relative susceptibility of genotype C compared with A+B was also estimated in Table 3 by the other odds ratio calculated for a 2 × 2 table in each of the dose levels; this odds ratio was consequently equal to the ratio of the two odds ratios for genotypes and dose at the same dose level.

The susceptibility of genotype C compared with other genotypes was remarkably high (odds ratio, 7.31; 95% CI, 2.13 to 25.12) at the lowest dose of less than 3 × 10<sup>5</sup> cigarettes and reduced to the odds ratios of 2.00 and 1.13 at the higher dose levels. The odds ratios of genotype C increased from 7.31 (95% CI, 2.13 to 25.12) to 13.17 (95% CI, 3.42 to 50.68) and 13.17 (95% CI, 2.99 to 58.05) with increased cigarette dose, although this increase was not statistically significant. On the other hand, the odds ratio of genotypes A+B increased linearly with the dose (*P* < 0.001;  $\chi^2 = 24.319$  for trend) from the baseline of 1.0 to 6.58 (95% CI, 2.39 to 18.12) and 11.70 (95% CI, 4.33 to 30.25).

DISCUSSION

Genetically determined variation among individuals in the metabolic activation of chemical carcinogens is first in line to explain the individual difference in susceptibility to chemical carcinogenesis. We investigated the genetic difference in susceptibility to lung cancer in terms of the polymorphisms of the P450IA1 gene (11, 15). We inferred that the DNA sequence polymorphisms of cytochrome P450IA1 might influence the activation of its substrates, benzo(a)pyrene, and other procarcinogens in cigarette smoke and thus contribute to the differing susceptibility to the cancer. Following our first approach summarized in Table 1, we analyzed the cumulated cigarette dose among the patients with the squamous cell carcinoma and showed that the patients with “susceptible” genotype C contracted the carcinoma with a smaller dose than those with other genotypes. Comparison of their smoking status at the time of interview did not provide any statistical significance, although the proportion of never- and exsmokers in genotype C was 0.42 (5 of 12) and higher than 0.27 (9 of 33) in genotypes A+B (the subjects of exsmoking within 1 yr before interviewing were regarded as current smokers). The distribution of the total amounts of cigarette consumption in the genotypes among the patients with the adenocarcinoma showed no differences, and the numbers of the patients with other histological types were too small for analysis. We also compared other lung cancer risk factors of occupational career, intake of green and yellow vegetables, and familial history of cancer between the patients of different genotypes, although no significant differences were found.

In Table 1 the healthy controls were randomly selected be-

Table 3 Relative risk estimate on the basis of the distribution of patients and controls by genotypes and cigarette dose

	Consumption of the following cigarette doses (×10 <sup>5</sup> ) by genotype					
	<3		3-4		>4	
	A + B	C	A + B	C	A + B	C
Patients	6	5 (0.455) <sup>a</sup>	11	4 (0.267)	16	3 (0.158)
Controls	79	9 (0.102)	22	4 (0.153)	18	3 (0.143)
Odds ratios for genotype and dose	1.0	7.31 <sup>b</sup> [2.13-25.12] <sup>f</sup>	6.58 <sup>c</sup> [2.39-18.12]	13.17 <sup>d</sup> [3.42-50.68]	11.70 <sup>e</sup> [4.53-30.25]	13.17 <sup>f</sup> [2.99-58.05]
Susceptibility of C to A + B		7.31 <sup>b</sup> [2.13-25.12]		2.00 <sup>g</sup>		1.13 <sup>i</sup>

<sup>a</sup> Numbers in parentheses, frequency.

<sup>b</sup>  $\chi^2 = 9.994, P < 0.005$ .

<sup>c</sup>  $\chi^2 = 13.308, P < 0.001$ .

<sup>d</sup>  $\chi^2 = 14.050, P < 0.001$ .

<sup>e</sup>  $\chi^2 = 25.785, P < 0.001$ .

<sup>f</sup>  $\chi^2 = 11.596, P < 0.001$ .

<sup>g</sup> Numbers in brackets, 95% CI.

<sup>h</sup>  $\chi^2 = 0.771$ .

<sup>i</sup>  $\chi^2 = 0.018$ .

cause the frequencies of the genotypes did not vary between sexes and ages. On the other hand, the controls in our case-control study had to be matched to the patients by sex and age because the cumulated cigarette amount obviously depended on these matching conditions. Our case-control study evaluated the risk of individuals with the susceptible and nonsusceptible genotypes in genotype-exposure interactions or dose response. Here we ascertained the fact that the environmental exposure, namely, cigarette dose, was independent of the genotypes among controls. Otherwise the risk as response to exposure could not be estimated correctly (19). Our results showed that the susceptibility of genotype C to the carcinoma was remarkably high at a low dose level of cigarette smoking and that the difference in susceptibility of the genotypes was reduced at high dose levels. Considering the biological role of P450IA1 enzyme in carcinogenesis, one of the most plausible interpretations for the high susceptibility of genotype C to the carcinoma may be that the susceptible individuals have different metabolic activation of carcinogens catalyzed by the enzyme. Less difference of risk among the genotypes at high dose levels may be reflected by a dose-response relationship of the enzymatic reaction, because this kind of saturation of metabolic activation was known for the effective dose of vinyl chloride in target tissues and because a rapid elevation of metabolic response, reaching a saturated level quickly, to increased dose among susceptible individuals was speculated upon by Omen (20). Another possible interpretation would be that the individuals with genotype C have a genetically high risk of the carcinoma independent of cigarette smoking. We did not have enough never-smoking patients to determine which interpretation is correct. On the other hand, a correlation of the high inducibility phenotype of P450IA1 enzyme (aryl hydrocarbon hydroxylase activity) with an increased risk of bronchogenic carcinomas was indicated (3, 4). Very recently cosegregation of the *MspI* polymorphisms and the inducibility phenotype of P450IA1 was reported, suggesting that the susceptible genotype may involve an increased metabolism of procarcinogens in cigarette smoke (21). However, the association of the high inducibility phenotype with lung cancer incidence still needs to be confirmed, because a lack of the association was also reported (8). Further study is also needed to confirm whether the high susceptibility observed in this paper is ascribed to the different metabolic activation of procarcinogens or to some other mechanisms.

It is essential in our study to investigate the mechanism of how the *MspI* polymorphisms influence the phenotypic expression of the P450IA1 gene. We recently found that single-point mutation occurred in the coding region of the P450IA1 gene in a strong linkage with this *MspI* polymorphism, and that this mutation resulted in an amino acid substitution in the heme-binding region, indicating at least two types of P450IA1 protein with different primary structures (12). A further biological and epidemiological investigation will be required to clarify the

mechanism and to establish the model for individual susceptibility to chemical carcinogenesis.

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