RESEARCH COMMUNICATION

Glutathione S-transferase M1 Gene Polymorphism in Thai Nasopharyngeal Carcinoma

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Abstract

Nasopharyngeal carcinoma (NPC) is a serious health problem in Thailand. It is caused by the combined effects of Epstein-Barr virus (EBV), carcinogens and genetic susceptibility. The glutathione S-transferase M1 gene (*GSTM1*) encodes a phase II enzyme responsible for detoxifying carcinogenic electrophiles. Polymorphic null forms of the gene *GSTM1* lack enzyme activity and have been associated with susceptibility to several cancers including NPC. To examine the association between *GSTM1* polymorphism and NPC susceptibility in Thais, *GSTM1* genotypes (normal and null genotypes) in 78 NPC patients and 145 age-matched healthy controls were determined using PCR assays. Overall, no statistically significant differences were observed in the frequency of *GSTM1* genotypes between cases and controls, nor among NPC patients compared on the basis of sex and clinical stage of disease. Carriers with the *GSTM1* null genotype had a 2.9-fold increased risk for NPC of WHO type III when compared to those with *GSTM1* normal genotype ($P < 0.05$ with OR =2.9, 95% CI = 1.2-6.8). When cases and controls were categorized into 3 age groups (>40, >45 and >50 years), the frequencies of *GSTM1* null genotype in cases the >45 and >50 age groups were significantly different from controls ($P < 0.05$). In addition, carriers of the *GSTM1* null genotype in age groups >45 and >50 years had a 2-fold and 3-fold increased risk for NPC when compared to those with *GSTM1* normal genotype (OR = 2.2, 95% CI = 1.1-4.7 and OR = 3.0, 95% CI = 1.2-7.5). We suggest that *GSTM1* polymorphism may be associated with NPC susceptibility in Thais, especially for *GSTM1* null genotype carriers of age higher than 45 years. The *GSTM1* null genotype may be a useful genetic marker for predicting Thai NPC and for screening of early stages of Thai NPC.

Key Words: nasopharyngeal carcinoma - Risk factor - *GSTM1* gene - Polymorphism - Thailand.

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Introduction

Nasopharyngeal carcinoma (NPC) is a great killer epithelial malignancy in southern China and Southeast Asia and causes uncountable losses in these regions including Thailand annually. NPC is the top ten leading cancer among Thai males and shows the highest rate in males in Bangkok City with age standardized rate = 4.5/105 persons/year, which is about 2.8-fold higher than females. The age distribution of Thai NPC initially rose in young adults and gradually increased up to age 65 years with the peak age at 40-50 years (McDermott et al., 2001; Parkin et al., 1997; Deerasamee et al., 1999). Clinically, the early stage (treatable stage) of NPC has no specific warning signs, resulting in late diagnosis when cancer is incurable (Tune et al., 1999). Screening of early stage NPC patients in high-risk populations for intensive counseling and immediately efficient treatment is suggested to be the best way to prevent this undesirable cancer. To date, several Epstein-Barr virus (EBV)-associated tumor markers including serological markers and genomic DNA detection have been reported to be useful tools for early detection, prognosis and management of NPC (Zong et al., 1992; Tiwawech et al., 2003; Wolf et al., 1973; Mutirangura et al., 2001). However, research on molecular markers for the cancer predisposition and susceptibility could be of both fundamental and clinical interest. Thus, the potential risk factor that could be predicting and screening of NPC high-risk populations for intensive early stage NPC detection and treatment is urgently required to better control of NPC.

Epidemiological and etiological evidence suggests that three major risk factors play a crucial role in NPC

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tumorigenesis. These are EBV infection, chemical carcinogen exposure and individual genetic susceptibility. However, the mechanism of NPC induction by these factors is still unknown. Among the three, EBV is well recognized as a causal factor for NPC (Zong et al., 1992; Wolf et al., 1973). On the other hand, a minority of EBV-infected persons have been developed NPC, suggesting that other risk factors may be involved in its development. It has been suggested that these may include environmental factors such as lifestyle and occupational exposure to chemical carcinogens and tumor promoters (Jeannel et al., 1990; Hildesheim et al., 1992; Nam et al., 1992; IARC, 1997; Armstrong et al.,1998; Ho et al., 1999; Mirabeelli et al., 2000; Vaughan et al., 2000). In addition, host susceptibility genes including HLA-regions, interferon-alpha and p53 alleles and a number of other polymorphic genes that encode enzymes involved in metabolic activation (phase I) and detoxification (phase II) of xenobiotics have been reported as remarkable risk factors (Lu et al., 1990; Golovleva et al., 1997; Nazar-Stewart et al., 1999; Kongruttanachok et al., 2001).

With respect to human phase II detoxification enzymes, at least five classes (alpha, mu, pi, sigma and theta) of cytosolic glutathione S-transferases (GSTs) have been identified. However, only enzymes in three classes including GST-M (mu), GST-P (pi) and GST-T (theta) class play a key role in the detoxification of carcinogenic electrophiles such as aflatoxin and polycyclic aromatic hydrocarbons (PAHs) in tobacco smoke (e.g., benzo[a]pyrene and other procarcinogens of PAHs). The absence of a homozygous allele of GSTM1 gene (e.g., the *GSTM1* null genotype) yields a complete loss of enzyme activity for binding with genotoxic substrates including epoxides derived from aflatoxin and PAHs (Hayes and Pulford, 1995). Actually, the frequency of *GSTM1* null genotype varies among different ethnic groups. For example, it is 20-30% in African-Americans (Ford et al., 2000), 45-56% in Asians (Gao and Zhang, 1999; Kiyohara et al., 2002), and 40-58% in Caucasians (Strange et al., 2000). Due to the lack of function to detoxify carcinogens, it is believed that individuals of the *GSTM1* null genotype are more prone to develop NPC than those of the *GSTM1* normal genotype.

Cumulative data from molecular epidemiological studies has demonstrated that individuals with the *GSTM1* null genotype are susceptible to cancer in various organs including the skin, prostate gland, colorectal system, nasopharynx, gastric system, oral cavity, ovaries, cervix, lungs, breasts, bladder and liver (Heagerty et al., 1994; Autrup et al., 1999; Gawronska-Szklarz et al., 1999; Nazar-Stewart et al., 1999; Setiawan et al., 2000; Kietthubthew et al., 2001; Spurdle et al., 2001; Sierra-Torres et al., 2003; Sweeney et al., 2003; van der Hel et al., 2003; Srivastava et al., 2004; Deng et al., 2005). However, results of many other studies dealing with association between *GSTM1* polymorphism and some of these cancers are still contradictory (Kelsey, et al., 1997; Lallas et al., 2000; Kim et al., 2002; Cheng et al., 2003; Zheng et al., 2003).

A previous study by Nazar-Stewart et al. on *GSTM1* null genotype and NPC susceptibility in Caucasian populations revealed that the *GSTM1* null genotype was moderately associated with increased risk of NPC (OR = 1.9, 95% CI = 1.0-3.3 for all cases and OR = 1.7, 95% CI = 0.8ñ3.5 for squamous cell cases) (Nazar-Stewart et al., 1999). However, a recent study by Cheng et al. on a Taiwanese group claimed that there was no association between *GSTM1* null genotype and risk of NPC (Cheng et al., 2003). Since the results of these two studied are inconsistent and an association between the *GSTM1* polymorphism and NPC susceptibility has not been studied in Thai populations, we conducted a case-control study on Thais to determine whether *GSTM1* polymorphism could be a risk factor in predicting NPC susceptibility or could be used to evaluate cancer status.

Materials and methods

Study Subjects

DNA samples from peripheral blood leukocytes of 78 ethnic Thai NPC patients (48 males and 30 females with a mean age of 50 years, range 29-84 years) who had admitted at the National Cancer Institute of Thailand (NCIT) were examined. All were histologically proven to have NPC based on the criteria of WHO. There were 12 cases of WHO type I, 34 cases of WHO type II and 32 cases of WHO type III. Tumor staging was based on the TNM tumor classification by the 1997 AJCC system (Fleming et al., 1997). There were 11 cases of NPC stages I & II and 67 cases of NPC stage III & IV. DNA samples were also obtained from 145 age-matched healthy individuals as controls (46 males and 99 females with a mean age of 49 years, range 26-82 years) that visited the NCIT for general health check-ups.

The incidence of NPC in Thailand is highest in Bangkok City and shows a peak at age 40-50 years (Deerasamee et al., 2000), suggesting that Thai people over 40 years may possibly represent an NPC high-risk group. Thus, in this study, both cases and controls were divided into 3 groups of ages >40, >45 and >50 years, in order to determine whether there was an age dependent association between *GSTM1* polymorphism and NPC.

GSTM1 genotyping

GSTM1 genotypes were determined by the PCR method described elsewhere (Nazar-Stewart et al., 1999) using primers 5'-GAA CTC CCT GAA AAG CTA AAG C-3' and 5'-GTT GGG CTC AAA TAT ACG GTG G-3'. Co-amplification of human β-globin using primers 5'-AAC TTC ATC CAC GTT CAC C-3' and 5'-GAA GAG CCA AGG ACA GGT AC-3' was used to confirm the true GSTM1 null genotype and not a failure in the PCR assay. Only samples that gave β-globin PCR positive results were recruited in the study. Briefly, the reaction mixture (50 μl) was incubated at 95 ˚C for 5 min prior to the PCR and then further processed for 40 cycles at 94 ˚C for 10 sec, 60 ˚C for 20 sec and 72 ˚C for 45 sec followed by extension at 72 ˚C for 5 min. The amplified products were subjected to electrophoresis on 2.5% agarose gel (Sigma), stained with ethidium bromide,

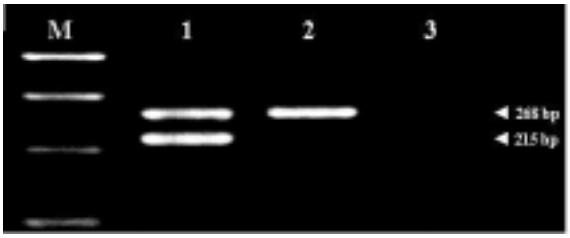

Figure 1. PCR Analysis of *GSTM1* in NPC Patients. The normal [lane 1: 268 bp (β-globulin) + 215 bp (*GSTM1*)] and null [lane 2: 268 bp (β-globulin)] genotypes of *GSTM1* are distinguishable on 2.5% agarose gel electrophoresis by the presence and absence of the *GSTM1* band. Lane 3: a negative control. M: a 100 bp size marker.

and visualized under ultraviolet light. The PCR products of *GSTM1* and β-globin were 215 and 268 base pairs (bp) in length, respectively (Figure 1). Double distilled water was used as the negative control.

Statistical Analyses

The Chi-square test was used to compare the prevalence of *GSTM1* genotypes between cases and controls. A P value of less than 0.05 was used as the criterion for a significant difference. OR and 95% CI were calculated to determine NPC risk between the *GSTM1* normal and null genotypes of cases and controls. Statistical analyses were carried out using EpiCalc 2000 statistical software version 1.02 (EpiCalc 2000).

Results

The overall frequency of the *GSTM1* null genotype in controls and cases were 51% and 64% respectively (Table 1). Although the *GSTM1* null genotype was found more frequently in NPC patients than in controls, the difference was not statistically significant (P > 0.05). Similarly, individuals with the *GSTM1* null genotype showed a moderate risk for NPC with OR = 1.7 (95% CI = 0.9-3.0), although there was no significant difference from controls.

With regard to sex, histological type and clinical stage, there were no statistically significant differences in *GSTM1* null genotype frequencies between cases and controls (P > 0.05) with an exception of undifferentiated carcinoma (WHO type III) of which frequency of *GSTM1* genotypes was significantly different from that of controls (P < 0.01). Individuals who carried the *GSTM1* null genotype had a 2.9-fold increased risk of developing WHO type III NPC (OR = 2.9, 95%CI = 1.2-6.8).

Among *GSTM1* null genotype carriers, males had a higher risk for NPC than females (OR for males : OR for females = 2.2 : 1.4). In addition, *GSTM1* null genotype carriers had a higher risk of developing WHO type III than WHO type I and WHO type II, respectively (OR for WHO type III : OR for WHO type I : OR for WHO type II = 2.9 : 1.9 : 1.1). Furthermore, *GSTM1* null genotype carriers have a higher risk of developing NPC stage I & II than stage III & VI (OR for stage I & II : stage III & VI = 2.6 : 1.6).

When cases and controls were categorized into 3 age groups of >40, >45 and >50 years, the frequency of *GSTM1* null genotypes in the two groups >45 and >50 years were significantly different from those of controls (P < 0.05), whereas the group >40 showed no difference (Table 2). The *GSTM1* null genotype carriers age groups >45 and >50 years had 2-fold and 3-fold higher risk, respectively of developing NPC (OR = 2.2, 95% CI = 1.1-4.7 and OR = 3.0, 95% CI = 1.2-7.5) when compared to *GSTM1* normal genotype carriers.

Discussion

NPC remains one of the major causes of mortality in southern China and in most parts of Southeast Asia including

Table 1. Frequency of *GSTM1* Genotypes in Healthy Controls and NPC Patients

Group	No. of sample	Mean age	*GSTM1* genotypes, n (%)		P [a]	OR[b] (95%CI)
			normal	null		
Controls	145	49	71 (49.0)	74 (51.0)		
Sex						
Male	46	48	24 (52.2)	22 (47.8)		
Female	99	50	47 (47.5)	52 (52.5)		
NPC	78	50	28 (35.9)	50 (64.1)	>0.05	1.7 (0.9-3.0)
Sex						
Male	48	49	16 (33.3)	32 (66.7)	>0.05	2.2 (1.0-5.0)
Female	30	52	12 (40.0)	18 (60.0)	>0.05	1.4 (0.6-3.1)
Histological type						
WHO I	12	59	4 (33.3)	8 (66.7)	>0.05	1.9 (0.6-6.7)
WHO II	34	50	16 (47.1)	18 (52.9)	>0.05	1.1 (0.5-2.3)
WHO III	32	47	8 (25.0)	24 (75.0)	<0.01	2.9 (1.2-6.8)
Clinical stage						
Stage I & II	11	48	3 (27.3)	8 (72.7)	>0.05	2.6 (0.7-10.0)
Stage III & IV	67	51	25 (37.3)	42 (62.7)	>0.05	1.6 (0.9 -2.9)

[a] Frequency of *GSTM1* genotypes was compared between NPC cases and controls.
[b] ORs were calculated to evaluate the association of *GSTM1* null genotype and NPC risk.

Table 2. Characteristics of Controls and Cases by *GSTM1* Genotype and Age

Age (years)	Controls, n (%)		NPC, n (%)		P [a]	OR[b] (95% CI)
	normal	null	normal	null		
>40	52 (48.6)	55 (51.4)	20 (38.5)	32 (61.5)	>0.05	1.5 (0.8-2.9)
>45	39 (54.9)	32 (45.1)	17 (35.4)	31 (64.6)	<0.05	2.2 (1.1-4.7)
>50	28 (52.8)	25 (47.2)	10 (27.0)	27 (73.0)	<0.05	3.0 (1.2-7.5)

[a]Frequency of *GSTM1* genotypes was compared between NPC cases and controls.
[b]ORs were calculated to evaluate the association of *GSTM1* null genotype and NPC risk.

Thailand. The identification of an effective potential risk factor that can predict NPC susceptibility and be used to detect persons at risk but without clinical symptoms would be useful. It can lead to major goals in controlling this cancer by earlier identification and more efficient therapy and medical counseling as well. Our goal was to determine whether recent advances in molecular biology might provide a highly sensitive and specific genetic assay for rapid detection of individuals at high-risk of developing NPC.

Although the process of NPC development is still unclear, it has been hypothesized that NPC tumorigenesis may begin with metabolic activation of carcinogenic compounds by Phase I enzymes such as cytochrome P4502E1 (CYP2E1) to yield carcinogens such as an epoxide form of benzo(a)pyrene and aflatoxin that can further interact with host DNA. Support for this hypothesis was found in a study (Kato et al., 1995) that showed an association between *CYP2E1* polymorphism and higher DNA adduct levels in lung tissue. On the other hand, epoxide forms of carcinogens may be detoxified by phase II enzymes, particularly GSTs, resulting in cancer inhibition. Therefore, NPC susceptibility to carcinogens may in part depend on the metabolic balance between phase I and phase II enzymes in each particular individual. It was found that persons who carried genotypes for high activity of phase I enzymes and for low activity of phase II enzymes were at high risk of developing NPC (Hayashi et al., 1991; Kihara et al., 1995).

Our results showed that there was no overall association between the *GSTM1* polymorphism and NPC susceptibility and clinical stage of cancer ($P > 0.05$), and were thus in agreement with the recent study by Chen et al. in Taiwanese populations (Cheng et al., 2003). They did not support the results of Nazar-Stewart et al. (1999) who reported a difference in *GSTM1* null genotype frequency between NPC patients and controls. On the other hand, our results did show that males were at a higher risk for NPC than females (OR for males : OR for females = 2.2 : 1.4) and this correlated with incidence for NPC that has been reported in Thai populations. This results contrasted with those of Nazar-Stewar et al. (1999) who found that females with *GSTM1* null genotype were at a higher risk for NPC than males (OR for females : OR for males = 3.4 : 1.4).

Unexpectedly, we found that *GSTM1* null genotype was associated with the most aggressive histological type of NPC, WHO type III (OR = 2.9, 95%CI = 1.2-6.8). This indicated that *GSTM1* null genotype carriers might be at high risk of developing WHO type III NPC. Since WHO type III NPC has been reported to be more strongly associated with EBV infection than cigarette and alcohol consumption, the interpretation of this result is still unknown.

Although we found an increased risk for development of NPC squamous cell carcinoma WHO type I (OR = 1.9, 95%CI = 0.6-6.7) for *GSTM1* null genotype carriers, the difference from controls was not significant ($P > 0.05$). This differed from the results of Nazar-Stewar et al. (1999) who reported an increase risk of NPC squamous cell carcinoma for *GSTM1* null genotype carriers (OR = 1.7, 95%CI = 0.8-3.5).

Interestingly, a clear association between *GSTM1* polymorphism and NPC susceptibility was found when cases and controls were compared by age groups >45 and >50 years. Since cancer occurs mostly in aged population, age selection and grouping of study subjects strongly influenced the outcome of statistical analysis. Therefore, in order to perform case-control cancer studies, we need to be aware of this age-grouping effect and include large numbers of young subjects together with age-grouped cases and controls. We demonstrated that *GSTM1* null genotype carriers in age groups >45 to >50 years had 2- to 3-fold higher risk of developing NPC than *GSTM1* normal genotype carriers. This indicated that individuals with *GSTM1* null genotype in age groups >45 to >50 years were at high-risk for NPC. This was in agreement with the results of Nazar-Stewar et al. (1999) who found an association between the *GSTM1* null genotype and NPC susceptibility in Caucasians populations, particularly for persons of age >50 years.

The mechanism by which *GSTM1* null genotype increases susceptibility of NPC development is still undefined. It has been reported that heavy cigarette and alcohol consumption can be considered risk factors for NPC (Ho et al., 1999). Most of the Thai NPC patients had previously been exposed to aflatoxin B1 and nitrosamines for a long period via the diet and they were also former heavy cigarette and alcohol consumers (personal observtion). Therefore, we propose that the *GSTM1* null genotype may increase susceptibility to NPC development in middle-aged Thai people (>45 years) as a result of increased levels of the accumulated epoxide form of aflatoxin B1, benzo(a)pyrene and other procarcinogens of PAHs that induce DNA damage in epithelial cells of the nasopharynx.

Since we found that not all but about 36% of the *GSTM1* normal genotype developed NPC, it is clear that other factors may also increase the risk of NPC development in healthy controls. For example, human papillomavirus (HPV16)

infection has been reported to reduce *GSTM1* enzyme activity and *GSTM1* mRNA levels in human cervical keratinocytes in culture (Chen and Nirunsuksiri, 1999).

We suggest that inconsistent results among different studies may be due to differences in sample size, gender, age grouping and ethnic frame of the subjects, misuse of the statistical methods, and other differences in etiological conditions such as viral infection and prior carcinogen exposure that may affect the outcome of each study. In conclusion, despite the lack of overall association between *GSTM1* polymorphism and NPC development, we have shown a significant difference in *GSTM1* polymorphism between NPC patients and healthy controls in age groups >45 and >50 years and we have demonstrated that individuals with *GSTM1* null genotype in these two age groups had a higher risk of developing NPC than did those with *GSTM1* normal genotype. Therefore, we conclude that *GSTM1* polymorphism may be associated with NPC susceptibility and the *GSTM1* null genotype may be a potential risk factor for NPC in Thais. The investigation of *GSTM1* polymorphism in families and relatives of NPC patients and in people who develop chronic head and neck symptoms will be valuable for predicting and screening early stages of NPC.

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