INTRODUCTION

Cancer of the oral cavity is a serious health problem in Thailand. The age standardized incidence rates (ASR) range from 2.7 to 6.5 per 100,000 population in both sexes. The incidence is highest in the Southern region of the country where oral cancer is the second leading cancer in males and the sixth in females. The age standardized incidence rates (ASR) in the Songkhla region are 9.8 and 3.5 per 100,000 population in males and females, respectively (Deerasamee et al., 1999). The current understanding is that poor dental hygiene is a major risk factor for oral cancer for both genders (Meyer et al., 1996; Genco, 1996; Lamont et al., 1995). The other major risk factors are smoking and alcohol consumption that are common among males, and betel chewing that is common among females in this region (Kerdpon and Sriplung, 1997). The habit of betel chewing is different among individuals. Some combine the betel quid with smokeless tobacco and others add spices to their betel quid. The association of betel chewing and oral cancer risk has been documented in other Asian countries like India and Taiwan (Sankaranarayanan et al., 1990; Ko et al., 1995). In India, where oral cancer is the most common malignancy, betel chewing is very common among Indian males. The chewing habit is also common among males in Taiwan but not in Thailand where most of the chewers are females.

A review of the literature indicates that the mentioned environmental risk factors do not account for the variations in risk for the development of oral cancer. Like lung cancer, the variations can be contributed by the existence of susceptible

genetic factors that interact with environmental factors for the development of disease (World Health Organization, 1993; Lin et al., 1994; Au et al., 1999 a-c). Most environmental carcinogens are metabolized by the phase I (activating) and phase II (detoxifying) enzymes (Guengerich et al., 1992; Hayes and Pulford,1995; Wormhoust et al., 1999) and the genes for some of these enzymes are polymorphic. Therefore, inheritance of certain variant alleles may impose susceptibility to environmental cancer. The relationship between genetic and environmental interactions for oral cancer has not been investigated in Thailand previously. Therefore, this is the focus of our investigation.

During the initial phase of the investigation, we targeted two polymorphic glutathione S-transferase genes (GSTM1 and GSTT1) because the variant alleles of these two genes have supposedly high prevalence in the normal population (approximately 50 and 30%, respectively). This high prevalence would allow us to conduct reliable association studies using small population sizes (Au et al., 1999). GSTM1 and GSTT1 are involved in the detoxification of a wide range of environmental and tobacco-specific carcinogens. Inheritance of the null (homozygous deletion) GSTM1 and/or GSTT1 alleles have been shown to be associated with the development of cancer in many organ systems, particularly among cigarette smokers. Examples are lung cancer (El-Zein et al, 1997 a-c), bladder cancer (Bell 1993; Anwar et al., 1996), head and neck cancer (Matthias et al., 1998; Jourenkova et al., 1998) and oral cancer (Deakin et al., 1997; Hung et al., 1997; Park et al., 1997; Jourenkova-Mironova et al., 1999; Nair et al., 1999, Sato et al., 1999). It should also be

emphasized that the reported positive association between inheritance of susceptible genes and cancer is not universally substantiated (D'Errico et al., 1996). Perhaps, geographical and ethnic differences in the distribution of the variant alleles are responsible for the discrepant observations. Therefore, our investigation in Thailand is needed. The evidence of positive association suggests that inheritance of the null (susceptible) alleles can cause cigarette smokers to have increased body burden of reactive metabolites from cigarette smoke to increase the risk for cancer. This suggestion is supported by the demonstration that the susceptible individuals and/or their cells had more DNA damage and chromosome aberrations than the non-susceptible individuals (Ichiba et al., 1994; El-Zein et al., 1997; Salama et al., 1999).

Here we report, for the first time in a Thai population, the effect of GSTM1 and GSTT1 polymorphisms on the risk of cancer of the oral cavity and their relationship with some environmental risk factors.

MATERIALS AND METHODS

Study Population:

Patients with cancer in the oral cavity were sequentially recruited before they had chemo- and/or radio-therapy from the Department of Radiology, Songkhlanagarind Hospital from August 1998 to December 1999. All individuals participated in the study voluntarily and they have provided informed consent. Participants were personally interviewed with a questionnaire that

was approved by the university ethic committee. The collected information was on individual's demographic background; cigarette smoking, betel chewing, and alcohol consumption habits; occupational exposure; nutritional behavior; oral hygiene as well as personal and family history of various cancers. Based on the collected information, the criteria for recruitment of normal and healthy controls were determined: matching with the patients according to age (+/- 5 years), gender and smoking or betel chewing habits. The matched controls were recruited from residents living in the Songkhla province and its vicinity.

Collection and preparation of specimens:

Peripheral blood samples from the qualified participants were collected into syringes and immediately transferred into polyethylenes tube that contained sodium heparin as an anticoagulant. The collected samples were coded and maintained in cool containers for transportation to the laboratory. The time taken for the transportation was usually less than 3 hours. In the laboratory, each blood sample was centrifuged at 2,000 rpm and the plasma was discarded. The remaining blood cells were kept frozen (-20^{o}C) until whole blood DNA extraction was performed.

GSTM1 and GSTT1 Genotyping:

DNA used for genetic analysis were isolated from 10 ml of frozen heparinized blood using a non-organic DNA extraction procedure (Sambrook et al., 1989). Amplification of the GSTM1 and GSTT1 genotypes were obtained simultaneously in a single assay approach using a multiplex PCR method (Abdel-Rahman et al., 1996). Briefly each 50 μl reaction mixture contained 200 μM

dNTPs, 5µl 10X PCR buffer (10X 500 mM KCl, 100 mM Tris-HCl, pH 9.0), 1.5 mM $MgCl_2$ and 2 U Amplitaq DNA polymerase (PE Applied Biosystems, New Jersey, USA). 50 ng of DNA was amplified together with 30 pmol of each pairs of the following primers; GSTM1 primers (5' GAA CTC CCT GAA AAG CTA AAG C 3' and 5' GTT GGG CTC AAA TAT ACG GTG G 3'), GSTT1 primers (5' TTC CTT ACT GGT CCT CAC ATC TC 3' and 5' TCA CCG GAT CAT GGC CAG CA 3') and CYP1A1 primers (5' GAA CTG CCA CTT CAG C TG TCT 3' and 5' CAG CTG CAT TTG GAA GTG CTC 3') were co-amplified as an internal control. The PCR was performed in a Perkin-Elmer GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, USA). The reaction conditions were: initial denaturation at 95°C, 9 min, followed by 35 cycles of melting (94°C, 1 min), annealing (59°C, 1 min) and extension (72°C, 1 min). A final elongation step (72°C, 10 min) terminated the process. The amplified products were determined by electrophoresis on ethidium bromide-stained 2% agarose gel . The presence of a band at 312 bp (corresponding to CYP1A1) indicated a successful amplification. The presence or absence of a band at 215 bp and a band at 480 bp determined a normal or deletion genotypes of GSTM1 and GSTT1, respectively.

Statistical Analysis:

The data was analysed by using a statistical analysis software (Stata version 6.0). Chi-square test were used for the comparison of proportion of characteristic variables and t-test was used to measure for the comparison of age. Stratified analysis and

logistic regression method were applied appropriately to obtained odd ratios (ORs) and their 95% confidence intervals.

RESULT

The case control study consisted of 53 cases of primary oral cancer (base of tongue 15, tongue 6, floor of mouth 9, palate 7, buccal 7, gum 7, and lip 2). All cases were confirmed by pathologists to be oral squamous cell carcinomas. A summary of the characteristics of the studied population is shown in Table 1. The 35 male and 18 female patients were strictly pair-matched by age (+/-5 year) and gender. Smoking, alcohol drinking and betel chewing habits were frequency matched. The mean age of the cases and controls are 67.0 +/- 10.1 (SD) and 67.3 +/- 10.3 (SD), respectively. There is no significant difference between the case and the control groups based on the comparison parameters. The proportion of Muslims was marginally different (p=0.079) between the two groups. However, Muslims are represented by only three individuals therefore their inclusion probably had no influence on the result of the analysis.

Table 1 Characteristics of cases and controls

	Cases (53)	Controls (53)	p-value
Sex			1.000
male	35	35	
female	18	18	
Religion			0.079
Buddhist	50	53	
Muslim	3	0	

Age			0.857
mean	67.0	67.3	
SD	10.1	10.3	
Smoking			0.677
yes	35	37	
no	18	16	
Alcohol			0.696
yes	30	28	
no	23	25	
Betel			0.698
yes	25	27	
no	28	26	
Smokeless tobacco			0.567
yes	15	11	
no	38	42	

A summary of the association of the GSTM1 and GSTT1 genotypes and oral cancer is shown in Table 2. The GSTM1 null genotype has a significant effect on oral cancer risk (OR=3.0, 95%CI 1.4-6.7) whereas the GSTT1 revealed no association (OR=0.6, 95%CI 0.3-1.3). Adjusted for exposure variables, GSTM1 null genotype significantly increases the risk for oral cancer (adjusted OR=2.6, 95%CI 1.04-6.5) while the GSTT1 null genotype is not independently associated with oral cancer risk (adjusted OR=0.6, 95%CI 0.2-1.5)(data not shown). As is shown in Table 3, the effect of the GSTs susceptible genotypes on oral cancer risk is not increased with the combined deletion of GSTM1 and GSTT1 (OR=2.0, 95%CI 0.5-7.8).

Table 2 Crude odds ratio (cOR) of GSTs and oral cancer

	case no. (%)	control no. (%)	cOR	95%CI	p-value
GSTM1					
Wild type	23 (43.4)	37 (69.8)			
null	30 (56.6)	16 (30.2)	3.0	1.4 - 6.7	0.006
GSTT1					
Wild type	35 (66.0)	28 (52.8)			
null	18 (34.0)	25 (47.2)	0.6	0.3 - 1.3	0.166

Table 3 Risk of GSTM1 and GSTT1 genes on oral cancer, adjusted for exposure variables

GSTM1	GSTT1	aOR	95%CI	p-value
Wild type	Wild type	1.0		
Wild type	null	0.7	0.2-2.3	0.508
null	Wild type	3.6	1.0-12.9	0.045
null	null	2.0	0.5-7.8	0.313
GSTM1 null or GSTT1 null		1.6	0.6 - 4.4	0.328
GSTM1 null and GSTT1 null		2.0	0.5-7.8	0.313

aOR= adjusted odds ratio

The association between genetic susceptibility and environmental risk factors for oral cancer is also investigated. Table 4 shows that with respect to the tobacco smoking habit, the GSTM1 wild type and GSTM1 null genotypes has no influence on oral cancer among the non-smokers and the occasional smokers. However,

frequent smokers with the GSTM1 null have a significantly increased risk for oral cancer (OR=4.1; 95%CI 1.5-11.3). The GSTM1 null genotype shows a significant increased risk among frequent alcohol drinkers (OR=4.3; 95%CI 1.4-13.2), but not among non- and minimal drinkers. With respect to the betel chewing habit, the GSTM1 null demonstrates an increased risk among frequent chewers (OR=4.0; 95%CI 1.3-12.9). Interestingly, for individuals who chew betel without smokeless tobacco, the risk is raised to 22 folds (95%C.I 2.2-222.0). After the adjustment for all other variables (i.e. smoking, drinking, betel chewing, local beans consumption), the effect of the GSTM1 null genotype on oral cancer development is more prominent among, drinkers and chewers (OR=7.2, 95%CI 1.5-33.8; OR=4.4, 95%CI 1.1-17.8, respectively). A strikingly increased trend is noted among chewers, who inherited susceptible GSTM1 genotype and those who do not use smokeless tobacco (OR =26.9, 95%CI 2.1-339.8).

Table 4 Risk of GSTM1 null genotype for oral cancer by cigarette smoke, alcohol and betel exposure

	case		control					
	null	normal	null	normal	cOR	95%CI	aOR	95%CI
tobacco								
no or minimal	11	9	7	10	1.7	0.5-6.4	2.0	0.4-10.2
frequent	19	14	9	27	4.1	1.5-11.3	4.0	1.2-13.7[a]
alcohol								
no or minimal	13	13	8	17	2.1	0.7-6.6	1.5	0.4-6.2
frequent	15	10	8	20	4.3	1.4-13.2	7.2	1.5-33.8[a]
betel								

no or minimal	13	15	7	20	2.5	0.8-7.7	2.6	0.6-11.9
frequent	17	8	9	17	4.0	1.3-12.9	4.4	1.1-17.8[a]
with ST	6	7	4	7	1.5	0.3-7.8	2.3	0.2-23.4
without ST	11	1	5	10	22.0	2.2-222.0	26.9	2.0-339.8[a]

a $p<0.05$ ST= smokeless tobacco

The interaction of the GSTT1 genotype with cigarette smoking, betel chewing, alcohol consumption and consumption of three local beans for oral cancer is demonstrated in Table 5. There is no significant influence on the GSTT1 null allele and oral cancer among smokers and non-smokers, drinkers and non-drinkers, and chewers and non-chewers. This indicates that there is no association between the GSTT1 genotypes and the risk for oral cancer development with respect to the major lifestyle risk factors collected in this study.

Table5 Risk of GSTT1 null genotype for oral cancer by cigarette smoke, alcohol and betel exposure

	case		control					
	null	normal	null	normal	cOR	95%CI	aOR	95%CI
tobacco								
no or minimal	7	13	8	9	0.6	0.2-2.3	0.5	0.1-2.9
frequent	11	22	17	19	0.6	0.2-1.5	0.8	0.2-2.8
alcohol								
no or minimal	9	17	12	13	0.5	0.2-1.5	0.5	0.1-2.1
frequent	9	18	12	16	0.7	0.2-2.0	1.3	0.3-5.3

betel							
no or minimal	7	21	12	15	0.4	0.1-1.3	0.7	0.1-2.9
frequent	11	14	13	13	0.8	0.3-2.4	0.7	0.2-2.9
with ST	4	9	6	5	0.4	0.1-2.0	0.4	0.0-4.1
without ST	7	5	7	8	1.6	0.3-7.4	3.1	0.4-25.1

DISCCUSSION

Polymorphism of GSTM1 and GSTT1 and their effect on oral cancer have been studied recently in several countries (Hung et al., 1997; Park et al., 1997; Jourenkova-Mironova et al., 1999; Nair et al., 1999; Sato et al., 1999). We report the first of such investigated in a Thai population. The frequencies of GSTM1 and GSTT1 null among our non-cancer population are 30.2% and 47.2% respectively (n=53). The results show that individuals with a susceptible version of GSTM1 genotype (the null genotype) have 2.6 folds increase risk for oral cancer independent of environmental risks exposure. There is no increased risk when this allele is combined with the GSTT1 null allele. However, the estimate of the risk in this latter case may not be precise due to the limited sample sizes. In addition, we could not find an association of GSTT1 null allele and oral cancer risk.

This report supports the evidence found in India where the null GSTM1 had 22 folds (95%CI 9.2-57.0) estimated risk for oral leukoplakia, a precancerous lesion, (Nair et al., 1999). It also showed concordance with a study in the Japanese which showed that deletion of the GSTM1 gene increased oral cancer risk by 2.2 folds (95%CI 1.4-3.6)(Sato et al., 1999). Our study did not support the results from the study in English Caucasians (Deakin

et al., 1996), which found no risk associated with GSTM1 null. Another study in Swiss Caucasians showed 2.4 folds increased risk with GSTT1 null (95%CI 1.0-5.5) but no association with the GSTM1 null (Jourenkova-Mironova et al., 1999). A study in the United States detected no association of the GSTM1 and oral cancer risk (Park et al., 1997). One possible explanation for the disagreements is the ethnic differences in allelic frequency of the GSTM1 and GSTT1 polymorphisms. In this regard, we carefully compared our result with data from the previous reports. Interestingly, we found that the baseline frequency of GSTM1 null from controls has a strong influence on oral cancer risk, i.e. when the frequencies were high, approaching or above 50%, their effect on oral cancer risk was low or minimal. Example can be found in the reports from Deakin et al (1996), Park et al (1997), Jourenkova-Mironova et al (1999) and Hung et al (1997) when the GSTM1 null frequencies are 55, 51, 52 and 58%, respectively. In contrast, when the frequencies of the genotypes were low (17% as observed by Nair et al.,), their influence on the risk was strikingly high. Another possible explanation of the discrepancy is the difference in environmental and life-style risk factors. As described earlier, the betel quid may have significantly different mixtures from various countries. The higher cancer risk among betel quid chewers without smokeless tobacco compared with those who consumed both is very interesting and may suggest some antagonistic effects between them. This observation requires further investigation. Tobacco smoking, alcohol drinking and betel chewing are known risk factors of oral cancer in Southern Thailand (Kerdpol and Sriplung, 1997). When the interaction between GSTM1 null and the lifestyle risk factors is

analyzed, our data demonstrates that these environmental factors enhance the risk from GSTM1 null (OR=4.0, 7.2, 4.4 for habitual tobacco smokers, alcohol drinkers and betel chewers, respectively). We found no difference in the age of onset on the oral cancer whether the patients inherited susceptible or normal versions of the GSTs genes.

We compared our data with the study on Taiwanese's (Hung et al., 1997) whose ethnicity and exposures are similar to ours. The influence of the GSTM1 null on oral cancer is stronger based on our study when the association is conducted separately for GSTM1 and GSTT1 (OR=3.6 to 1.3). However, when two genes were combined in the analysis we could not detect the association (OR=2.0; 95%CI 0.5-7.8), whereas the Taiwanese detected an increased risk (OR=4.9). The age of onset for our patients was 67 years old, the Taiwanese cases was 54.1 years old. The observed discrepancies can possibly be explained by the difference in tobacco smoking and betel chewing habits. Our oral cancer patients who smoked also drank alcohol, but rarely chewed betel whereas most of Taiwanese chewers in the Hung's study were smokers. In addition, betel chewing style and ingredients may be different also.

Although we have collected oral hygiene information from our patient and control groups, the information is not adequate for us to conduct an association analysis. This is because the majority of them had many types of oral problems and about half of the patients have lost all of their teeth.

CONCLUSION

We found that there is a gene-environmental interaction on the risk of oral cancer in Thailand: GSTM1 null with cigarette smoking, alcohol consumption and betel chewing. Since the development of oral cancer like other cancers is based on multifactorial contributions, therefore additional environmental and genetic factors should be explored. For this reason, a study with a larger sample size is ongoing in our laboratory for further exploration of gene-gene and gene-environment interactions on the development of oral cancer.