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Genetic polymorphism of drug-metabolizing enzymes and susceptibility to oral cancer

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An individual difference in the susceptibility to chemical carcinogens is one of the most important factors in the estimate of risk of human cancer. Recently, it has been reported that genetic risk for tobacco-related cancers is associated with polymorphisms of the *CYP1A1* and *GSTM1* genes in terms of genotype frequencies and cigarette smoking dose. In this study, we investigated the inter-individual difference in genetically determined susceptibility to oral squamous cell carcinoma (SCC) in relation to cigarette smoking dose in a Japanese population. DNA samples were obtained from both patients and controls. We identified individuals at high risk genetically for oral SCC in terms of polymorphisms of the *CYP1A1* and *GSTM1* genes. This study then compared the estimated total number of cigarettes smoked by patients with those smoked by controls. In this case–control study, we estimated the odds ratios of susceptible to non-susceptible individuals. *CYP1A1* genotype *C* and *GSTM1* deficiency were frequently found among oral SCC patients. Patients with genotype *C* and *GSTM1* deficiency contracted carcinoma after fewer cigarettes than those with other genotypes. Individuals with these two genotypes were at remarkably high risk at a low dose level of cigarette smoking. Individual differences in polymorphisms of the *CYP1A1* and *GSTM1* genes is one important factor in the estimate of risk of oral SCC at a low dose level of cigarette smoking.

Introduction

It is now accepted that a large proportion of human cancers are caused by synthetic or natural chemical compounds in the environment (1). Carcinogenic risks from exposure to exogenous chemical carcinogenes depend not only on the intrinsic nature and dose of each chemical, but also may depend on inter-individual variability in sensitivity to the carcinogens (2). Most chemical carcinogens require metabolic activation by Phase I enzymes (cytochromes P-450) and detoxification by conjugation via the various Phase II enzymes (epoxide hydrolase, glutathione *S*-transferase, *N*-acetyltransferase, sulfotransferase, etc.) (3). Thus, the coordinated expression and regulation of Phase I and Phase II drug-metabolizing enzymes and their metabolic balance may be an important host factor in determining whether exposure to carcinogens results in cancer or not (4–6). At present, it is accepted that most of the carcinogens in our environment are activated mainly by a restricted number of P-450 species, including CYP1A1, CYP1A2, CYP2E1 and CYP3A (7,8). *CYP1A1* and *GSTM1* polymorphisms have been associated with an increased risk for tobacco-related diseases such as lung cancer (9–11). Oral cancer is also a tobacco-related disease that represents a significant problem based upon its high incidence in many parts of the world (12,13). Some studies on genetic markers for individual susceptibility to head and neck cancer have also been reported (14–17). In this study, we report that polymorphisms of the *CYP1A1* and glutathione *S*-transferase genes are associated with susceptibility to oral squamous cell carcinoma (SCC) in relation to cigarette smoking dose in a Japanese population.

Abbreviation: SCC, squamous cell carcinoma.

Materials and methods

Experimental

Blood samples were obtained from 142 oral SCC patients (tongue 62, gingiva 27, buccal mucosa 24, oral floor 17 and other cases 12) and 142 healthy controls. DNA was isolated from the peripheral lymphocytes in these specimens.

Genotype identification of the *CYP1A1* gene was carried out by PCR amplification followed by digestion with *Msp*I, detecting substitution of CCGG for CTGG in the *Msp*I site at base 264 from the additional polyadenylation [poly(A)] signal in the 3′-flanking region. Genotype *A* is the predominant homozygote in which the *Msp*I site is absent at the 3′-end. A homozygous rare allele was named genotype *C*. Genotype *B* is heterozygous for both alleles (18). To determine if the *Msp*I genotypes were associated with cancer, the frequencies of the three genotypes in oral SCC patients and healthy controls were compared. Two synthetic oligonucleotide primers (5′-TAGGAGTCTTGTCTGATGCCT-3′ and 5′-CAGTGAAGAGGTGTAGCCGCT-3′) were used according to the methods of Hayashi *et al*. (19). Thirty cycles of PCR were carried out under the following conditions: 1 min denaturation at 95°C; 1 min primer annealing at 57°C; 1 min primer extension at 72°C. The amplified fragments including the *Msp*I site were digested with *Msp*I for 2 h at 37°C and products were subjected to electrophoresis in 2% agarose gels.

GSTM1, one of the μ class isozymes, detoxifies benzo[*a*]pyrene, including the epoxide and hydroxylated forms. The *GSTM1*-deficient phenotype was shown to be due to a homozygous null *GSTM1* gene (20). The genotypes of *GSTM1* were determined by PCR according to methods described previously (9). Three oligo primers for the exons 4 and 5 region of the gene were used. Primer 1 (5′-GAAGGTGGCCTCCTCCTTGG-3′) and primer 2 (5′-AATTCTGGATTGTAGCAGAT-3′) could also anneal to another μ class gene (*GSTM4*), while primer 3 (5′-TTCTGGATTGATGCAGATCA-3′) was specific for the *GSTM1* gene. When the three primers were used together in a PCR assay, the single *GSTM4* band (158 bp fragment) was consistently found, whereas the polymorphic 231 bp fragment could only be seen in the *GSTM1*-positive genome. The constant 158 bp fragment was amplified as an internal control, excluding the possibility of a false interpretation due to failure in the amplification reaction. PCR was carried out for 30 cycles under the following conditions: 1 min denaturation at 95°C; 1 min primer annealing at 50°C; 1 min primer extension at 72°C. The PCR products were subjected to electrophoresis in 2% agarose gels (Figure 1). The incidence of the homozygous or heterozygous allele of the complete *GSTM1* gene [*GSTM1*(+)] or the homozygous deficient gene [*GSTM1*(–)] was compared between oral SCC patients and controls.

Epidemiology

All of the 142 oral SCC patients were histopathologically diagnosed in the Saitama Cancer Center Hospital (78 males and 64 females). Patients consenting to participate in our study were interviewed at the time of their first admission using a standardized questionnaire concerning their cigarette and alcohol

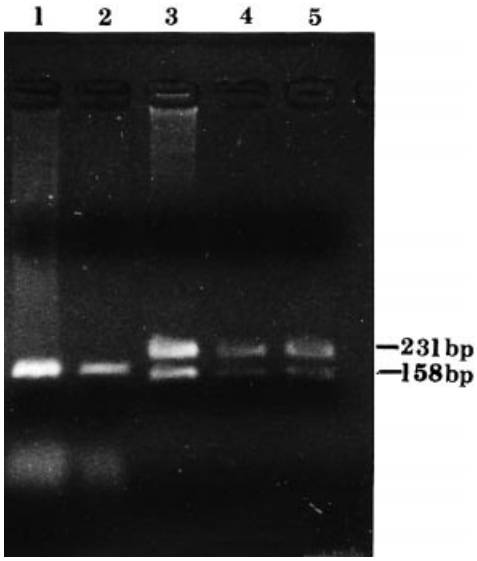

Fig. 1. *GSTM1* genotypes by PCR. Lanes 1 and 2, *GSTM1*(–) genome; lanes 3–5, *GSTM1*(+) genome.

consumption history. The only exclusion criterion used was co-existing malignancies in the patients group; nine cases were missed by this criterion. They do not comprise an apparent sub-group of those with the least or most severe disease. We also collected DNA samples from 142 consenting controls who were individually matched to the patients with respect to sex and age (±1 year). The 142 age- and gender-matched controls were randomly selected from a pool of 648 apparently healthy individuals free of malignancy who were interviewed using the same questionnaire at the time of their admission for a complete physical examination to hospitals near the Saitama Cancer Center Hospital. In this study, we summarize the smoking history of the patients and controls as the estimated total number of cigarettes consumed over their lifetime until interview. To compare the two medians of cigarette consumption among patients and controls with different genotypes, the Mann–Whitney distribution was calculated to test for statistical significance. In this case–control study of 142 patients with oral SCC and matched non-cancer controls, we evaluate the relative risk for individuals with susceptible genotypes in relation to the cumulative cigarette smoking dose.

Results

MspI genotypes of CYP1A1 and GSTM1 genotypes and oral SCC incidence

CYP1A1 genoypes *A* (*m1/m1*), *B* (*m1/m2*) and *C* (*m2/m2*) were found in 62 (43.6%), 65 (45.8%) and 15 (10.6%) individuals, respectively, among the healthy controls. This result gave a good fit to the Hardy–Weinberg equilibrium with a gene frequency of 0.665 for *m1* and 0.335 for *m2*, in which the relative frequencies p^2, $2pq$ and q^2 of the genotypes estimated from the gene frequencies p and q must be equal to that observed. On the other hand, types *A* (*m1/m1*), *B* (*m1/m2*) and *C* (*m2/m2*) were found in 56 (39.5%), 55 (38.7%) and 31 (21.8%) oral SCC patients, respectively. The frequency of genotype *C* among SCC patients differed from that among healthy controls with a statistical significance of $P < 0.01$ ($\chi^2 = 5.28$, d.f. = 1). The odds ratio estimate revealed that the individuals with genotype *C* had an ~2.3-fold higher risk of developing cancer than those with the *A* genotype (95% CI ~1.1–4.7).

The frequency of *GSTM1*(–) in healthy controls was 64 (45.1%). The frequency of *GSTM1*(–) was 92 (64.8%) in oral SCC patients, which was higher than that of *GSTM1*(+) ($P < 0.001$, $\chi^2 = 11.2$, d.f. = 1). The odds ratio estimate revealed that the individuals with *GSTM1*(–) had an ~2.2-fold higher risk of developing cancer than those with *GSTM1*(+) (95% CI ~1.4–3.6) (Table I).

Table I. Distribution of *Msp*I genotypes of *CYP1A1* and *GSTM1* genotypes among oral SCC patients and healthy controls

Genotype	Population		Odds ratio (95% CI)
	Healthy controls	Oral SCC	
CYP1A1			
A (*m1/m1*)	62 (43.6)	56 (39.5)	1.0
B (*m1/m2*)	65 (45.8)	55 (38.7)	0.9 (~0.6–1.7)
C (*m2/m2*)	15 (10.6)	31 (21.8)	2.3[a] (~1.1–4.7)
Total (%)	142 (100)	142 (100)	
GSTM1			
GSTM1(+)	78 (54.9)	50 (35.2)	1.0
GSTM1(–)	64 (45.1)	92 (64.8)	2.2[b] (1.4~3.6)
Total (%)	142 (100)	142 (100)	

[a] $P < 0.01$.
[b] $P < 0.001$.

Combined genotyping of the CYP1A1 and GSTM1 genes and oral SCC incidence

Six genotypes could be analyzed by combining the three *Msp*I genotypes of *CYP1A1* and the two of *GSTM1* and comparing the combined frequencies between oral SCC patients and healthy controls. The frequency of the combined genotypes *C* and *GSTM1*(–) was 2.1% in healthy controls. Among oral SCC patients, the frequency of the combined genotypes *C* and *GSTM1*(–) was 14.1% ($P < 0.001$, $\chi^2 = 12.6$, d.f. = 1) (Table II).

Cumulative cigarette dose in MspI genotypes of CYP1A1 and GSTM1 genotypes

The estimated total number of cigarettes consumed over a lifetime did not show statistical significance among the controls with genotypes *A*, *B* and *C* (data not shown). There are no differences in the distributions between genotypes *A* and *B* among the patients. However, the cigarette numbers for patients with genotype *C* was fewer than those for the other two genotypes ($P < 0.001$, Mann–Whitney *U*-test), even though age showed no differences among the patients of different genotypes. The estimated number of cigarettes smoked by the patients with *GSTM1*(–) was less than those with *GSTM1*(+) ($P < 0.001$, Mann-Whitney *U*-test). The estimated numbers of cigarettes consumed by patients with the combined genotypes *C* and *GSTM1*(–) were lower compared with those of the other combined genotypes ($P < 0.001$, Mann–Whitney *U*-test) (Table III).

Relative risk associated with smoking level by MspI genotype of CYP1A1

The distribution of patients and controls by genotype for the *Msp*I polymorphism is shown in Table IV. The estimated cumulative cigarette dose is divided into three categories: those that consume at least 4×10^5 cigarettes (a higher dose than 4×10^5 cigarettes is commonly regarded as a heavy smoking level), those consuming ~2–4×10^5 cigarettes and those consuming $<2\times10^5$ cigarettes (a value at least half that of the highest group dose). Odds ratios were calculated for the *Msp*I genotypes and cigarette dose, setting the risk of the first category with genotype *A* and *B* combined as *A* + *B* at the lowest dose level as the baseline of 1.0. The baseline of exposure could not be set as non-smokers because there were

Table II. Distribution of combined genotyping of *CYP1A1* and *GSTM1* genes in oral SCC patients and healthy controls

Population	Genotype								
	CYP1A1 A (*m1*/*m1*)			*CYP1A1 B* (*m1*/*m2*)			*CYP1A1 C* (*m2*/*m2*)		
	GSTM1(+)	*GSTM1*(–)	Total	*GSTM1*(+)	*GSTM1*(–)	Total	*GSTM1*(+)	*GSTM1*(–)	Total
Healthy controls	37 (26.0)	35 (17.6)	62 (43.6)	29 (20.4)	36 (25.4)	65 (45.8)	12 (8.5)	3 (2.1)[a]	15 (10.6)
Oral SCC	20 (14.1)	36 (25.4)	56 (39.5)	19 (13.4)	36 (25.3)	55 (38.7)	11 (7.7)	20 (14.1)[a]	31 (21.8)

[a]$P < 0.001$.

Table III. Characteristics of patients with *Msp*I genotypes of *CYP1A1* and *GSTM1* genotypes

Genotype	No. of subjects (frequency)	Age (years; median ± IQR)	Cigarette consumption ($\times 10^5$; median ± IQR)
A	56 (0.39)	58.5 ± 6.9	2.8 ± 1.7
B	55 (0.38)	60.0 ± 9.0	2.4 ± 1.5
C	31 (0.22)	60.0 ± 9.5	0.4 ± 1.2
GSTM1(+)	50 (0.35)	56.5 ± 9.3	2.9 ± 2.8
GSTM1(–)	92 (0.65)	59.5 ± 7.4	1.4 ± 1.7
A/*GSTM1*(+)	20 (0.14)	60.5 ± 7.4	3.6 ± 4.1
A/*GSTM1*(–)	36 (0.26)	58.0 ± 6.5	2.1 ± 1.6
B/*GSTM1*(+)	19 (0.13)	54.0 ± 9.0	3.2 ± 3.6
B/*GSTM1*(–)	36 (0.25)	60.0 ± 7.5	1.4 ± 1.9
C/*GSTM1*(+)	11 (0.08)	57.0 ± 15.0	1.0 ± 1.4
C/*GSTM1*(–)	20 (0.14)	62.0 ± 8.4	0.2 ± 1.1

not enough non-smokers among the patient cohort. These odds ratios are designated in Table IV as odds ratios for genotype and dose. The relative susceptibility of genotype *C* compared with *A* + *B* is also estimated in Table IV by the other odds ratio calculated for a 2×2 table at each of the dose levels. This odds ratio was consequently equal to the ratio of the two odds ratios for genotype and dose at the same level. The susceptibility of genotype *C* compared with other genotypes was 4.3-fold higher (95% CI ~1.9–10.1, $P < 0.001$) at the lowest cigarette dose and reduced to odds ratios of 1.9 and 0.2 at the higher dose levels. The odds ratios of genotype *C* also decreased from 4.3 (95% CI ~1.9–10.1) to 3.1 (95% CI ~0.9–11.1) and 1.8 (95% CI ~0.2–12.9) with increased cigarette dose. On the other hand, the odds ratio of genotypes *A* + *B* increased with dose from the baseline of 1.0 to 1.6 (95% CI ~0.9–3.0) and 11.3 (95% CI ~4.2–30.8, $P < 0.001$). The odds ratios were not statistically significant between genotype *C* and other genotypes at the higher cigarette dose.

Relative risk associated with smoking level by GSTM1 genotypes

The odds ratio of the *GSTM1*(–) genotype increased from 3.1 (95% CI ~1.6–5.9) to 3.9 (95% CI ~1.6–9.1) and 16.2 (95% CI ~4.3–61.0) with increased cigarette dose; this increase was statistically significant. On the other hand, the odds ratio of the *GSTM1*(+) genotype increased with cigarette dose from the baseline of 1.0 to 1.9 (95% CI ~0.8–4.4) and 10.8 (95% CI ~3.2–36.2). The odds ratios were not statistically significant between the *GSTM1*(–) and *GSTM1*(+) genotypes at the higher cigarette dose. The susceptibility of genotype *GSTM1*(–) compared with other genotypes was 3.1-fold higher (95% CI ~1.63–5.91, $P < 0.001$) at the lowest cigarette dose and reduced to odds ratios of 2.0 and 1.5 at the higher dose levels (Table V).

Discussion

The *CYP1A1* and *GSTM1* polymorphisms have been associated with an increased risk for smoking-related cancers such as lung, bladder and head and neck cancer (10,11,14–16,21–25). In this study, we investigated whether an association of oral SCC with an *Msp*I restriction site polymorphism of the *CYP1A1* gene and polymorphism of *GSTM1* exists. Further, we tested whether a genetic risk for oral SCC was associated with these two polymorphisms in terms of genotype frequencies and cigarette smoking dose in a Japanese population.

The *C* genotype was found in 22% of oral SCC patients, a statistically significant incidence, about twice as high as that among healthy controls. This observation is consistent with a report of SCC of the lung (26). It is suggested that the *C* genotype confers high susceptibility to oral SCC as well as to SCC of the lung, known to be closely associated with cigarette smoking.

We then compared the incidence of at least one *GSTM1* gene [*GSTM1*(+)] or its complete deletion [*GSTM1*(–)] between patients and controls. The frequency of the *GSTM1*(–) genotype in controls was 45% and that in patients was 65%, in good agreement with values reported previously for Japanese populations (10,11). However, two Caucasian studies have reported a lack of association between the *GSTM1* polymorphism and oral cancer (15,23). This discrepancy may be ascribable to an ethnic difference in allelic frequency of the *GSTM1* polymorphism or factors such as diet, alcohol consumption, smoking habit, etc.

The frequency of the combined genotypes *C* and *GSTM1*(–) was 14.1% among oral SCC patients, a statistically significant increase compared with the frequency seen among healthy controls. However, it is not certain whether these two genes work synergistically to enhance the risk of oral SCC, because there were insufficient subjects for an adequate analysis on the basis of odds ratios.

Next, estimating the cumulative cigarette dose for patients exhibiting the *Msp*I genotypes of *CYP1A1*, it was found that SCC patients with genotype *C* had a statistically significantly lower dose than patients with genotypes *A* and *B*. Also, the estimated number of cigarettes for the patients with *GSTM1*(–) was fewer than for *GSTM1*(+) patients. It was suspected that the genotypes *C* and *GSTM1*(–) play an important role in individual differences in susceptibility to oral SCC, especially at the lowest cigarette dose level.

Also, in the present case–control study our results show that the susceptibility of patients with genotypes *C* and *GSTM1*(–)

Table IV. Relative risk associated with smoking levels by *Msp*I genotypes of *CYP1A1*

	Consumption of the following cigarette doses by genotype					
	⩽2×10^5		~2–4×10^5		⩾4×10^5	
	A + B	*C*	*A + B*	*C*	*A + B*	*C*
Patients	52	22	27	7	32	2
Controls	92	9	30	4	5	2
Odds ratio for genotype and dose	1.0	4.3[a]	1.6	3.1	11.3[a]	1.8
(95% CI)		(~1.9–10.1)	(~0.9–3.0)	(~0.9–11.1)	(~4.2–30.8)	(~0.2–12.9)
Susceptibility of *C* compared with *A+B*	4.3[a]		1.9		0.2	
(95% CI)	(~1.9–10.1)		(~0.5–7.4)		(~0.02–21.4)	

[a]$P < 0.001$.

Table V. Relative risk associated with smoking level by *GSTM1* genotype

	Consumption of the following cigarette doses by genotype					
	⩽2×10^5		~2–4×10^5		⩾4×10^5	
	GSTM1 (+)	*GSTM1* (–)	*GSTM1* (+)	*GSTM1* (–)	*GSTM1* (+)	*GSTM1* (–)
Patients	20	54	14	20	16	18
Controls	54	47	20	14	4	3
Odds ratio	1.0	3.1[a]	1.9	3.9[b]	10.8[a]	16.2[a]
(95% CI)		(1.6~5.9)	(0.8~4.4)	(1.6~9.1)	(3.2~36.2)	(4.3~61.0)
Susceptibility of *GSTM1* (–) compared with *GSTM1* (+)	3.1[a]		2.0		1.5	
(95% CI)	(1.6~5.9)		(0.8~5.4)		(0.3~7.7)	

[a]$P < 0.001$.
[b]$P < 0.005$.

to oral SCC was remarkably higher at a lower dose level of smoking (2×10^5 cigarettes). Nakachi *et al.* stated that the lower difference in risk among the genotypes at high dose levels may reflect a dose–response relationship of the enzymatic reaction and another less likely interpretation is that individuals with genotype *C* have a genetically high risk of carcinoma independent of cigarette smoking (18). Although we did not have enough non-smoking patients to determine which interpretation is correct, genotype *C* was found among 35% of them and *GSTM1*(–) was found among 91% of them in this study. It is likely that the individuals with genotypes *C* and *GSTM1*(–) have a genetically high risk of carcinoma independent of cigarette smoking in the Japanese population.

To summarize, our study in a Japanese patient cohort indicates that individuals with specific polymorphisms in both the *CYP1A1* and *GSTM1* genes have a genetically high risk of oral SCC. This suggests that an individual difference in the susceptibility to chemical carcinogens is one of the most important considerations in the risk assessment of oral SCC.

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