RESEARCH COMMUNICATION

Combined Effects of Isothiocyanate Intake, Glutathione s-Transferase Polymorphisms and Risk Habits for Age of Oral Squamous Cell Carcinoma Development

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Abstract

Dietary isothiocyanates (ITCs) found in cruciferous vegetables (Brassica spp.) has been reported to reduce cancer risk by inducing phase II conjugating enzymes, in particular glutathione S-transferases (GSTs). This case-control study was aimed at determining associations between dietary ITCs, GSTs polymorphisms and risk habits (cigarette smoking, alcohol drinking and betel-quid chewing) with oral cancer in 115 cases and 116 controls. Information on dietary ITC intake from cruciferous vegetables was collected via a semi-quantitative food frequency questionnaire (FFQ). Peripheral blood lymphocytes were obtained for genotyping of GSTM1, GSTT1 and GSTP1 using PCR multiplex and PCR-RFLP. Chi-square and logistic regression were performed to determine the association of ITC and GSTs polymorphism and risk of oral cancer. When dietary ITC was categorized into high (greater than/equal to median) and low (less than median) intake, there was no significant difference between cases and control group. Logistic regression yielding odd ratios resulted in no significant association between dietary ITC intake, GSTM1, GSTT1 or GSTP1 genotypes with oral cancer risk overall. However, GSTP1 wild-type genotype was associated with later disease onset in women above 55 years of age (p=0.017). Among the men above 45 years of age, there was clinical significant difference of 17 years in the age of onset of oral cancer between GSTP1 wild-type + low ITC intake and GSTP1 polymorphism + high ITC intake (p=0.001). Similar conditions were also seen among men above 45 years of age with risk habits like drinking and chewing as the earlier disease onset associated with GSTP1 polymorphism and high ITC intake (p<0.001). This study suggests that combination effects between dietary ITCs, GSTP1 polymorphism and risk habits may be associated with the risk of oral cancer and modulate the age of disease onset.

Keywords: Oral squamous cell carcinoma - GST polymorphisms - ITC intake

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Introduction

Oral cancer is a serious public health problem in the world with over 390,000 new cases reported annually worldwide (Sankaranarayanan, 2003). Despite the advancement in several treatment modalities, 50% of patients afflicted will still die within 5 years of diagnosis (Johnson, 2003). It has been recognized worldwide that tobacco smoking, alcohol consumption and betel-quid chewing are the three main risk factors found to be associated with oral cancer (Johnson, 2003). Although the distinct risk factors for oral cancer are well-recognized, little is known about the role of diet and molecular mechanisms responsible for this malignancy.

Both genetic and environmental factors are involved in the development of cancer. Its interactions on carcinogenesis has been well demonstrated by phase I and II enzymes that are involved in the metabolism of carcinogens. Dietary carcinogens such as polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines (HAs) and nitrosamines require metabolic activation to cause DNA-damage and cancer. The activation of carcinogens is primarily catalysed by phase I enzymes such as cytochrome P450 (Steinkellner et al., 2001). Protection can be accomplished by the inhibition of activating enzymes and/or by induction of phase II which leads to detoxification and accelerated excretion of carcinogens.

One of the most important systems in detoxification is the glutathione s-transferase (GST) family of enzymes.

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Human GSTs enzymes can be subdivided into five main classes, alpha (α), mu (μ), pi (π), theta (θ) and zeta (ζ) (Cho et al., 2006). Induction of phase II detoxification enzyme (GSTM1/GSTT1/GSTP1) is a useful strategy for achieving protection against carcinogenesis, mutagenesis and other forms of toxicity of electrophiles and reactive forms of oxygen. Since consumption of large quantities of fruit and vegetables is associated with a striking reduction in the risk of developing a variety of malignancies, it is of interest that a number of edible plants contain substantial quantities of compounds that regulate human enzymes of xenobiotic metabolism (Fahey et al., 1997).

Isothiocyanates (ITCs) found predominantly in cruciferous vegetables demonstrated strong cancer-prevention activity in animal models (Hecht, 1999). Human studies also show an inverse association between consumption of ITC and risk of cancer through their ability to alter detoxification pathways (Zhang and Talalay, 1998; Hecht, 1999), leading to decreased activation of pro-carcinogens and an increased excretion of carcinogens.

Specifically, ITCs are tasked to inhibit phase I activating enzymes, and induce phase II detoxification enzymes in various target tissues (Zhang and Talalay, 1994; Fahey et al., 1997; Hecht, 1999). Induction of phase II detoxification enzymes reduces exposure of the target tissue to DNA damage, thus exerting a 'blocking effect' on the initiation stage of chemical carcinogenesis (Steinkellner et al., 2001; Rouzaud et al., 2004).

No reports to date have explored the influence of dietary ITC and GSTM1, GSTT1 and GSTP1 polymorphisms on the risk of oral cancer. Therefore, this study was carried out to determine the association between dietary ITC and GSTs polymorphisms on oral cancer risk.

Materials and Methods

Study population

This is a hospital-based case-control study where the cases and controls recruited were individuals registered between June 2006 and January 2007 into the Malaysian Oral Cancer Database and Tumor Bank System (MOCDTBS) coordinated by the Oral Cancer Research & Coordinating Centre, University of Malaya (OCRCC-UM). The MOCDTBS includes an oral cancer data bank comprising of socio-demographic, clinical and pathological information from patients who attended nine selected centres in Malaysia.

The cases that were included in this study are newly diagnosed and histologically confirmed patients with squamous cell carcinoma of the oral cavity (OSCC) and the controls were recruited among those who do not have oral cancer, potentially malignant lesions and as well as other cancers. For both case and control groups, patients with complete diet data and genomic DNA in the nuclei acid bank (at OCRCC-UM and CARIF) were included in the study. Non-Malaysian citizens, recurrent cases and individuals currently undergoing treatment were excluded. The ethical approval was obtained as part of a major project in OCRCC-UM (Medical Ethics code no. DF OP0306/0018/(L)) and endorsed by the Ministry of Health Malaysia.

Data collection

a) Socio-demographic profile: The socio-demographic information used in this study were patients' habits for tobacco smoking, alcohol drinking and betel-quid chewing, occupation, medical history, family history of cancer, age, gender and ethnicity.

b) Dietary ITC intake: Information on dietary intake was collected through a semi-quantitative food frequency questionnaire (FFQ). Oral cancer patients were asked to estimate their usual dietary intake 1 year prior to diagnosis, whereas controls were asked to estimate their usual dietary intake for the previous year. For each individual food, subjects were given an option to select from nine frequency categories (ranging from 'never' to 'more than six times a day'). For each of the food items in the FFQ, a score based on the daily equivalent was tabulated to determine the estimated intake of food by the patient.
Of the 21 vegetables listed in FFQ, there were three members of the Brassicaceae family. Malaysians commonly consumed these three cruciferous vegetables. These were cabbage (Brassica oleracea var. capitata), cauliflower (Brassica oleracea var. botrytis) and kai lan/choy sum (Brassica oleracea var. alboglabra, also known as Chinese kale/Brassica oleracea var. parachinensis, also known as Chinese flowering cabbage). Watercress (Nasturtium officinale) and broccoli (Brassica oleracea var. italica) were infrequently consumed in this population thus were not included in the questionnaire. The information obtained from the FFQ was used to quantify the total energy and daily nutrient intake including ITC using NutrieMart Version 2 (Custommedia, Malaysia). In the analysis, all foods and nutrients were expressed as weight per 1000kcal to adjust for total energy intake.

Determination of GSTM1, GSTT1 and GSTP1 genotype

The presence of GSTM1 and GSTT1 were determined using a modified multiplex PCR approach for simultaneous amplification of both genes as previously reported by others (Nair et al., 1999). The co-amplification of an albumin gene fragment served as internal positive control for a successful amplification reaction. Meanwhile, the genotyping of GSTP1 gene codon 105 polymorphism was performed using PCR-restriction fragment length polymorphism (RFLP) analysis, following procedures described by Park et al., (1999).

Statistical analysis

Data analysis was done using Statistical Package for Social Sciences (SPSS) Version 12.0. To determine the association between dietary ITC intake, GSTs polymorphisms and risk habits with oral cancer risk, logistic regression was employed to obtain odds ratios (ORs) and their 95% confidence intervals (CI). Patients were categorized into high and low ITC intake based on the median value of the study subjects. For analysis of genotype, null genotypes of GSTM1 and GSTT1 was compared with the non-null while the GSTP1 polymorphism (ile/val and val/val genotypes) against the GSTP1 wild-type (ile/ile). The differences in age of OSCC onset between GSTP1 genotypes were examined

by Mann Whitney test followed by Kruskall-Wallis test for multiple comparisons. Results were expressed as median and interquartile range (IQR: the difference between the 25 and 75 percentile). P values of <0.05 were considered statistically significant.

Results

A total of 115 cases of oral cancer and 116 controls were included in this analysis. Regarding selected socio-demographic profiles of cases and controls, the mean age of the cases and controls were 59.6 ± 12.0 and 40.8 ± 11.7 years, respectively. There were more females in the group of cases (63.6%) as compared to the males (36.5%) and the differences between the cases and controls was statistically significant (p = 0.039). Among the different ethnic groups, Indian was the majority in cases (42.6%). For both habits on alcohol drinking and betel-quid chewing, the proportion in non-drinkers and non-chewers was significantly higher in controls than those in the cases (p<0.001). Because the age, gender, ethnicity and habits were significantly different between cases and controls, these confounding factors were taken into consideration during computation of odds ratios to compare between genotypes.

The prevalence of the GSTM1 and GSTT1 null genotype among controls was 56.0% and 41.4%, respectively, and that of the GSTP1 polymorphism (ile/val and val/val genotypes) was 58.6%. The OR for GSTM1 null genotype was 0.92 (95% CI=0.546–1.542), relative to GSTM1 non-null, and that for the GSTT1 null genotype was 0.94 (95% CI=0.559–1.597) relative to GSTT1 non-null genotype. The OR for GSTP1 polymorphism, relative to the wild-type genotype, were 0.65 (95% CI=0.385–1.088). Although all OR values of GSTs polymorphisms indicated reduced risk against oral cancer, neither GSTM1 or GSTT1 null nor the GSTP1 polymorphism was significantly associated with oral cancer risk. Further analysis combining the GSTM1 and GSTT1 genes and GSTM1, GSTT1 and GSTP1 genes, polymorphisms of these combined genotypes was also found not significantly associated with the risk of having oral cancer.

The median dietary intake of ITC was slightly lower among controls (median 2.26μmol/1000kcal, IQR 3.62) than among cases (median 2.38μmol/1000kcal, IQR 3.86) (data not shown). Interestingly, it was noted that when dietary ITC was grouped into high (greater than median) and low (less than/equal to median) intake, high ITC intake was observed to be associated with increased risk of having oral cancer by 13% (OR=1.13, 95% CI=0.674-1.891), although this was not statistically significant (p=0.645).

Using multivariate analysis (adjusted for age, gender, ethnicity and habits), we showed that neither dietary ITC nor GST polymorphisms have significant association with oral cancer. In stratification by GST genotypes, no interaction was also observed between GSTM1, GSTT1 and dietary ITC in relation to oral cancer risk. However, in stratified analysis high ITC intake conferred a 20% reduction in risk among those with GSTP1 polymorphism (OR 0.80, 95% CI=0.387–1.635), although this did not reach statistical significance (data not shown). Because GSTP1 may be a key enzyme in metabolism of ITCs in human, this study embarked further analysis on the age of diagnosis according to the GSTP1 status in cases.

The distribution of GSTP1 genotypes was stratified by age and gender among the cases. The impact of GSTP1 genotypes on age of disease onset was analyzed by comparing the genotypes in two subgroups namely ≤55 years and >55 years at diagnosis was found to be statistically significant between these groups (p=0.003). Further stratification by gender showed that among the female >55 years, the age of OSCC onset was earlier on individuals with GSTP1 polymorphism (p=0.017).

Subsequent analysis was also done to look at the effects of GSTP1 genotypes and dietary ITC intake on the age of OSCC onset by analyzing its status in both the gender and different age groups (Table 1). Regardless of high or low intake of ITC, among patients above 55 years old, the age of disease onset was later on individuals with GSTP1 wild-type (p=0.009). Notably, among the men above 45 years of age, there was clinical significant difference of 17

Table 1. Age of Disease Onset According to GSTP1 Genotype and Dietary ITC Intake Status Stratified by Age and Gender in Oral Cancer

Subgroups		GSTP1 wild-type				GSTP1 polymorphism				p-value[a]
		Low ITC intake		High ITC intake		Low ITC intake		High ITC intake		
Gender	Male	9	67.0 (21.0)	14	51.0 (22.0)	13	64.0 (11.0)	6	54.5 (11.0)	0.012
	Female	14	63.4 ± 8.2[b]	23	61.2±13.9[b]	20	62.2±7.0[b]	16	55.7 ± 12.7[b]	0.225[c]
Age (years)	≤ 45	2	-	8	37.0 (9.0)	1	-	4	33.5 (15.0)	0.381
	> 45	21	66.1 ± 8.4[b]	29	63.3±9.6[b]	32	63.2±7.1[b]	18	58.6 ± 7.8[b]	0.053[c]
	≤ 55	6	50.5 (10.0)	15	45.0 (15.0)	4	49.5 (13.0)	11	51.0 (15.0)	0.577
	> 55	17	69.0 (5.0)*	22	66.0 (10.0)	29	65.0 (10.0)	11	59.0 (9.0)*	0.009
Gender/Age										
Male	≤ 45	2	-	4	37.0 (9.0)	1	-	1	-	0.303
	> 45	7	71.4 ± 35.3*[b]	10	57.8±8.6[b]	12	64.8±7.4[b]	5	54.4±2.2*[b]	0.001[c]
	≤ 55	2	-	9	47.0 (14.0)	1	-	5	54.0 (17.0)	0.349
	> 55	7	71.0 (12.0)	5	65.0 (8.0)	12	65.0 (10.0)	1	-	0.102
Female	≤ 45	0	-	4	36.5 (10.0)	0	-	3	39.0 (-)	0.858
	> 45	14	63.6 ± 8.2[b]	19	66.3±9.0[b]	20	62.2±6.9[b]	13	60.2±8.6[b]	0.206[c]
	≤ 55	4	51.5 (4.0)	6	41.5 (18.0)	3	50.0 (-)	6	44.5 (16.0)	0.253
	> 55	10	69.0 (7.0)	17	66.0 (11.0)	17	65.0 (10.0)	10	59.0 (10.0)	0.082

Data recorded were Numbers and Median (IQR); [a]Kruskal Wallis; [b]mean ± SD; [c]analysis of variance (ANOVA); *<0.05

Table 2. Age of Disease Onset According to GSTP1 Genotype and Dietary ITC Intake Status Stratified by Male Age and Risk Habits for Oral Cancer

Subgroups	GSTP1 wild-type				GSTP1 polymorphism				p-value[a]
	Low ITC intake		High ITC intake		Low ITC intake		High ITC intake		
Male									
Cigarette smoking status									
Yes	7	67.0 (14.0)*	8	51.0 (29.0)*	10	63.0 (14.0)	5	55.0 (4.0)	0.028
No	2	57.0 (-)	6	51.0 (13.0)	3	66.0 (-)	1	-	0.276
Alcohol consumption status									
Yes	6	69.0 (20.0)	8	60.0 (27.0)	6	60.5 (26.0)	3	55.0 (-)	0.242
No	3	67.0 (-)	6	49.5 (9.0)*	7	66.0 (7.0)*	3	54.0 (-)	0.025
Betel-quid chewing status									
Yes	1	-	2	49.5 (-)	5	60.0 (21.0)	-	-	0.132
No	8	69.0 (28.0)*	12	53.5 (25.0)*	8	65.0 (10.0)^	6	54.5 (11.0)^	0.047
Male > 45 years									
Cigarette smoking status									
Yes	6	69.0 (13.0)*	5	65.0 (15.0)	9	64.0 (16.0)	5	55.0 (4.0)*	0.007
No	1	-	5	52.0 (15.0)	3	66.0 (-)	-	-	0.203
Alcohol consumption status									
Yes	5	71.0 (14.0)	5	65.0 (9.0)	5	64.0 (21.0)	3	55.0 (-)	0.054
No	2	69.0±2.8*[b]	5	51.0±3.8[b]	7	63.7 ± 4.5^[b]	2	54.5±0.7*[b]	<0.001[c]
Betel-quid chewing status									
Yes	1	-	2	49.5 (-)	5	60.0 (21.0)	-	-	0.132
No	6	72.5±5.7*[b]	8	59.9±8.3[b]	7	64.3 ± 4.2[b]	5	54.4±2.2*[b]	<0.001[c]

Data recorded were Numbers and Median (IQR); [a]Kruskal Wallis; [b]mean ± SD; [c]analysis of variance (ANOVA); *<0.05 ;^<0.05

years in the age of onset of oral cancer between GSTP1 wild-type with low ITC intake and GSTP1 polymorphism with high ITC intake (p=0.001).

Together with risk habits, the outcome of the GSTP1 genotypes and dietary ITC intake on the age of OSCC onset was further evaluated (Table 2). Among the male smokers with GSTP1 wild-type, the age of disease onset was later on individuals with low ITC intake (p=0.028). Even among the non drinkers, the age of OSCC onset was delayed on individuals with GSTP1 polymorphism and low intake of ITC as compared to individuals with GSTP1 wild-type and high ITC intake (p=0.025). Further investigation discovered earlier OSCC onset occurred on smokers with GSTP1 polymorphism and high ITC intake particularly in men above the age of 45 years (p=0.007). Similar trends were also seen among men above 45 years of age without risk habits like drinking and chewing as the earlier disease onset associated with GSTP1 polymorphism and high ITC intake (p<0.001).

Discussion

ITCs can inhibit cancer development through blocking DNA damage by both inhibition of carcinogen activation of phase I enzymes and detoxification of reactive carcinogens through induction of phase II enzymes. This dual action is thought to reduce the production of electrophilic intermediates with carcinogenic activity and to enhance the detoxification and clearance of carcinogens. Hence, low intake of ITC and polymorphisms of the GSTs may results in increased of cancer risk. In the present study, high level of dietary ITC intake seemed to confer 13% increased in oral cancer risk, although this finding was not statistically significant. It could be due to many confounding factors such as ethnicity which leads to various diets and lifestyles and as well as different practicing habits. It was reported that Chinese people are among the most frequent consumers of cruciferous vegetables in the world (Seow et al., 1998). While this study reveals that there is no association betwe en the high dietary ITC intake and oral cancer risk, other studies on different cancers suggest otherwise. Several investigations suggest that high ITC intake and GSTs polymorphisms has been associated with lower risk to lung (London et al., 2000; Zhao et al., 2001) and colorectal cancer (Seow et al., 2002).

Overall, only a few studies on GSTM1, GSTT1, GSTP1 polymorphism and oral cancer risk has been reported. Occasionally, polymorphisms of the GSTM1, GSTT1 and GSTP1 genotypes have been associated with oral cancer (Jourenkova-Mironova et al., 1999; Sikdar et al., 2004) but most studies to date had reported no association (Hung et al., 1997; Olshan et al., 2000; Sreelekha et al., 2001; Sugimura et al., 2006). This study did not find any association between GSTM1, GSTT1 and GSTP1 polymorphisms and the risk of oral cancer. The observed lack of an association between GSTs polymorphisms and susceptibility to oral cancer is consistent with previous findings (Hung et al., 1997; Oude-Ophuis et al., 1998; Olshan et al., 2000; Sreelekha et al., 2001; Gronau et al., 2003; Sugimura et al., 2006). The effect of the GST-susceptible genotypes on oral cancer risk is not increased with the combined polymorphism of either combination of GSTM1 and GSTT1 or GSTM1, GSTT1 and GSTP1 (Olshan et al., 2000; Seow et al., 2002). The lack of association between GSTs polymorphism and risk of oral cancer demonstrated in this study may suggest that other dominant genetic changes that supersede the GSTs polymorphism may have occurred in these patients. The effectiveness of GSTs in modulating risk to cancer has been shown to depend on the CYP1A1 polymorphism status (Scully et al., 2000). Polymorphism of CYP1A1

was found to increase risk of colorectal cancer with Lynch syndrome as reported in Pande et al (2008). As the CYP1A1 polymorphism status is not included in this study, further analysis on the possibility that GSTs may modulate specific subpopulation of OSCC patients is warranted.

No information on the specific role of the GSTP1 gene in ITC metabolism has been reported. Interestingly, this study suggests that GSTP1 may be a key enzyme in the metabolism of ITCs in human. When stratified by GSTP1 genotype, a protective effect of high ITC intake was observed only among subjects with GSTP1 polymorphism genotype (OR=0.80, 95% CI=0.387-1.635). Although this finding was not significant, there could be a possibility of the interaction between the dietary ITC intake and GSTP1 polymorphism with oral cancer.

Due to its possible interaction between GSTP1 genotypes and dietary ITC with potential lower risk of oral cancer, their role on the age of OSCC onset was analyzed by comparing the genotypes and ITC intake in gender, different age groups and risk habits. In this study, the median age of onset for female OSCC patients above the age of 55 with the GSTP1 polymorphism genotypes was 5 years earlier than those with the GSTP1 wild-type genotype. Similarly, Gaspar and colleagues (2004) were also able to identify strong association between the presence of GSTP1 wild-type genotype and later onset of the papillary thyroid cancer. The polymorphism of GSTP1 genotype will generally lower activity towards PAHs diol epoxides, and thus, has been predicted to have lower detoxification potential and greater risk for cancer.

Further analysis showed by contrast low ITC intake seems to be protective as it is associated with a delayed onset of OSCC by 10 years in individuals with the GSTP1 wild-type genotype, particularly above the age of 55 years. Similar scenario also seen among the males above the 45 years of age where the OSCC onset was at a much later age on individuals with GSTP1 wild-type genotype and low ITC intake as compared to individuals with GSTP1 polymorphism genotype and high ITC intake. Intriguingly, this association between genotype, ITC intake and median age of onset was not observed in males below the age of 45 years or in females. From the study, it was deduced that dietary ITC intake may not play significant role in affecting the age of disease onset even after stratified by age and gender among cancer patients. The bioavailability of dietary ITC intake upon ingestion in vivo remains unclear. The amount of ITCs relevant to human exposure is further complicated by the method of processing and cooking the vegetables. Evidence has shown myrosinases which catalyze the release of ITCs from glucosinolates in vegetables were inactivated by cooking in boiling water for as short as 3 minutes (Jiao et al., 1998). This probably explains that glucosinolates rather than their degradation products, ITCs, in these cooked vegetables that are ingested by humans.

Another novelty of the present study is that the onset age of OSCC among male smokers above 45 years of age with GSTP1 polymorphism genotype and high ITC intake was significantly younger than that with GSTP1 wild-type genotype and low ITC intake. Not taking into consideration on the ITC intake, worse prognosis on the age of disease onset seems to appear when combination of smoking habit and GSTP1 polymorphism on the oral cancer patients. Accumulation of carcinogen-induced DNA damage over a long period of time leads to carcinogenesis. Even in the presence of high cruciferous vegetable intake, smokers may still develop oral cancer, especially if they continuously smoke for a long time (Tang et al., 2010). Similar situation was also observed with later age of OSCC onset on individuals with GSTP1 wild-type genotype and low ITC intake notably among the male above 45 years old on both non-drinkers and non-chewers. It was obvious that GSTP1 played a stronger role in the determining the OSCC onset among the cancer patients. GSTP1 being the most abundant of the GST enzymes, has been shown to express in most human tissues including oral cavity (Sarkar et al., 1997). It was hypothesized that individuals carrying GSTP1 polymorphism genotype may have their oral mucosa more susceptible to carcinogen exposure (cigarette, alcohol, betel-quid) and resulted in the earlier onset of tumor formation.

The association between specific GSTs polymorphism and risk of oral cancer is a widely explored area of research. Since it is unlikely that any single genetic marker would completely explain the cancer risk in an individual, studying a wide array of susceptibility genes is expected to yield more complete picture of an individual's cancer risk profile.

In conclusion, data from this study suggest that dietary ITCs intake and GSTs polymorphisms do not confer an increased risk of oral cancer but together with the risk habits it may modulate the age of disease onset.

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