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Association of *GST* genotypes with age of onset and lymph node metastasis in oral squamous cell carcinoma

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BACKGROUND: Environment–gene interaction in oral carcinogenesis is well demonstrated by phase I and II enzymes that are involved in the metabolism of carcinogens. This study investigated the association of glutathione S-transferase (*GST*)T1 and *GST*M1 genotypes of phase II enzyme genes with risk for, age of onset, and neck lymph node metastasis (LNM) in areca-associated oral squamous cell carcinoma (OSCC).

METHODS: A total of 114 OSCC male patients and 100 male controls were recruited. All subjects were areca users and tobacco smokers. DNA was obtained from peripheral blood samples. Genotyping of *GST* T1 (non-null/null) and *GST*M1 (non-null/null) was determined by polymerase chain reaction (PCR) analysis using specific primers that only amplify non-null alleles.

RESULTS: No association was found between *GST* genotype and the risk of OSCC based on case–controls. Patients with the *GSTT*1 null genotype were older at onset ($P = 0.03$). Those with the *GST*M1 null genotype had a higher incidence of neck LNM than those with the *GST*M1 non-null genotype ($P = 0.01$). Patients with the *GST*M1/*GST* T1 null genotype appeared to have later onset and a higher incidence of neck LNM than those carrying the opposite genotype.

CONCLUSION: The *GST* genotypes may be important markers for the age of onset and risk of metastasis in OSCC. The data also suggest that the various *GST* isoforms may be differentially involved in development or progression of OSCC.

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Keywords: areca; genotype; *GST*M1; *GST* T1; mouth neoplasm

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Introduction

Oral squamous cell carcinoma (OSCC) is a disease with worldwide distribution and is one of the leading cancers in most Asian countries (1, 2). Malignant transformation to OSCC involves a series of genetic events (multihits) and chronic exposure to genotoxic agents (3). Tobacco and alcohol use are the major risk factors for OSCC in Western populations. Epidemiologic evidence has suggested that, in addition to tobacco and alcohol, areca (betel nut) exposure is the most potent risk factor for OSCC in Taiwan (4–6). It is likely that genotoxic insults plus imbalances in host response contribute to susceptibility to and development and progression of OSCC.

Environment–gene interaction in carcinogenesis is reflected by xenobiotic-metabolizing phase I enzymes (cytochrome p450 species) and detoxification by phase II enzymes, including epoxide hydrolase, *N*-acetyltransferase, sulfotransferase, glutathione S-transferase (*GST*), etc. (7). Enzymes in the cytochrome p450 (*CYP*) superfamily catalyze the oxidative metabolism of most endogenous and exogenous chemicals. The coordinated expression and regulation of phase I and II drug-metabolizing enzymes and their metabolic balance may be important host factors determining whether genotoxic insults result in cancer. Many *CYP* superfamily members are known to exist in variant forms that have differing activities. The *CYP*1A1 genotype, which results in loss of enzyme activity, has been reported to increase the risk for OSCC in Taiwanese, with such individuals often developing the cancer at an earlier age (8).

GST phase II enzymes catalyze the conjugation of reduced glutathione with a large number of electrophiles (7). These results in increased water solubility, allowing renal excretion of various carcinogens or epoxides formed during phase I detoxification. The *GST* superfamily includes four classes, α, μ, π, and θ. There are five closely related *GST*M isoenzymes (M1–M5) which have a high sequence homology. The *GST* T class consists of two genes, *GST* T1 and *GST* T2 (9). Null–*GST* genotypes for *GST*M1 and *GST* T1 result in absence of enzyme activity. Because of the lack of the gene

product responsible for detoxification of metabolic intermediates of tobacco carcinogens and endogenous lipid peroxides, these genotypes are considered important for tumor susceptibility and are being intensively investigated as risk factors for various neoplasms related to tobacco use. However, the association between *GST* genotypes and the risk for OSCC or head and neck squamous cell carcinoma (HNSCC) is equivocal (7, 10–19). Areca can induce oxidative stress in the local environment of the oral cavity (20). However, the association between the *GST* genotype and the risk for areca-associated OSCC has not been fully addressed (13).

*GST*s also contribute to detoxification either by direct conjugation of the drug with glutathione, increasing its secretion via bile and urine, or by neutralization of reactive compounds induced by a cytotoxic drug (21). This detoxification can protect cells from injury by chemotherapy (22). The role of these genes in monitoring therapeutic effects and survival in lung carcinoma has been studied (23). In this study, we investigated the association of *GST* genotypes with the risk for and clinical pattern of OSCC associated with areca use.

Material and methods

Subjects

A total of 114 patients with pathologically proven primary OSCC from the Oral and Maxillofacial Department at Taipei Mackay Memorial Hospital were enrolled. All patients received the surgical treatment in our Department during 2002–2003. Pathologic examination was performed for all patients including that the neck lymph node metastasis (LNM) was diagnosed according to the initial surgical specimen. About 100 case–control male subjects were selected from people who came for routine physical checkups, non-neoplastic minor operations, or maxillofacial trauma. Those with autoimmune disorders, blood disease, or previous malignancy were excluded. All the cases and controls were male with areca chewing and tobacco smoking. Although 60 controls and 75 OSCC cases were alcohol drinkers, around 20% subjects had questionable alcohol use history. Thereby, the alcohol consumption was excluded from our analysis. The study was approved by an ethics review committee. After informed consent was obtained, blood was drawn from the subjects and genomic DNA was extracted from fresh or frozen peripheral blood leukocytes by standard protocols.

PCR genotyping

The polymerase chain reaction (PCR) was done to amplify and assay *GST*M1 and *GST* T1 in genomic DNA. The PCR analysis employed both *GST*M1 (220 bp) and *GST* T1 (480 bp) specific primer pairs in the same amplification mixture and included the third primer pair for *β-globin* (268 bp) as an internal control (Table 1) (16). PCR was carried out in a total volume of 50 μl containing 0.5–1.0 μg genomic DNA, 120 ng each of the upstream and downstream primers (Table 1), 200 μM each of dNTP, 2.5 U *Taq* polymerase (Promega, Madison, WI, USA) and 5 μl of PCR buffer. Amplification conditions consisted of an initial denaturing step at 94°C for 3 min followed by 30 cycles at 95°C for 30 s and at 64°C for 1 min. This was followed by a prolonged extension at 72°C for 5 min. PCR products were resolved on a 2% agarose gel and visualized by an imaging system (Viber Lourmat, Marne La Valle, France). The absence of a 220 bp or 480 bp fragment indicated *GST*M1 or *GST* T1 null phenotypes. Subjects with those PCR products were defined as having a non-null genotype. The genotyping was a blind analysis with all experiments performed in duplicate. All results were also confirmed by repeated experiments.

Table 1 Primers used for the PCR

Genes	*Primers (5′–3′)*
*GST*M1	
Forward	GAACTCCCTGAAAAGCTAAAGC
Reverse	GTTGGGCTCAAATATACGGTGG
GST T1	
Forward	TTCCTTACTGGTCCTCACATCTC
Reverse	TCACCGGATCATGGCCAGCA
β-Globin	
Forward	CAACTTCATCCACGTTCACC
Reverse	GAAGAGCCAAGGACAGGTAC

PCR, polymerase chain reaction; GST, glutathione S-transferase.

Statistical analysis

Evaluation of the Hardy–Weinberg equilibrium was performed by comparing observed and expected heterozygotes and homozygotes, as well as observed and expected genotypes, using the chi-square analysis. *GST* genotype and risk of OSCC genesis was estimated by odds ratio (OR) and associated 95% confidence interval (CI), which were calculated by unconditional logistic regression models. An unpaired *t*-test was used to evaluate differences in age at onset. The various clinical parameters were compared by Fisher's exact test to obtain *P*-value, OR, and 95% CI. Differences were considered to be statistically significant if the *P*-value was < 0.05.

Results

Subjects

The age range of the 114 patients with OSCC was 30–75 years (mean = 51.4) and for the controls was 28–81 years (mean = 49.8). The most common primary OSCC site was the buccal mucosa, followed by the tongue, gingiva, palate, and floor of the mouth (Table 2). Fifty-four (47.3%) patients presented with stage IV tumors, while the remaining 60 had stages I–III, according to the staging system of the American Joint Committee on Cancer (Table 1) whereas 34 (29.8%) patients had LNM.

Genotypings

The allelic distribution of *GST* in controls fulfilled Hardy–Weinberg equilibrium. The age-adjusted uncon-

Table 2 Clinical parameters

Parameters	*Case (n = 114)*	*Control (n = 100)*
Age (years)		
Mean ± SE	51.4 ± 0.8	49.8 ± 1.1
Range	30–75	28–81
Location		
Buccal mucosa	67	
Tongue	27	
Gingiva	13	
Palate	4	
Floor of the mouth	3	
Stage		
I–III	60	
IV	54	
LNM		
Absence	80	
Presence	34	

GST, glutathione S-transferase; LNM, lymph node metastasis.

Table 3 *GST* genotypes

Genotype	*Case*	*Control*	*OR (95% CI)*
GSTM1			
Non-null	48 (42)	45 (45)	1
Null	66 (58)	55 (55)	0.81 (0.53–1.63)
GST T1			
Non-null	63 (55)	63 (63)	1
Null	51 (45)	37 (37)	0.77 (0.44–1.36)
GSTM1/*GST* T1			
Non-null	28 (47)	27 (59)	1
Null	30 (53)	19 (41)	0.69 (0.34–1.43)

Data given as unconditional logistic regression (%).
GST, glutathione S-transferase; OR, odds ratio; CI, confidence interval.

ditional logistic regression analysis showed no significant difference in the genotypic distribution between cases and controls (Table 3). The data indicated the null genotypes for *GSTM1*, *GST* T1 or both isoforms were not associated with the risk of OSCC.

Among patients, no association between age at onset of OCSS and *GSTM1* genotype was identified (Table 4). However, *GST* T1 null patients presented at a significantly older age than did those with the *GST* T1 non-null genotype (Table 4). In addition, a cross-analysis also indicated that patients with combined *GSTM1*/*GST* T1 null genotypes had a significantly higher age at onset compared to those carrying with both non-null genotypes (Table 4).

Because clinical stage and the LNM status have been the most important parameters in determining the survival and progression of OSCC, we looked for association between *GST* genotype and these parameters. None was found between the *GST* genotype and stage. Interestingly, however, the *GSTM1* null genotype was significantly associated with the presence of LNM (Table 5). Similarly, patients with a combined *GSTM1*/*GST* T1 null genotype appeared to have a higher risk for LNM compared to those with the opposite genotype (Table 5).

Table 4 Glutathione S-transferase (*GST*) genotypes related to age of onset (unpaired *t*-test)

Genotypes	*n*	*Age of onset (mean ± SE)*	*P-value*
GSTM1			
Non-null	48	50.2 ± 1.2	NS
Null	66	52.6 ± 1.1	
GST T1			
Non-null	63	49.8 ± 1.2	0.03
Null	51	53.2 ± 1.3	
GSTM1/*GST* T1			
Non-null	28	47.2 ± 1.6	0.01
Null	30	52.6 ± 1.4	

The *GST* genotype did not differ significantly with respect to the site of the tumor (detailed analysis not shown).

Discussion

Failure to detoxify electrophilic, DNA-damaging agents or chemical carcinogens may contribute to the process of carcinogenesis by an accumulation of genetic changes. Although the *GST* null genotypes associated with the lack of enzyme activity have been linked to the risk of OSCC in several studies (10, 16, 17, 19), no association between the frequency of *GST* genotype and primarily areca-related OSCC was found in this study (Table 3). This is in agreement with the majority of studies denoting the lack of association between *GST* genotypes and OSCC mainly associated with tobacco (7, 12–15). However, both *GSTM1* null and *GST* T1 null genotypes were strongly implicated in the risk for precancerous lesions related to areca use in an Indian population (24). There is a great ethnic diversity in the incidence of the *GST* null genotype (7, 10, 24–26), occurring in around 20% of Indians (10, 24) but in 50–60% of Chinese populations studied (10, 24–26). In this study *GSTM1* null genotype incidence is 58% and *GSTT1* null type genotype incidence is 45%, compatible with the previous studies in Chinese population (10, 24–26). Hardy–Weinberg equilibrium analysis further excluded the sampling bias or analytical errors in our study. In most Asian countries, areca chewing is combined with the use of tobacco (chewed or smoked), which complicates our attempts to understand the carcinogenic process (1, 2, 6, 24). The genotoxic factors influencing pathways for inactivation of metabolic intermediates of areca carcinogens is unclear. A large-scale, stratified, multicountry study may be needed in elucidate the role of *GST* genotypes in areca-associated OSCC risk.

It was interesting to note that patients with the *GST* T1 non-null genotype had earlier onset of areca-associated OSCC. A relationship between *GST* polymorphism and age of onset has been identified for carcinoma of prostate and colon (27, 28). High *GST* expression in tumors compared to normal tissues, together with their high level in plasma from cancer patients, suggests that *GST* might be useful as a tumor

Table 5 *GST* genotypes related to stage and LNM

Genotype	n	Stage			LNM		
		I–III	*IV*	*OR (95% CI)*	*Absence*	*Presence*	*OR (95% CI)*
*GST*M1							
Non-null	48	25 (52)	23 (48)	1	41 (85)	7 (15)	1
Null	66	35 (53)	31 (47)	0.96 (0.45–2.02)	43 (65)	23 (35)	3.13 (1.24–8.08)*
GST T1							
Non-null	63	32 (51)	31 (49)	1	49 (77)	14 (23)	1
Null	51	27 (52)	24 (48)	0.91 (0.44–1.92)	36 (70)	15 (30)	1.77 (0.88–3.55)
*GST*M1/*GST* T1							
Non-null	28	14 (50)	14 (50)	1	22 (78)	6 (22)	1
Null	30	16 (53)	14 (47)	0.87 (0.32–2.45)	15 (50)	15 (50)	3.66 (1.15–11.61)**

Fisher's exact test: $*P = 0.01$, $**P = 0.03$ (%).
GST, glutathione S-transferase; LNM, lymph node metastasis; OR, odds ratio; CI, confidence interval.

marker and a therapeutic target (21). The findings of cases with combined a *GST*M1/*GST* T1 non-null genotype conferring earlier onset tumors might also support the role of the *GST* genotype as a potential marker for OSCC presenting at an earlier age, despite that the number of variants is small (Table 4). The deficiency of *GST* enzymes secondary to the null genotype may result in higher levels of glutathione because of reduced consumption in *GST*-catalyzed reactions. In addition to its role in detoxification, intracellular glutathione has also been implicated in the control of cell proliferation and apoptosis (29). Accordingly, whether the increasing glutathione levels influence inhibition of oral keratinocyte proliferation and enhanced apoptosis should be carefully addressed. The presence of activated oncogenes and/or inactivated tumor suppressor genes may result in constitutive activation of multiple transcription factors for carcinogenesis. This may be especially true in the early stages of tumorigenesis (30). A possible linkage of *GST* genotypes to molecular alterations or tumor markers might represent interesting topics for future investigation.

Neck LNM occurring soon after development of a tumor is frequent in OSCC because of the rich lymphatic drainage from the oral cavity. Proteins related to angiogenesis, cell proliferation, and migration contribute to metastasis (4, 5). *GST*s are also involved in metabolism of several cytotoxic drugs, including anticancer drugs. The presence of *GST* enzyme activity might reflect biologically distinct, more aggressive disease, with enhanced potential for chemoresistance and worse survival. However, the impact of *GST* genotypes on therapeutic outcomes and survival of HNSCC have not been clearly defined (18). The incidence of primarily neck LNM from OSCC range from 25 to 36% (31–34). Although our analysis limited to a cohort of only 114 OSCC, the incidence of neck LNM (29.8%) in this cohort was rather consistent with that in other studies. It suggests that the sampling of the present study was random. Our analysis indicates that *GST*M1 null and combined *GST*M1/*GST* T1 null genotypes have a significantly higher potential for LNM (Table 5). Advanced tumors are in a stage of uncontrolled tumor growth and may consequently exist in a stress microenvironment, with hypoxia, acidosis, and free radical overproduction (30). This microenvironment may enhance aberrant activation of multiple metastasis-related proteins; bestow survival and growth advantages for emerging metastatic variants. It is believed that *GST* can facilitate the removal of free radicals generated by oxidative stress. This is in keeping with our findings that *GST* null patients were at higher risk for LNM. Better understanding of the interaction between the expression of metastasis-related molecules and *GST* activity may shed more insight on the control of OSCC metastasis.

In conclusion, our analysis indicated that *GST*M1 and *GST* T1 genotypes may be independent predictors, respectively, for age of onset of and LNM in OSCC. The mechanisms involved should be further studied. Whether the *GST*M1/*GST* null genotype may have a novel role for selecting OSCC patients for prevention and monitoring should be more comprehensively studied.

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