

Detection of *GSTM1* Polymorphism in Patient with Nasopharyngeal Carcinoma by Real-Time PCR

Danai Tiwawech, Dr.* Somjin Chindavijak, M.D.,** Anant Karalak, M.D.,*** Takafumi Ishida, Dr.,****

Yaninee Jarratwisrutpom, B.Sc.***

*Research, **Otolaryngology, ***Pathology Division, National Cancer Institute, Bangkok 10400, Thailand.****Unit of Human Biology and Genetics, Department of Biological Sciences, School of Science, University of Tokyo, Tokyo, Japan.

ABSTRACT

Objective: To investigate whether the real-time polymerase chain reaction (R-PCR) assay with SYBR green I and melting curve analysis could be used for glutathione S-transferase M1 gene (*GSTM1*) polymorphism detection in Thai nasopharyngeal carcinoma (NPC) patients by comparing the results of this assay with the conventional PCR (C-PCR) assay.

Methods: DNA samples from peripheral blood leukocytes of 60 Thai NPC patients were investigated in this study. *GSTM1* polymorphism [*GSTM1* normal genotype (*GSTM1*+) and *GSTM1* null genotype (*GSTM1*-)] were examined by using the R-PCR assay with SYBR green I and melting curve analysis and the C-PCR assay.

Results: The results of *GSTM1* polymorphism detection by the R-PCR assay were in concordance with the C-PCR assay ($\kappa=1.0$). Twenty-six individuals with *GSTM1* in the R-PCR assay showed 2 peaks of melting point at 82.5°C and 87.5°C that correlated with the appearance of 2 DNA bands of *GSTM1* [215 base pair (bp)] and β -globin (268 bp) in the C-PCR assay, respectively. In addition, thirty-four individuals with *GSTM1*- in the R-PCR assay showed only 1 peak of melting point at 87.5°C that correlated with the appearance of 1 DNA band of β -globin (268 bp) in the C-PCR assay. Moreover, we found that the R-PCR assay was a faster and safer method for detection of *GSTM1* polymorphism than the C-PCR assay.

Conclusion: The present study suggests that the R-PCR assay with SYBR Green I and melting curve analysis may be a useful screening tool for more convenient, rapid, reliable, and safer detection of *GSTM1* polymorphism in Thai NPC as compared to the C-PCR assay.

Keywords: Glutathione S-transferase M1 gene; melting curve; nasopharyngeal carcinoma; real-time polymerase chain reaction; SYBR green I

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Nasopharyngeal carcinoma (NPC) is a very harmful cancer in southern China and Southeast Asia including Thailand. This cancer ranks the 6th in Thai males with the peak age at 40-50 years.¹⁻³ Epidemiologically, NPC is a multi-factorial disease caused by a combination of Epstein-Barr virus (EBV) infection, exposure to carcinogens and genetic susceptibility⁴⁻⁵. Clinically, early stages of NPC are treatable; but most patients are diagnosed at later stages when they are incurable⁶. Early detection can therefore contribute significantly to the management and effective treatment of NPC. Currently, early detection of NPC relies on screening for anti-EBV IgA that targets viral capsid antigens (VCA) and early antigens (EA), or

for other molecular biomarkers such as EBV-DNA copy, EBV genotypes and individual cancer-susceptibility gene polymorphisms.^{4,7-9} Among these, the studies on the role of individual cancer-susceptibility gene polymorphisms result in the discovery of diagnostic tools for early detection and monitoring of Thai NPC and may contribute a potential genetic risk marker to predict the non-symptomatic risk person who will develop NPC in the future. This, in turn, may lead to a faster detection of early stage of NPC for curative treatment and counseling for NPC prevention that will provide the reduction of the morbidity and mortality rate of NPC worldwide.

Concerning the individual cancer-susceptibility gene, the *GSTM1* gene (*GSTM1*) that is located on the chromosome 1p13.3¹⁰ is an important gene that plays a crucial role in the prevention of cancer development by

Correspondence to: Danai Tiwawech

E-mail: tdanai@hotmail.com

encoding GSTM1 enzyme to detoxify the ultimate carcinogen form from the pro-carcinogen. Genetic polymorphism of the *GSTM1* has been reported to be associated with several cancers including NPC. The absence of a homozygous allele of *GSTM1* (*GSTM1* null genotype or *GSTM1*-) resulting in a lack of enzyme activity for binding with genotoxic substrates including epoxides that are derived from aflatoxin B1 and polycyclic aromatic hydrocarbons (PAHs).¹¹ Thus, individuals with *GSTM1*- are suggested to be more prone to develop cancers than individuals with *GSTM1* normal genotype (*GSTM1*+). Several molecular epidemiological studies have demonstrated that individuals with *GSTM1*- are susceptible to cancer of the skin, leukocyte, esophagus, prostate gland, colorectal system, gastric system, oral cavity, ovaries, cervix, lungs, breasts, bladder, liver and nasopharynx.¹²⁻²⁵ Therefore, *GSTM1*- is claimed to be a potential genetic risk marker for human cancers. Previously, we have reported that Thai *GSTM1*- carriers with age >45 and >55 years had a 2-fold and 3-fold increased risk for NPC compared to those *GSTM1*+ carriers (OR = 2.2, 95% CI = 1.1-4.7 and OR = 3.0, 95% CI = 1.2-7.5)²⁶ and suggested that *GSTM1*- might be a useful genetic risk marker for a mass screening of the NPC high-risk group, particularly the family members and relatives of NPC patients with age \geq 30 years old. Thus, a mass screening of the NPC high-risk group by using *GSTM1*- detection is really needed for a better control of NPC in Thailand.

Actually, *GSTM1* polymorphism is commonly detected by using the conventional polymerase chain reaction (C-PCR) assay. However, this assay is not suitable for a mass screening since it is time consuming and is not safe as it uses a toxic chemical. Recently, the real-time R-PCR assay has been suggested to be an advantageous method for detection of genetic polymorphism of various cancer-susceptibility genes with a faster and safer performance. Ko et al., have developed the real-time R-PCR assay using the hybridization probes technology for a rapid detection of *GSTM1*, *GSTT1* and *GSTP1*.²⁷ Although, this method is highly sensitive and specific, it is not practical for the mass screening since the probes are quite expensive. Therefore, an inexpensive R-PCR assay by using SYBR green I fluorescence and melting curve analysis technology is proposed to be an appropriate method for mass screening for the NPC high-risk group. The specific aim of this study is to establish the R-PCR assay by using SYBR green I fluorescence and melting curve analysis for *GSTM1* polymorphism detection in Thai NPC and to confirm the results of this assay with the results of the C-PCR assay.



Fig 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)] of *GSTM1* are examined 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.

MATERIALS AND METHODS

Study subjects

DNA samples from peripheral blood leukocytes of 60 ethnic Thai NPC patients who were admitted at the National Cancer Institute of Thailand (NCIT) were examined. All patients were histologically proven to have NPC based on the criteria of WHO.²⁸ DNA extraction was performed using QIA amp[®] DNA Blood Mini Kit (Qiagen). All samples were kept at -20°C prior to analysis.

GSTM1 genotyping by C-PCR assay

GSTM1 genotypes were determined by the PCR method as described elsewhere²⁵ 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 the 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*- but not a failure in the PCR assay. All samples that showed β -globin PCR positive results were recruited in this study.

Briefly, the reaction mixture (50 μ l) 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, 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 (Fig 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 used 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 to optimize the assay condition.

Briefly, the reaction mixture (20 μ l) was incubated at 95°C for 10 min prior to the PCR and further processed for 40 cycles at 95°C for 10 sec, 58°C for 5 sec and at

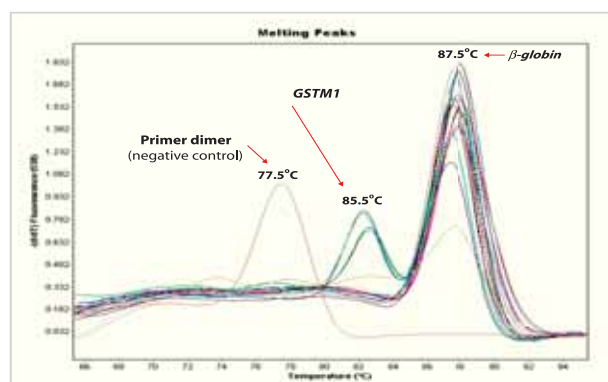


Fig 2. R-PCR assay with SYBR green I and melting curve analysis of *GSTM1*. The normal genotype [the peak of melting point at 87.5°C (β -globin) + 82.5°C (*GSTM1*)] and null genotype [the peak of melting point at 87.5°C (β -globulin)] of *GSTM1* are distinguished on melting point presentation by the presence and absence of the *GSTM1* peak at 82.5°C. The peak of melting point at 77.5°C is primer dimer that present in the negative control.

72°C for 10 sec. The amplicons were identified using melting curve analysis, by increasing the temperature of the reaction mixtures up to 95°C, by 0.1°C/sec, starting at 68 °C for 15 sec. The fluorescent 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 showed the melting point at 82.5 and 87.5°C, respectively (Fig 2). DDW was used as the negative control.

Statistical analysis

Statistical analysis was performed by using the QuickCals online calculators for scientists (<http://graphpad.com/quickcalcs/kappa1.cfm>).²⁹ 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.²⁹

RESULTS

The distribution of the *GSTM1* genotypes, consumption time and the toxic chemical used in the R-PCR and C-PCR assays are shown in Table 1. With respect to the genotype frequency, the results of *GSTM1* polymorphism detected in 60 NPC samples by using the R-PCR assay were identical to the C-PCR assay. We identified 26 (43.3%) *GSTM1+* and 34 (56.7%) *GSTM1-* individuals in the R-PCR assay. An excellent degree of agreement between the *GSTM1* status revealed by R-PCR and C-PCR was observed ($\kappa= 1.0$, 95% CI = 1.0 to 1.0).

All of 26 cases with *GSTM1+* in the R-PCR assay showed 2 peaks of melting point at 82.5°C and 87.5°C that correlated with the characteristics of 2 DNA bands of *GSTM1* [215 base pair (bp)] and β -globin (268 bp) found in the C-PCR assay. In addition, the other 34 cases with *GSTM1-* in the R-PCR assay showed a single peak of melting point at 87.5°C that correlated with the characteristics of 1 DNA band of β -globin (268 bp) presented in the C-PCR assay (Fig 3).

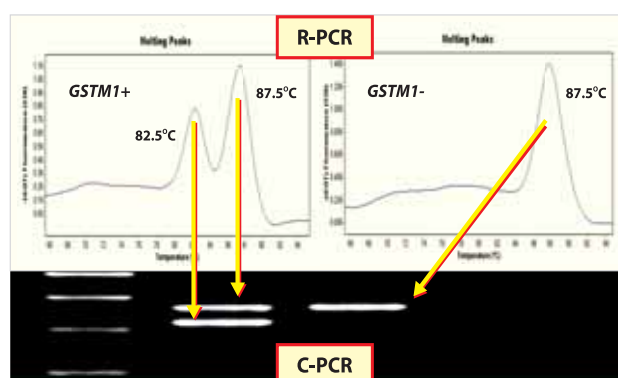


Fig 3. Concordance of R-PCR and C-PCR assay for *GSTM1* detection in NPC patients. Sample with *GSTM1+* in the R-PCR assay that showed 2 peaks of melting point at 82.5°C and 87.5°C has the corresponding 2 DNA bands of *GSTM1* (215 bp) and β -globin (268 bp) in the C-PCR assay. While sample with *GSTM1-* in the R-PCR assay that showed 1 peak of melting point at 87.5°C has the corresponding 1 DNA band of β -globin (268 bp) in the C-PCR assay.

TABLE 1. Frequency of *GSTM1* genotypes in NPC patients by R-PCR and C-PCR assays.

Assay	<i>GSTM1</i> <i>GSTM1+</i>	genotypes ^a <i>GSTM1-</i>	Time consuming (minutes)	Carcinogen used (ethidium bromide)
R-PCR	26	34	60	No
C-PCR	26	34	360	Yes

^aThe *GSTM1* genotypes are detected by R-PCR and C-PCR assays in the same individuals.

Total time consumption for R-PCR assay was 60 minutes after DNA extraction, whereas for C-PCR assay it was about 360 minutes. Moreover, the R-PCR assay was safer than C-PCR assay since ethidium bromide, a carcinogen, was not used in the assay (Table 1).

DISCUSSION

An obvious problem to fight against NPC is that most of patients are diagnosed at incurable late stages since the treatable early stages NPC have no specific warning symptoms to be identified.⁶ Screening for early stage NPC patients in the NPC high-risk population without clinical symptoms followed by early effective treatment is believed to be a promising strategy to control this harmful cancer. To date, several genetic markers are suggested to be a useful tool for screening of the NPC high-risk group. Hence, an application of genetic markers, particularly *GSTM1-* for a mass screening of the NPC high-risk group in the family members and relatives of NPC patients as well as people with chronic diseases of head and neck aged over 30 years old is very urgent.

GSTM1 is recognized as an important detoxifying enzyme for AFB1 and smoke-derived carcinogens, e.g. polycyclic aromatic hydrocarbon and aromatic amines,^{11,30,31} which are risk factors of NPC. The *GSTM1* encoding cytosolic GST class μ enzyme includes a deletion polymorphism that, in the homozygous state (*GSTM1-*), results in the total absence of a functional gene product.³² Several investigators have demonstrated high agreement between the *GSTM1-* and a lack of GST class μ function.³³⁻³⁶ In general, *GSTM1* is detected in various organs, mainly liver, kidney and adrenal glands³⁷. The frequency of the *GSTM1-* varies among different ethnic groups and was reported to be 20-30% in African-Americans³⁸, 45-56% in Asians^{39,40} and 40-58% in Caucasians.⁴¹ It is believed that individuals with *GSTM1-* are more prone to develop NPC than those of the *GSTM1+*. Recently, *GSTM1-* is suggested to be a useful predictive marker for warning of NPC development in the high-risk groups, those without any clinical symptoms (persons who will develop early stages of NPC). By this way, the *GSTM1-* carriers will be informed to visit physicians for early stage NPC detection and immediate effective treatment as well as to prevent themselves from the cancer-related risk factors. Since about 50% of the Thai populations are *GSTM1-* carriers, this indicates that at least half of Thai populations are prone to develop various kinds of cancer including NPC. Thus, the *GSTM1-* biomarker may have a great impact for the regulation of cancer development in the Thais. Therefore, a mass screening of NPC high-risk persons (family members and relatives of NPC patients with age ≥ 30 years old) is reasonably and really needed. In order to achieve this goal, we have designed a rapid, reliable, and safe molecular biological assay for *GSTM1* polymorphism detection.

The C-PCR assay for *in vitro* amplification of DNA

has been used successfully for detecting *GSTM1* polymorphism in the DNA sample from peripheral blood leukocytes of patients with several cancers including NPC.¹²⁻²⁶ Although the C-PCR assay is a sensitive method, it is limited by the complexity, time consumption and safety. Recently, the R-PCR assay based on the detection and quantification of a fluorescent reporter is an advanced method to solve the limitations of C-PCR.^{42, 43} Three major fluorescence-monitoring systems for DNA amplification which includes hydrolysis probes, hybridising probes and DNA-binding agents are well established. Among these, the cheapest system is the double-stranded DNA binding dye chemistry, which detects the amplicon production by the use of a non-sequence specific fluorescent intercalating agent (e.g., 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.^{44, 45} However, SYBR green-based R-PCR has some disadvantages including the requirement for extensive optimisation and the non-specific amplifications that required follow-up assay (melting point or dissociation curve analysis) for amplicon identification.⁴⁶ Actually, SYBR green I was used in single-plex reactions, but when coupled with melting point analysis it can be used for multiplex reactions.⁴⁷

In this study, we found a considerable concordance between *GSTM1* genotypes ($\kappa = 1.00$) as detected by R-PCR and C-PCR assay, indicating similar sensitivities. However, R-PCR assay consumed lesser time and does not use toxic chemicals. This implied that our R-PCR assay has more advantage in terms of time consumption and safety as compared to the C-PCR assay. In addition, this newly established R-PCR assay is cheaper than the previous one established by Ko et al. In conclusion, we suggest that R-PCR assay based on SYBR green I fluorescence and melting curve analysis may be a useful screening tool for more convenient, rapid, reliable and safer detection of *GSTM1* polymorphism in Thai NPC. However, further studies using larger sample sizes to validate the results of the present study are needed.

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