RESEARCH COMMUNICATION

Real-Time PCR Assay for Rapid Detection of *GSTM1* Polymorphism in Nasopharyngeal Carcinoma Patients

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Abstract

Nasopharyngeal carcinoma (NPC) is a common public health problem in Thailand. Glutathione S-transferase M1 gene deletion (*GSTM1* null genotype) carriers have been reported to be at increased risk and therefore this parameter is a potential marker for screening of NPC high-risk individuals. However, the conventional polymerase chain reaction (C-PCR) assay commonly used for *GSTM1* null genotype detection is not suitable for mass screening since it is inconvenient, time consuming and unsafe due to the use of a toxic chemical. Currently, real-time PCR (R-PCR) assay is recommended for quicker and safer detection of various genetic polymorphisms. The aim of this study was to develop a SYBR green I R-PCR assay combined with melting curve analysis for *GSTM1* polymorphism detection in Thai NPC patients. The results were compared to those from the C-PCR assay using DNA samples from peripheral blood leukocytes of 120 Thai NPC patients. The frequencies of *GSTM1* polymorphism detected by the R-PCR and the C-PCR were the same. Forty-eight individuals that were *GSTM1+* in the R-PCR assay showed 2 peaks with melting points of 82.5˚C and 87.5˚C that correlated with the appearance of 2 DNA bands in the C-PCR assay (i.e., one for *GSTM1* at 215 base pairs (bp) and one for *β-globin* at 268 bp). By contrast, 72 individuals that were *GSTM1-* in the R-PCR assay showed 1 peak with a melting point of 87.5˚C that correlated with the appearance of 1 DNA band for *β-globin* at 268 bp in the C-PCR assay. The R-PCR assay using SYBR Green I and melting curve analysis for *GSTM1* polymorphism detection was as reliable as the C-PCR assay but was quicker and safer and more amenable to large scale screening in Thai NPC cases.

Key Words: *GSTM1* gene - real-time PCR - nasopharyngeal carcinoma - SYBR green I - melting curve

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Introduction

Nasopharyngeal carcinoma (NPC) is a serious cancer in southern China and Southeast Asia including Thailand. This cancer ranks 6th in Thai males with the peak age at 40-50 years (Parkin et al., 1997; Deerasamee S, et al., 1999; McDermott et al., 2001). Epstein-Barr virus (EBV) infection, exposure to carcinogens and genetic susceptibility are the epidemiological risk factors that play a crucial role in NPC development (Wolf et al., 1973; Zong et al., 1992). Generally, early stages of NPC are treatable, but most patients are diagnosed at incurable late stages (Tuneet al., 1999). Early detection may provide significant improvement in the management and effective treatment of NPC. Actually, early detection of NPC relies on screening for EBV-DNA load and for anti-EBV IgA against viral capsid antigens and early antigens. Other biomarkers suggested for inclusion in new screening tests for NPC include EBV genotypes and individual cancer-susceptibility gene polymorphisms (Wolf et al., 1973; Zong et al., 1992; Mutirangura 2001; Tiwawech et al., 2003). Recent studies on the role of individual cancer-susceptibility gene polymorphisms in Thai NPC resulted in the discovery of potential diagnostic markers for early identification of non-symptomatic individuals at risk for development of NPC. Identificaiton of such individuals could lead to counseling for NPC prevention and also to sufficiently early detection of NPC to permit curative therapy leading to a reduction in morbidity and mortality.

The cancer-susceptibility gene *GSTM1* is located on chromosome 1p13.3 (Pearson et al., 1993). It is an important gene in preventing cancer development because it encodes GSTM1, a cytosolic GST class μ1 enzyme that detoxifies electrophiles derived from procarcinogens. For example, it detoxifies aflatoxin B1 (AFB1) and smoke-derived carcinogens such as polycyclic aromatic hydrocarbon and aromatic amines (Ketterer et al., 1992; Hirvonen et al., 1994; Hayes et al., 1995). Genetic polymorphism of *GSTM1* has been associated with several cancers including NPC.

Deletion of homozygous *GSTM1* alleles (i.e., in the *GSTM1* null genotype or *GSTM1-*) results in lack of enzyme activity that binds genotoxic substrates such as epoxides derived from aflatoxin B1 and polycyclic aromatic hydrocarbons (PAHs) (Seidegard et al., 1988; Hayes et al., 1995). Thus, *GSTM1-* individuals are more

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likely to develop cancers than individuals with the normal *GSTM1* genotype (*GSTM1+*). There is high agreement between *GSTM1-* and lack of GST class μ function (Seidegard. et al., 1986; Shea et al., 1990; Zhong et al., 1991; Brockmoller et al., 1992). *GSTM1* is detected in a variety of organs, but mainly in the liver, kidney and adrenal gland (Ye et al., 2006). The frequency of *GSTM1-* individuals varies among different ethnic groups and was reported to be 20-30%, 45-56% and 40-58% in African-Americans (Ford et al., 2000), Asians (Gao et al., 1999; Kiyohara et al., 2002) and Caucasians (Strange et al., 2000), repectively.

Several investigators have reported that *GSTM1-* individuals are more susceptible to cancers of skin, leukocytes, the esophagus, the prostate gland, the colorectal system, the gastric system, the oral cavity, ovaries, the cervix, lungs, breasts, the bladder, the liver and the nasopharynx. (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; Wang et al., 2002; Sierra-Torres et al., 2003; Sweeney et al., 2003; Van der Hel et al., 2003; Srivastava et al., 2004; Deng et al., 2005; Pakakasama et al., 2005). Hence, *GSTM1-* is believed to be a potential genetic risk marker for human cancers. Previously, we found that *GSTM1-* Thais of age >45 and >55 years, respectively, had a 2-fold and 3-fold increased risk for NPC when compared to those *GSTM1+* Thais (OR = 2.2, 95% CI = 1.1-4.7 and OR = 3.0, 95% CI = 1.2-7.5) (Tiwawech et al., 2005). We suggested that mass screening for *GSTM1-* Thais might be useful in identifying an NPC high-risk group, particularly with families and relative of NPC patients aged > 30 years. This could constitute an important element in an integrated program urgently required for better control of NPC in Thailand.

GSTM1 polymorphism can be detected by the conventional polymerase chain reaction (C-PCR) assay. C-PCR assay for in vitro amplification of DNA has been used successfully for detecting *GSTM1* polymorphism in DNA samples from peripheral blood leukocytes of patients with several cancers, including NPC (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; Wang et al., 2002; Sierra-Torres et al., 2003; Sweeney et al., 2003;Van der Hel et al., 2003; Srivastava eet al., 2004; Deng et al., 2005; Pakakasama et al., 2005; Tiwawech et al., 2005). However, this method is not suitable for a mass screening since it is time consuming and unsafe due to use of the carcinogen ethidium bromide in the post PCR assay procedure. Real-time PCR (R-PCR) assay has been proposed as a quicker and safer method to solve these problems (Lee et al., 1993; Livak et al., 1995). Three major fluorescence-monitoring systems for DNA amplification are well established and include the use of hydrolysis probes, hybridising probes and DNA-binding agents. Among these, the cheapest system is the double-stranded DNA binding dye chemistry, which detects amplicon production by the use of a non-sequence specific fluorescent intercalating agent SYBR green I.

SYBR green I is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal (1,000-fold greater fluorescence) upon binding to double-stranded DNA (Wittwer et al., 1997; Morrison et al., 1998). However, SYBR green-based R-PCR has some disadvantages including the requirement for extensive optimisation and the propensity for non-specific amplification that requires follow-up assay (i.e., melting point or dissociation curve analysis) for amplicon identification (Ririe et al., 1997).

Although it is usually employed in singleplex reactions, it can also be used for multiplex reactions when coupled with melting point analysis (Siraj et al., 2002). Other methods are more expensive. For example, Ko et al. (2000) have developed an R-PCR assay combined with hybridization probe technology for rapid detection of *GSTM1*, *GSTT1* and *GSTP1*. Although, this method is highly sensitive and specific, it is not very suitable for mass screening since the probes are expensive. Thus, the purpose of the present study was to develop an inexpensive method of *GSTM1* polymorphism analysis that employed R-PCR assay using SYBR green I fluorescence and melting curve analysis for mass screening of NPC high-risk individuals. Efficacy was assessed by comparison to the standard C-PCR assay.

Materials and Methods

Study subjects

Ethnic Thai NPC patients (120 cases) admitted to the National Cancer Institute of Thailand were recruited. All patients were proven to have NPC based on the criteria for WHO histological classification (Fleming et al., 1997). DNA was extracted from peripheral blood leukocytes of all these patients using QIA amp® DNA Blood Mini Kits (Qiagen) and stored at -20 ˚C until used.

GSTM1 genotyping by C-PCR assay

GSTM1 genotypes were determined by a PCR method described elsewhere (Tiwawech et al., 2005), 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 included as an internal control to confirm true GSTM1- status and to elimate the possibility of a simple failure of the PCR assay. Only samples that gave *β-globin* PCR positive results were used

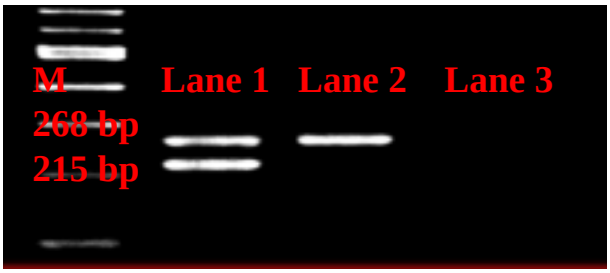

Figure 1. C-PCR assay of GSTM1. The normal genotype [lane 1: 268 bp (β-globin) + 215 bp (GSTM1)] and null genotype [lane 2: 268 bp (β-globin)] were examined on 2.5% agarose gel electrophoresis for the presence and absence of the GSTM1 band. Lane 3: a negative control. M: a 100 bp size marker

in this study. Briefly, the reaction mixture (50 µl) was incubated at 95 ˚C for 5 min prior to PCR for 40 cycles at 94 ˚C for 10 sec, 58 ˚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, 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 (DDW) was used as the negative control.

GSTM1 genotyping by R-PCR assay

The R-PCR assay was performed in a LightCycler model 1.5 machine using the same primer sets for detection of *GSTM1* and human *β-globin* as in the C-PCR assay. The LightCycler® FastStart DNA MasterPLUS SYBR green I (Roche) was used as the master mix. We used SYBR green I technology because it is inexpensive and easy to handle in routine applications and in assay optimization. Briefly, the reaction mixture (20 µl) was incubated at 95˚C for 10 min prior to the PCR for 40 cycles at 95˚C for 10 sec, 58 ˚C for 5 sec and at 72˚C for 10 sec. Amplicons were identified using melting curve analysis, by increasing the temperature of the reaction mixtures up to 95°C at a rate of 0.1°C/sec, starting at 68°C for 15 sec. The fluorescence signal of SYBR green I in each reaction was measured at a wavelength of 530 nm. Later, the melting curves were converted to display the first negative derivative (–dF/dT) versus the temperature. The amplicons of GSTM1 and β-globin had melting points of 82.5 and 87.5˚C, respectively (Figure 2). DDW was used as the negative control.

Statistical analysis.

Statistical analysis was performed by using QuickCals online calculators for scientists (http://graphpad.com/quickcalcs/kappa1.cfm). Kappa statistics were used to assess concordance between the two methods. Kappa values (κ) of 0-0.4 represent poor to fair agreement, values of 0.4-0.8 represent moderate to good agreement, and values of 0.8-1.0 represent excellent agreement.

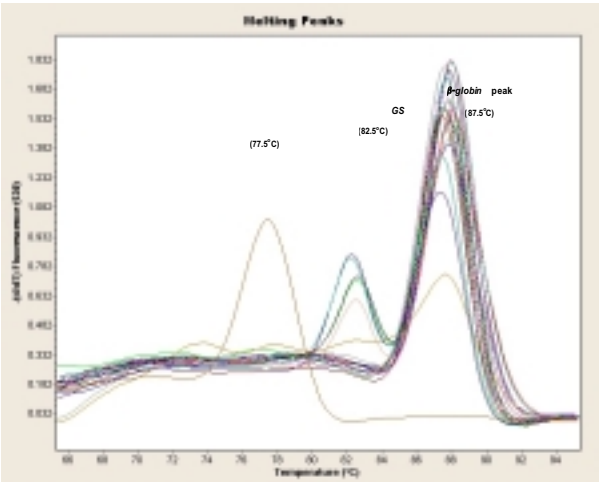

Figure 2. R-PCR assay with SYBR green I and melting curve analysis of *GSTM1*. The normal genotype give one peak with a melting point of 87.5˚C (*β-globin*) and another with a melting point of 82.5˚C (GSTM1) while the null genotype gives a single melting point peak at 87.5 0C (*β-globulin*). Thus, *GSTM1+* and *GSTM1-* can be distinguished easily by the presence or absence, respectively, of the peak at 82.5˚C. The melting point peak of 77.5˚C in the negative control arose from the primer dimer present

Table 1. Frequencies of *GSTM1* Genotypes in NPC Patients by C-PCR and R-PCR assays

Assay	Genotypes[a]		Time	Carcinogen	κ (95%CI)
	GSTM1+	*GSTM1-*	(minutes)		
C-PCR	48	72	360	yes	
R-PCR	48	72	60	no	1.0 (1.0-1.0)

[a]Detected by C-PCR and R-PCR assays

Results

The distribution of *GSTM1* genotypes, assay time and toxic chemical use in the R-PCR and C-PCR assays are compared in Table 1. With respect to genotype frequency, the results of *GSTM1* polymorphism detected in 120 NPC samples using R-PCR assay were identical to those from the C-PCR assay. We identified 48 (40.0%) GSTM1+ and 72 (60.0%) *GSTM1-* individuals. An excellent degree of agreement between the *GSTM1* status revealed by R-PCR and C-PCR was observed (κ = 1.0, 95% CI = 1.0-1.0).

In the R-PCR assay, all 48 GSTM1+ samples showed 2 peaks with melting points of 82.5˚C and 87.5˚C that correlated with 2 DNA bands, one for *GSTM1* at 215 bp and one for *β-globin* at 268 bp in the C-PCR assay, respectively. The other 72 *GSTM1-* samples gave a single peak with a melting point of 87.5˚C that correlated with the 1 DNA band for *β-globin* 268 bp in the C-PCR assay (Figure 3).

Excluding DNA extraction, the total time consumed for the C-PCR and R-PCR assays, was 360 and 60 minutes,

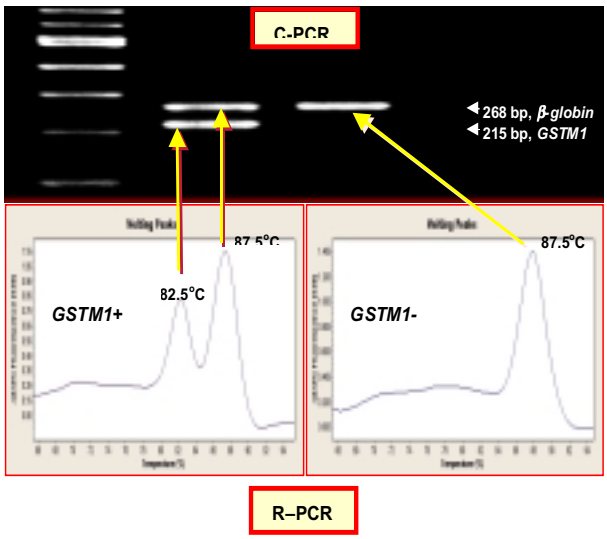

Figure 3. Concordance of R-PCR and C-PCR Assays for *GSTM1* Detection in NPC patients. *GSTM1+* samples in the R-PCR assay that showed 2 peaks with melting points of 82.5˚C and 87.5˚C gave 2 corresponding DNA bands in the C-PCR assay, one for *GSTM1* at 215 bp and another for *β-globin* at 268 bp. By contrast, *GSTM1-* samples that showed 1 peak with a melting point of 87.5˚C in the R-PCR assay gave 1 corresponding DNA band for *β-globin* at 268 bp in the C-PCR assay

respectively. In addition, the R-PCR assay was safer than the C-PCR assay since the carcinogen ethidium bromide was not used in the assay (Table 1).

Discussion

In this study, we have shown a complete concordance between *GSTM1* genotype detected by SYBR green I R-PCR assay and C-PCR assay. The R-PCR assay consumed less time, did not use toxic chemicals and was amenable to high throughput screening. In addition, the method was cheaper than a previous R-PCR method described by Ko et al (2000).

We have shown that R-PCR can be used successfully for rapid, safe and high-throughput screening for *GSTM1*- individuals. This makes it feasible to carry out molecular mass screening for individuals at high risk for NPC and for individuals at early NPC stages when there are no specific warning symptoms (Tune et al., 1999). This could allow for timely NPC detection at early curable stages as opposed to late incurable stages when most patients are currently diagnosed. The method would be particularly useful for mass screening of family members and relatives of NPC patients as well as people at ages over 30 with chronic diseases of the head and neck. In addition to early diagnosis of NPC, *GSTM1*- individuals without symptoms of NPC could be informed of the need to visit a physician at least twice a year for early stage NPC diagnosis. Should any NPC lesions be detected in these visits, immediate effective treatment for NPC could be initiated. These individuals could also be encouraged to alter their life-styles to reduce cancer-related risk activities such as smoking. Since approximately 50% of Thais are known to be *GSTM1*-, such a program could have an important impact on the regulation of cancer development in Thailand.

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