Prevalence of GSTT1, GSTM1 and NQO1 (609C>T) in Filipino children with ALL (acute lymphoblastic leukaemia)\(^1\)

Marilyn G. RIMANDO*†, Mary N. CHUA†, Ernesto d’J. YUSON§, Gloria de CASTRO-BERNAS∥ and Takashi OKAMOTO*‡2

*Department of Biological Sciences, College of Science, University of Santo Tomas, Manila, The Philippines, †Department of Molecular and Cellular Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan, ‡Department of Pediatric Oncology–Hematology, University of Santo Tomas Hospital, Manila, Philippines, §Hematology Unit, The Children’s Medical Center, Quezon City 1100, The Philippines, and ∥Research Center for the Natural Sciences, University of Santo Tomas, Manila, The Philippines

Synopsis

In the present paper, we examined the incidence of polymorphic genes involved with the detoxification of exogenous chemicals, including carcinogens, namely GSTT1 (glutathione transferase θ1), GSTM1 (glutathione transferase μ1) and NQO1 (NAD(P)H:quinone oxidoreductase 1) in 60 Filipino children with ALL (acute lymphoblastic leukaemia). We found a significantly high incidence of the GSTM1 null genotype in ALL children (71.7%) compared with 51.7% in the control group of children (\(P < 0.05\)). The GSTT1 null genotype was observed in 35.0% and 33.3% of the ALL cases and the control subjects respectively, without significant difference. Screening for NQO1 (609C>T) mutant alleles showed a high incidence of the NQO1 C/C genotype (NQO1 homozygous wild-type allele genotype) in 60.0% of ALL cases and was significantly higher than in the control group (23.3%) (\(P < 0.01\)). These GSTM1 null and NQO1 wild-type genotypes are independently associated with the risk of ALL in Filipino patients. When these two genotypes, GSTM1 null and NQO1 C/C, were combined, the hazard rate for childhood leukaemia was significantly increased (\(P < 0.001\)). We also noticed that the incidences of GSTM1 null mutations and the NQO1 C/C genotype were significantly higher among Filipinos. These findings suggest a possible role of the GSTM1 null and NQO1 C/C genotypes in the susceptibility of paediatric ALL cases in the Philippines.

Key words: acute lymphoblastic leukaemia (ALL), carcinogen, genotype, glutathione transferase (GST), multiplex PCR, NAD(P)H:quinone oxidoreductase 1 (NQO1).

INTRODUCTION

Leukaemia, in particular ALL (acute lymphoblastic leukaemia), is the most common form of childhood malignancy [1]. The peak age incidence of ALL is 3–4 years [2] and it is most prevalent among Hispanics, followed by Caucasians and Asians, and lowest among Afro-Americans [1,3]. Despite the rates of successful induction of remission or cure by treatment improving significantly in ALL [4,5], the incidence of ALL cases has been increasing [1,6].

The aetiology of leukaemia is largely unknown, although it is considered to be multifactorial [7]. The involvement of certain environmental exposures has been linked to leukaemia, such as contact with industrial chemicals like benzene [8], industrial and household chemicals [9,10] and chemotherapeutic drugs, such as topoisomerase inhibitors [11] and ionizing radiation [12]. In addition, leukaemogenesis is considered to be affected by interactions between genes and environments [13]. In particular, it was reported that potential carcinogens from the environment or their metabolites, such as dipyrene [9] and permethrin [10], cause DNA damage that induces irreparable chromosomal breaks and thus leads to initiation and progression of leukaemia [13,14].

Individuals, however, display varying degrees of susceptibility as well as severity of illness [15] towards potential carcinogens, indicating differences in genetic background among individuals, such as variations in enzyme expression involved in either detoxification or DNA repair. GSTT1 (glutathione transferase θ1), GSTM1 (glutathione transferase μ1) and NQO1 [NAD(P)H:quinone oxidoreductase 1] belong to the phase II

Abbreviations used: ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CI, confidence interval; GSTM1, glutathione transferase μ1; GSTT1, glutathione transferase θ1; NQO1, NAD(P)H:quinone oxidoreductase 1; NQO1 C/C genotype, NQO1 homozygous wild-type allele genotype; NQO1 C/T genotype, NQO1 heterozygous wild-type and mutant 609C>T allele genotype; NCR, National Capital Region; OR, crude odds ratio; PCR–CTPP, PCR confronting two-pair primer; WBC, white blood cell.

\(^1\) This paper is dedicated to the memory of Dr Gloria Bernas, in appreciation of her contributions to science education and research in the Philippines.

\(^2\) To whom correspondence should be addressed (email tokamoto@med.nagoya-cu.ac.jp).
group of common detoxification enzymes which facilitate the conversion of toxic metabolites (initially metabolized by phase I enzymes) into more water-soluble products that can easily be eliminated [16]. Genes coding for these enzymes are reported to be polymorphic, thereby the presence of both alleles renders an individual with the capacity to eliminate potential carcinogens more efficiently than those with a deletion or mutation of the gene.

GSTT1 and GSTM1 are dimeric soluble proteins of approx. 25 kDa [17]. A wide variety of electrophilic compounds, including well-known environmental carcinogens such as benzopyrene, epoxides and PAH (polycyclic aromatic hydrocarbons), and other industrial carcinogens such as organophosphates, alkylating agents, dihalomethanes, buta-1,3-diene and ethylene oxides [18,19] are detoxified by these enzymes.

Previous studies have clarified the correlation of deletion of alleles of either GSTT1 and GSTM1 or a null genotype with the risk of developing ALL [20–24], other leukaemia subtypes [25–27] and other malignancies such as cervical, lung, prostate, esophageal, colon and breast cancers [28–32]. Genotype frequencies for both GSTT1 and GSTM1 also vary with ethnic background, with the highest percentage of the null genotype observed in 80% of Asians compared with 20% of Caucasians [18]. Among Asians, the GSTT1 null genotype is more frequent among Japanese, Korean and Chinese populations [33,34].

NQO1 is a dimeric flavoprotein which specifically catalyses the reduction of numerous quinines [35]. It is an important chemoprotective enzyme against quinones or quinones derived from the oxidation of phenolic metabolites of benzene [36]. It also functions in generating antioxidant forms of vitamin E, quinone and ubiquinone, thereby protecting cells from oxidative stress [37], scavenging toxic superoxides within the cell [38] and stabilizing p53 [39]. By contrast, apart from detoxification, NQO1 is known to promote carcinogenesis by activating carcinogens such as nitrosamines and heterocyclic amines present in tobacco smoke and food [36]. Currently, 22 single-nucleotide polymorphisms in the NQO1 gene have been reported [40], including a cytosine to thymine point mutation in exon 6 (609C>T), which leads to a proline to serine amino-acid residue substitution (P187S) and results in low enzymatic activity [41]. Genotypes can either be NQO1 C/C (NQO1 homozygous wild-type allele genotype), NQO1 C/T (NQO1 heterozygous wild-type and 609C>T allele genotype) or NQO1 T/T (NQO1 homozygous 609C>T mutant allele genotype). Association of the NQO1 609C>T polymorphism with the risk of developing ALL and various cancer types have been reported previously [42–44]. Similarly to GSTT1 and GSTM1, the frequency of the NQO1 609C>T genotype varies with race, with a high frequency of the mutant allele observed among Hispanics and Asians [45].

Considering that leukaemia is the leading malignancy among Filipino children [46] and that a high prevalence of gene deletion or mutation among Asians has been reported, we investigated whether this high incidence would be present among leukemic children in the local region. The present study, therefore, was conducted to investigate the prevalence of these genes (GSTT1, GSTM1 and NQO1) among Filipino leukaemic children residing within the NCR (National Capital Region) in the northern part of the Philippines, including Manila, Quezon City, Pasig and Parañaque. This study also attempted to determine the independent and combined relative risks of GSTT1, GSTM1 and NQO1 genotypes attributable to the occurrence of ALL among Filipino children. In the present paper, we provide evidence of a positive association between GSTM1 null and NQO1 C/T genotypes and ALL susceptibility in the Philippines.

MATERIALS AND METHODS

Sample collection and clinical profiles

Genotype screening for GSTT1, GSTM1 and NQO1 was conducted in 60 unrelated ALL paediatric cases and 60 randomly-selected normal children, as controls, with ages less than 18 years old. Cases were newly diagnosed or receiving treatment at the University of Santo Tomas Hospital in Manila and the Children’s Medical Center in Quezon City during the period of 1 June to 30 October 2005. Controls were chosen from children receiving routine check-ups at the University of Santo Tomas Hospital, with a complete blood count falling within normal range, reported to be in good health and were not suspected to have any type of malignancy. All subjects represented natural-born Filipino children, who are not products of marriages of a Filipino with another race (eg. Caucasians or other Asians) and were residing within the NCR. Peripheral-blood samples for both case and control subjects were collected. Informed consent of the subjects was given, and approval of the local bioethics committees was obtained.

Clinical information about these ALL cases, including age at diagnosis, gender, WBC (white blood cell) count at diagnosis and ALL subtypes (pre-B-cell ALL, T-cell ALL and undetermined lineage) is summarized in Tables 1 and 2. Among the 60 ALL cases, 70.0% were undergoing chemotherapy, 11.7% had clinical remission at the time of this study and 13.3% were in remission.

Genotyping

Genomic DNA was obtained from peripheral-blood samples of leukaemia and control cases and was extracted using the Wizard® genomic DNA purification kit (Promega). All samples were genotyped in triplicate with consistent results confirming that the genotypes have no mixture of germline and somatic mutations. GSTT1 and GSTM1 genes were amplified using multiplex PCR as described previously [22,29,47,48], with co-amplification of the albumin gene as a positive internal control. A PCR mixture containing genomic DNA without the GSTT1 and GSTM1 primers was used as a negative control. A total reaction volume of 20 μl was prepared as described previously [47] containing 10 mM Tris/HCl (pH8.3), 50 mM KCl, 3.5 mM MgCl2, 3.0 μg/ml of each GSTM1 primer and 1.0 μg/ml of each GSTT1 primer, 0.6 μg/ml of each albumin.
primer, 200 μM dNTPs, 0.5 unit of Taq DNA polymerase. Genomic DNA (100 ng/μl) from each individual was used as a template with the following primer sequences: GSTT1: 5′-TTCTTACTGTCCTCACATCTC-3′ and 5′-TCACCGGATCATGGCCAGCA-3′ [47,48]; GSTM1: 5′-GAACTCCTGAAAGCTAAGC-3′ and 5′-GTGGGCTCCTAAATATACGGTG-3′ [47,48] and albumin: 5′-GCCCTCTGCTAACAAGTCCTAC-3′ and 5′-GCCCTAAAATGAAAAATGCCCAATC-3′ as reported previously [47]. All samples were amplified in duplicate using the following PCR conditions: 95°C (1 min), 66°C (1 min), 72°C (1 min) for 40 cycles, with a final extension step of 10 min at 72°C in a PTC-200 Peltier thermal cycler (MJ Research). Genotyping for NQO1 was conducted using primers constructed previously for the PCR–CTPP (PCR confronting two-
Figure 1 Representative PCR results of DNA
Genotypes were screened for GSTT1 (459 bp) and GSTM1 (219 bp) by multiplex PCR, with albumin (350 bp) amplified as a positive internal control. The PCR results for ten patients is shown. The arrows indicate the positions of GSTT1 (459 bp), Albumin (350 bp) and GSTM1 (219 bp) genes that are amplified. The presence (+) or absence (−) of GSTT1 and GSTM1 is indicated below. We cannot distinguish between heterozygous and homozygous genotypes using the current PCR protocol. Mr, 100 bp DNA ladder marker is shown on the left-hand side (in bp); Pt, patient.

Figure 2 Diagram of the PCR method used and representative results for NQO1 (609C>T) detection
(A) The PCR–CTPP method for multiplex detection of NQO1 wild-type and mutant alleles. The method has been described previously [33]. (B) Representative PCR results for ten patients (Pt) screened for the NQO1 wild-type allele (C; 161 bp) and the mutant allele (T; 283 bp) by multiplex PCR. Only patient 7 exhibited the NQO1 C/C genotype, whereas the other patients displayed the NQO1 C/T genotype. Mr, 100 bp DNA ladder marker is shown on the left-hand side (in bp).

A comparison of each allele and the genotype frequencies for GSTT1, GSTM1 and NQO1 in between ALL cases and the control group is summarized in Table 3. Since allele frequencies may be used directly to state probabilities in observing the same genotypes in succeeding generations, we determined the allele frequencies for GSTT1, GSTM1 and NQO1. The GSTT1 null allele frequency is 0.59 and 0.58 in the ALL and normal groups respectively. Although there is no significant difference in the GSTT1 null allele frequencies, the frequency of GSTM1 null alleles in the ALL group (0.85) is significantly higher than the controls (0.72) (P < 0.05). In addition, the NQO1 wild-type allele frequency is significantly higher among ALL patients (0.80) than the control group (0.61) (P < 0.05).

In terms of the genotype incidence of GSTT1, GSTM1 and NQO1 from both the ALL cases and the control group, we found...
a high incidence of the GSTM1 null (71.7%) and NQO1 C/C (60.0%) genotypes among ALL patients (Table 3). This high GSTM1 null genotype incidence among ALL patients is significantly higher ($P < 0.05$) than the incidence in the control group (51.7%). However, the incidence of the NQO1 C/C genotype is significantly higher in ALL children ($P < 0.05$) than in the control group (23.3%). In addition, the incidence of the NQO1 C/T genotype is 40% in ALL children, compared with 75.0% in the control group. The GSTT1 null genotype in individuals with leukaemia and normal individuals, on the other hand, accounts for 35.0% and 33.3% respectively, and is consistent with the Hardy–Weinberg equilibrium. However, GSTM1 and NQO1 gene frequencies were not completely consistent, suggesting the possibility of slight sampling bias or population stratification, although in every genotyping procedure, population stratification may be present, even in well-designed studies [50].

With an observed high GSTM1 null incidence among ALL children, the risk of developing ALL was further determined and we found a 2.37-fold increase in the risk (OR = 2.37; 95% CI = 1.11–5.04) for children possessing the GSTM1 null genotype. By further determining the relative risk for NQO1, we found a significant 4.82-fold increase in the risk of developing ALL (OR = 4.82; 95% CI = 2.19–10.6) due to a NQO1 C/C genotype. Since both GSTM1 null and NQO1 C/C genotypes are associated with an increased risk of ALL, we further determined the combined effect of these two genotypes. Table 4 showed that the relative risk of developing ALL was further increased by 11.9-fold (OR = 11.9; 95% CI = 3.5–41.1) when both GSTM1 null and NQO1 C/C genotypes were combined.

**DISCUSSION**

In the present paper, we report the genotype incidence of GSTT1, GSTM1 and NQO1 genes coding for phase II detoxification enzymes in children with ALL. We found a high incidence of deletion of both GSTT1 and GSTM1 genes and the presence of the NQO1 C/C genotype among Filipino ALL paediatric patients. A gross deletion of GSTT1 and GSTM1 or a single-base substitution in the NQO1 gene leads to limited or no expression and thus a diminished function of these enzymes [18,40]. To our knowledge, this is the first report of the incidence of GSTT1, GSTM1 and NQO1 among children diagnosed with ALL in the Philippines.

The positive association we observed in the GSTM1 null genotype with the risk of ALL coincides with three independent leukaemia studies of Canadian [21], Caucasian [26] and Thai populations [23]. Also, the high GSTM1 null prevalence (71.7%) we observed in this study coincides with previous reports based on

### Table 3 Comparison of GSTT1, GSTM1 and NQO1 allele and genotype frequencies in ALL and control Filipino children

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele frequency</th>
<th>Genotype frequency</th>
<th>Control group</th>
<th>Genotype frequency</th>
<th>OR (95% CI)</th>
<th>Significance (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTT1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>0.41</td>
<td>39 (65.0)</td>
<td>0.42</td>
<td>40 (66.7)</td>
<td>1.0*</td>
<td>0.85</td>
</tr>
<tr>
<td>Null</td>
<td>0.59</td>
<td>21 (35.0)</td>
<td>0.58</td>
<td>20 (33.3)</td>
<td>1.08 (0.51–2.29)</td>
<td>–</td>
</tr>
<tr>
<td>GSTM1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>0.15</td>
<td>17 (28.3)</td>
<td>0.28</td>
<td>29 (48.3)</td>
<td>1.0*</td>
<td>0.02</td>
</tr>
<tr>
<td>Null</td>
<td>0.85</td>
<td>43 (71.7)</td>
<td>0.72</td>
<td>31 (51.7)</td>
<td>2.37 (1.11–5.04)</td>
<td>–</td>
</tr>
<tr>
<td>NQO1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>0.80 (C allele)</td>
<td>36 (60.0)</td>
<td>0.61 (C allele)</td>
<td>14 (23.3)</td>
<td>4.82 (2.18–10.6)</td>
<td>0.0002</td>
</tr>
<tr>
<td>C/T</td>
<td>0.20 (T allele)</td>
<td>24 (40.0)</td>
<td>0.39 (T allele)</td>
<td>45 (75.0)</td>
<td>1.0*</td>
<td>–</td>
</tr>
<tr>
<td>T/T</td>
<td>0.00</td>
<td>0 (0)</td>
<td>0.00</td>
<td>1 (1.67)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Value used as a reference.

### Table 4 Comparison of relative risks of ALL among the ALL case and control groups with combinations of GSTM1 and NQO1 genotypes

<table>
<thead>
<tr>
<th>Genotype combinations</th>
<th>ALL group</th>
<th>Control group</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTM1 positive + NQO1 C/T</td>
<td>6 (10.0)</td>
<td>20 (33.3)</td>
<td>1.0*</td>
</tr>
<tr>
<td>GSTM1 positive + NQO1 C/C</td>
<td>11 (18.3)</td>
<td>7 (11.7)</td>
<td>5.24 (1.41–19.52)</td>
</tr>
<tr>
<td>GSTM1 null + NQO1 C/T</td>
<td>18 (30.0)</td>
<td>26 (43.3)</td>
<td>2.31 (0.77–6.88)</td>
</tr>
<tr>
<td>GSTM1 null + NQO1 C/C</td>
<td>25 (41.7)</td>
<td>7 (11.7)</td>
<td>11.9 (3.45–41.09)</td>
</tr>
</tbody>
</table>

* Value used as a reference.
Canadian (64.9%) [21] and Black patients (41.2%) [22]; however, the GSTM1 null incidence among Filipinos is slightly elevated compared with these two ethnicities. Furthermore, the GSTM1 null genotype frequency from other ethnicities with various malignancies (40.0%–64.0%) [19,25,26,29] is similarly prevalent.

Although a 2.37-fold risk of ALL is associated with the GSTM1 null genotype, we found that the GSTT1 null genotype was not associated with ALL among Filipinos. Our finding is similar with association studies reported previously with ALL in the U.S.A. [20,21,22] and Thailand [23], and with other cancer types such as AML (acute myeloid leukemia) [25,26], cervical [28] and lung cancers [19].

This association of the GSTM1 null genotype with ALL risk and not the GSTT1 null genotype can probably be explained by differences in catalytic properties among GST subtypes. Although GSTT1 and GSTM1 have overlapping substrate specificity, these enzymes exhibit different catalytic activities [18]. For instance, deletion of the GSTM1 gene results in an increase in the DNA-damaging effect of TSO (trans-stilbene oxide) in cultured human lymphocytes, but deletion of GSTT1 has no effect [51]. Other potential carcinogens which are specific substrates for GSTM1 and not GSTT1 may be present in the environment thus increasing the risk of leukaemogenesis in individuals with the GSTM1 null genotype.

Similar to our observation on the association of the GSTM1 null genotype with ALL risk, we found a positive association of the NQO1 C/C genotype with ALL. However, two previous reports on ALL observed the opposite results [43,52]. Krajinovic et al. [43] and Smith et al. [52] reported a positive association of the NQO1 C/T genotype with ALL. In contrast, other reports support our results with NQO1 genotype analysis. For example, a positive association of the NQO1 C/C genotype with CML (chronic myelogenous leukemia) [27] and lung cancer [29,53] was reported. In addition, another study [25] showed a negative association of the NQO1 C/T genotype with de novo AML risk. Although we do not currently know the reason, it is possible that these apparent discrepancies might reflect differences in the chemical carcinogens involved in leukaemogenesis in different countries. Furthermore, the association of the NQO1 C/C genotype with the risk of developing ALL may be attributable to the function of the enzyme in activating carcinogens aside from detoxification. A review by Ross et al. [36] postulated that some endogenous metabolites can be transformed by NQO1 to yield more active products that can produce reactive oxygen or alkylating species, thus attacking nucleophilic sites within essential biomolecules such as DNA. An example of this is the reduction and activation of nitro compounds found in cooked foods such as 4NNO (4-nitroquinoline-1-oxide) [36,54].

We also determined the possible enhanced risk of ALL with a combination of the GSTM1 null and NQO1 C/C genotypes, and observed that ALL susceptibility was enhanced by a combination of these genotypes. Although the extent of increased risk was expected, our findings support the idea that GSTM1 and NQO1 genotypes both contribute to ALL independently. The positive association of the combined GSTM1 null and NQO1 C/C genotypes with cervical cancer in Japan was reported previously [28].

However, no such previous genetic analysis of ALL has been reported.

From our findings, we therefore consider that the GSTM1 null and NQO1 C/C genotype combination may be likely to play a role in ALL leukaemogenesis among Filipino children. In addition, considering that NQO1 has the potential to activate probable substrates into carcinogens [36], including GSTT1, which is capable of transforming some substrates such as ethylene dibromide and halogenated methanes into mutagenic electrophiles [19], it is possible that the putative and so far unidentified carcinogen(s) involved may be activated by NQO1 and detoxified by GSTM1.

Further studies in combination with other genes for detoxification or DNA repair is recommended, for instance, CYP1A1, a phase 1 enzyme which, in combination with the GSTM1 null genotype, is known to be associated with an increased risk of ALL [26]. Similarly, CYP2E1 (cytochrome P450 2E1) and MPO (myeloperoxidase) in combination with NQO1 further increase the risk of malignancies [48]. It would be thus interesting to investigate combinations of phase 1 and phase 2 groups of enzymes, since the former are generally the first enzymatic defence against foreign compounds [55] and can generate more reactive molecules that are further metabolized by GSTs and NQO1.

In conclusion, we clarified the genetic contribution of GSTM1 null and NQO1 C/C genotypes among children living in the Philippines to the risk of increased ALL leukaemogenesis (11.9-fold). Although we still have not identified the carcinogenic substance involved, we assume that metabolic activation of potential carcinogen(s) might play a role. From this baseline information, we hope to stimulate further studies on ALL risk factors, particularly to investigate the influence of environmental exposure that can contribute to the awareness, preventive and management measures of ALL in the Philippines.

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