Glutathione S-transferases Null Genotype in Acute Myeloid Leukaemia

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ABSTRACT

Background: The glutathione S-transferase (GST) family of metabolising enzymes plays an important role in the detoxification of mutagens and carcinogens. The expression of many of these cancer susceptibility enzymes is genetically polymorphic. An increased frequency of GST-null genotypes has been associated with several malignancies.

Objective: To investigate the rate of GSTT1 and GSTM1 null genotypes in AML patients and to determine its importance in prognosis of the disease.

Methods: DNA was extracted by phenol/chloroform method from peripheral blood or bone marrow of 180 white Caucasian patients. A multiplex PCR method was used simultaneously to amplify regions of GSTM1, GSTT1, and β-globin genes in genomic DNA. The survival curves were analyzed by the Kaplan-Meier method and compared by the log-rank test (Mantel-Cox) using the SPSS software program.

Results: Of the total of 180 patients, 23 cases (12.8%) showed null genotypes in both genes, while in 52 patients (28.9%) both genes were wild-types. GSTM1 null-GSTT1 wild-type was detected in 91 patients (50.6%) and GSTM1 wild-type-GSTT1 null genotype was detected in 14 patients (7.8%). These rates are within the upper limit of the rates detected in the normal European population. There was no significant difference in the overall survival and in disease free survival between different groups.

Conclusion: These observations suggest that the inherited absence of the GSTT1 and GSTM1 carcinogen detoxification pathway may be related to carcinogenesis but it is not an important determinant of prognosis in AML.

Keywords: Glutathione S-Transferase, AML, Null Genotype

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INTRODUCTION

It has been known for several decades that those individuals who lack specific DNA repair genes are highly predisposed to cancer (1). In fact, the genome depends on mechanisms beyond DNA repair to protect its integrity. These protective mechanisms include processes for neutralizing or detoxifying mutagens that enter our bodies from outside or are generated as the products of normal metabolism. Cancer susceptibility genes are involved in encoding enzymes involved in the metabolism of carcinogens or environmental toxins (2). Any alteration in the activity of these enzymes would result in an altered susceptibility to cancer. The expression of many of these cancer susceptibility enzymes is genetically polymorphic and such polymorphisms may be related to an increased risk of cancer in some individuals.

The glutathione S-transferase (GST) family of metabolizing enzymes plays an important role in the metabolism and detoxification of mutagens and carcinogens. GST genes encode a family of phase II enzymes (molecular mass 17-28 kD) that have major roles in catalyzing the conjugation of glutathione to a wide variety of hydrophobic and electrophilic substrates and carcinogens such as benzo(a)pyrene (1) and reactive oxygen species (ROS) (3). GST levels can be induced by exposure to foreign substrates in vivo suggesting that they form part of a system adaptive to chemical stress (4).

Multiple isoforms of GST have been identified in various tissues, and some of them appear to be expressed at different developmental stages. There are four classes of dimeric cytosolic GSTs; α (GSTA), µ (GSTM), π (GSTP) and θ (GSTT). Members of the same family show a minimum of 65% amino acid sequence identity (5). Although polymorphic loci have been identified in each of the four classes of GST gene families, most interest in the possible consequences of GST polymorphism has focused on the polymorphisms at the GSTM1 and GSTT1 gene loci, which have been located on chromosome 22. GSTM1 isozymes catalyze the detoxification of a wide range of reactive toxic and mutagenic compounds. It is therefore reasonable to speculate that individuals homozygous for GSTM1 null genotype will be associated with an increased susceptibility to cancer and/or inflammatory pathologies. GSTT1 is also an attractive candidate gene as a susceptibility factor in cancer. It metabolizes various potential carcinogens such as monohalomethanes (e.g. methyl chloride) and ethylene oxide, present in cigarette smoke and ubiquitously used as methylating agents, pesticides and solvents in industry (6).

The incidence of null genotype for GSTT1 and GSTM1 is different in different racial groups. The GSTM1 null genotype has a frequency within the human population of nearly 50% in Caucasian whites (7), and 20-48.9% in the other ethnic groups (8-11). While the incidence of the GSTT1 null genotype is 10-20% in Caucasian whites (7), and 16.8-25.7% in the other ethnic groups (8, 9, 11).

However, these loci are not linked because individuals who are GSTT1-null are not necessarily GSTM1-null, and vice versa. Furthermore, individuals who are both GSTT1-null and GSTM1-null may be at heightened risk because they lack both enzymes (12), as it was shown in the cases with gastric cancer (13).

An increased frequency of GST-null genotypes has been associated with several malignancies, including smoking-related cancer (14, 15), lung cancer in lifetime non-smokers (16), stomach cancer (17), bladder cancer (18), colorectal cancer (19), astrocytoma (20), and esophageal squamous cell cancer (21). The influence of GST on susceptibility to cancer may be influenced by a variety of factors such as smoking,
diet and gender (22). The GSTT1-null genotype has been associated with an increased risk of lung cancer in non-smokers (23) while the GSTM1-null genotype has been associated with an increased risk of smoking-induced lung and bladder cancers (24-26). In hematological malignancies, an association has been shown between GST-null genotype and an increased risk of developing acute leukemia, especially acute lymphoblastic leukemia (ALL) (4), acute myeloid leukemia (AML) (27) and myelodysplastic syndrome (MDS) (28). A similar finding was observed in the group of chronic lymphoblastic leukemia (CLL) patients (29) and in male patients who developed myeloid malignancies as compared with male controls (30). However, some other studies have failed to confirm these findings (31-36). More investigation seems necessary on the effect of environmental factors in these groups, as it is possible that the malignant patients which are under more environmental carcinogenesis influence, show a higher rate of GST-null genotypes than the patients with less exposure to these agents.

The present study was conducted to investigate the rate of GSTT1 and GSTM1 null genotypes in AML patients and its importance in prognosis of the disease.

MATERIALS AND METHODS

GSTT1 and the GSTM1 null genotypes were investigated by a multiplex PCR in 180 AML patients attending the University Department of Hematology, Manchester Royal Infirmary, Manchester, UK. Of the total of 180 patients, 106 were females, and 74 were males. There were 25 s-AML and 155 de novo AML patients. Data of FAB types were collected for 174 of the AML patients studied; these data were not available in the remaining 6 patients. Based on FAB classification, 7 M0, 31 M1, 60 M2, 21 M3, 29 M4, 16 M5, 9 M6 and 1 M7 were included in this study.

DNA was extracted by phenol/chloroform method from the mononuclear cell (MNC) samples separated from peripheral blood (PB) or bone marrow (BM) of the patients. Two to 10 mL PB and 1-5 mL BM samples were collected from the patients, at presentation time, into an EDTA (1.5 mg/mL, pH 8.0) tube. Mononuclear cells (MNC) were obtained by gradient density separation of these PB or BM on Ficoll-Hypaque. A multiplex PCR method was used simultaneously to amplify regions of GSTM1, GSTT1, and β-globin genes in genomic DNA. PCR amplification was performed on 100 ng of genomic DNA, in a reaction mixture with the total volume of 30 µL that contained 2mM MgCl2 and 12.5 pM each of the forward and reverse GSTT1, GSTM1, and β-globin primers (Table 1) (33). The reaction was carried out, using 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min. The PCR product was run in 2% (w/v) agarose gel and the bands were visualized with UV light. DNA from patients with positive GSTM1, GSTT1, and β-globin alleles yielded 219bp, 480bp, and 268bp products, respectively.

The absence of GSTM1 or GSTT1 (in the presence of β-globin PCR product) indicates the respective null genotype for each (Figure 1). Samples positive for all three PCR products were considered ‘wild-type’. Co-amplification of human β-globin served as a positive control, to ensure that a null genotype was attributed to the absence of the respective gene and not because of a PCR failure (36).
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Table 1. Primer sequences for GST multiplex PCR

<table>
<thead>
<tr>
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<tr>
<td>GSTM1</td>
<td>F-GAA CTC CCT GAA AGG CTA AAG G</td>
<td>2401-2422</td>
<td>X68676</td>
</tr>
<tr>
<td></td>
<td>R-GTT GGG CTC AAA TAT ACG GTG G</td>
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<td>X79389</td>
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<tr>
<td></td>
<td>R-TCA CGG AT CAT GGC CAG CA</td>
<td>704-723</td>
<td></td>
</tr>
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<td>β-globin</td>
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<td>61992-62011</td>
<td>U01317</td>
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<tr>
<td></td>
<td>R-CCA CTT CAT CCA GGT TAC</td>
<td>62240-62259</td>
<td></td>
</tr>
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</table>

F: forward primer, R: reverse primer, Ann. seq.: nucleotide numbers for annealing with, Acc. No.: GeneBank accession no.

Figure 1. Multiplex PCR products of GSTT1, GSTM1 and β-globin. A 2% (w/v) agarose gel showing the PCR product. DNA from patients with positive GSTM1, GSTT1, and β-globin alleles yielded 219bp, 480bp, and 268bp products respectively. The absence of GSTM1 or GSTT1 (in the presence of β-globin PCR product) indicates the respective null genotype for each (3, double null; 5, GSTM1 null; 6, GSTT1 null). Samples positive for all three PCR products were considered ‘wild-type’ (2 & 4). Lane 1 is negative control and L is Molecular weight marker.

A number of samples were chosen randomly for a single gene PCR amplification of GSTT1 or GSTM1 to confirm the findings of the multiplex PCR.

The GSTT1 primers used in this multiplex PCR amplified the last exons of its transcript. To distinguish between possible partial deletion and complete deletion of GSTT1, another PCR protocol was designed to amplify the upstream region that covers part of exons 1 and 2 of the GSTT1 gene. The PCR was done on 250 ng of genomic DNA in a total volume of 30 µL containing 2 mM MgCl2 and 15 pM of each GSTT1 primers (F: 5’ AGCTCTACCTGGACCTGCTG 3’ and R: 5’ TTAATCAGATC-CACGATGCG 3’) plus β-globin primers. PCR amplification was performed at 30 cycles of 94°C for 40 sec, 62°C for 40 sec and 72°C for 1 min. PCR products were electrophoresed on a 2.5% (w/v) agarose gel. PCR amplification produced 96 bp and 268 bp fragments for GSTT1 and β-globin, respectively (Figure 2).

Overall survival was calculated from the date of presentation to the date of death or last follow-up. Median overall survival was 19.3 months (range 0.1-193.3) in these patients. Disease free survival (DFS) was calculated for the patients who achieved complete remission (CR), and was measured from the date of CR to the date of relapse, death or last follow-up. Of 180 AML patients, 29 (16.1%) achieved no remission. Median DFS was 14.2 months (range 0.4-192.5) in 151 AML patients who achieved remission.
Figure 2. PCR products of GSTT1 exon 1 and 2 were electrophoresed on a 2.5% (w/v) agarose gel. PCR amplification produced 96 bp and 268 bp fragments for GSTT1 and β-globin, respectively. The first series (GSTT1+) showing both bands are positive for GSTT1 exon 1 and 2. The second series showing bands only for β-globin and not for GSTT1 are negative for GSTT1 exon 1 and 2.

The survival curves were analyzed by the Kaplan-Meier method (37) and compared by the log-rank test (Mantel-Cox) using the SPSS software program version 10.1; p values less than 0.05 were considered significant.

RESULTS

For statistical analysis, our findings were categorized into 4 genotypes: (1) GSTM1 and GSTT1 wild-type, (2) GSTM1 null-GSTT1 wild-type, (3) GSTM1 wild-type-GSTT1 null, and (4) GSTM1 and GSTT1 null (30). Of the total of 180 patients, 23 cases (12.8%) showed null genotypes in both genes, while in 52 patients (28.9%) both genes were wild-types. GSTM1 null-GSTT1 wild-type was detected in 91 patients (50.6%) and GSTM1 wild-type-GSTT1 null genotype was detected in 14 patients (7.8%). Among the 25 s-AML patients, both genes were wild-types in 10 patients (40%), but only 2 cases (8%) showed null genotypes in both genes. In addition, GSTM1 null-GSTT1 wild-type was detected in 12 patients (48%) while GSTM1 wild-type-GSTT1 null genotype was detected only in one patient (4%).

Of the 52 patients in the first group (GSTM1 and GSTT1 wild-type), 16 were male and 36 were female. The FAB classification was not known in 2 of them and in the rest the rate was as follows: no M0, 11 M1, 17 M2, 9 M3, 8 M4, 3 M5 and 2 M6. The overall survival was 24 months (0.1-176.5). There was no CR in nine (17.3%) of these patients and DFS was 21.4 months (0.4-175.5) in the rest.

In the second group (GSTM1 null-GSTT1 wild-type), of the 91 patients 45 were male and 46 were female. The FAB classification was not known in two of them and in the rest the rate was as follows: 6 M0, 11 M1, 32 M2, 7 M3, 15 M4, 10 M5, 7 M6 and 1 M7. The overall survival was 15.3 months (0.1-193.5). There was no CR in 17 (18.7%) of these patients and DFS was 9.9 months (0.4-192.5) in the rest.

In the third group (GSTM1 wild-type-GSTT1 null), of the 14 patients 4 were male and 10 were female. The FAB classification was as follows: no M0, 2 M1, 7 M2, 2 M3, 2 M4 and 1 M5. The overall survival was 22.2 months (8.4-171.1). All of the patients in this group achieved CR and DFS was 11.6 months (1.5-170.1) in them.
Finally, in the last group (\textit{GSTM1} and \textit{GSTT1} null), of the 23 patients 9 were male and 14 were female. The FAB classification was not known in 2 of them and in the rest the rate was as follows: 1 M0, 7 M1, 4 M2, 3 M3, 4 M4 and 2 M5. The overall survival was 26.6 months (0.1-165). Three (13\%) of these patients did not achieve CR and DFS was 14.6 months (0.4-164) in the rest.

<table>
<thead>
<tr>
<th>\textit{GSTT1} null</th>
<th>\textit{GSTM1} null</th>
<th>Both null</th>
<th>Both WT</th>
</tr>
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<tbody>
<tr>
<td>OS (months)</td>
<td>22.2</td>
<td>15.3</td>
<td>26.6</td>
</tr>
<tr>
<td>(range)</td>
<td>(8.4-171.1)</td>
<td>(0.1-193.5)</td>
<td>(0.1-165)</td>
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<tr>
<td>DFS (months)</td>
<td>11.6</td>
<td>9.9</td>
<td>14.6</td>
</tr>
<tr>
<td>(range)</td>
<td>(1.5-170.1)</td>
<td>(0.4-192.5)</td>
<td>(0.4-164)</td>
</tr>
</tbody>
</table>

WT: Wild type, OS: overall survival, DFS: disease free survival

Survival rates in different groups are shown in table 2. The overall survival and DFS in patients were analyzed by the Kaplan-Meier method and compared in different categories using the log-rank test; p values less than 0.05

\textbf{Figure 3.} Kaplan-Meier survival plot for AML patients with different \textit{GST} genotypes. 1; \textit{GSTM1} and \textit{GSTT1} wild-type is shown by the broken line, 2; \textit{GSTM1} null-\textit{GSTT1} wild-type, by the dotted line, 3; \textit{GSTM1} wild-type-\textit{GSTT1} null, by the solid line and 4; \textit{GSTM1} and \textit{GSTT1} null by the bold line.

The overall survival was 24 months (0.1-176.5) in the first group, 15.3 months (0.1-193.5) in the second group, 22.2 months (8.4-171.1) in the third group and 26.6 months (0.1-165) in the fourth group.

Survival rates in different groups are shown in table 2. The overall survival and DFS in patients were analyzed by the Kaplan-Meier method and compared in different categories using the log-rank test; p values less than 0.05
were considered significant. The results showed that there was no significant difference ($p=0.1661$) in the overall survival (Figure 3) and in DFS ($p=0.2615$) between the different groups (Figure 4).

![Figure 4. Kaplan-Meier disease free survival plot for AML patients with different GST genotypes.](image)

**DISCUSSION**

There are a complex variety of mechanisms in humans by which they protect themselves from insult by environmental agents such as ultraviolet (UV) light, inhaled cigarette smoke and incompletely-defined dietary factors. Human beings vary in their ability to metabolize carcinogens and people with diminished ability to detoxify chemicals may be at increased risk of cancer. It has been suggested that glutathione may be important in antitumorogenesis as a free-radical trap; also it may confer resistance to chemotherapeutic drugs and may have diagnostic value as tumor markers (38). Glutathione S-transferases (GST) protect the cells against toxicants by conjugating them to glutathione. The glutathione adducts are often less toxic and generally have greater water solubility than the free compounds, which facilitates their removal from the cell. Humans are polymorphic in their ability to detoxify such intermediates, which in theory may explain the differences in risk of leukemia as a result of exogenous exposures. The $GSTT1$ and $GSTM1$ exhibit genetic polymorphism in their population distribution, with a large percentage of individuals displaying a homozygous deletion of the structural genes or the “null” genotype. This deletion could be detected by PCR-based tests of somatic cell DNA. It was shown that in individuals with $GSTT1$ null and $GSTM1$ null genotypes there is no enzymatic functional activity of the
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respective enzyme (39, 40). Genes coding for the GSTM1 and GSTT1 proteins are absent or homozygous null, in 10-60% of different ethnic populations (8, 33, 41, 42). In a study by Nelson et al. (43) there were major differences in the prevalence of GSTT null genotype attributable to ethnicity. Therefore, they suggested that ethnic origin even among Caucasians should be considered in studies of GST genes. The information on the frequency of GSTM1 null and GSTT1 null genotypes in different populations and ethnic groups was summarized in a review by Cotton et al. (44). This review was later updated by Geisler & Olshan (45). Based on these reviews the frequency of the GSTM1 null genotype ranges from 23%-48% in African populations, from 33%-63% in Asian populations and 39%-62% in European populations. The frequency of the GSTT1 null genotype ranges from 15%-26% in African populations, from 16%-64% in Asian populations and 10%-21% in European populations. Several investigations have found a higher rate of the GSTM1 and GSTT1 genes null genotypes in the patients with such cancers, as carcinoma of lung, bladder, gastrointestinal tract, cervix, and breast (26, 41, 46-49). Several studies have investigated the frequencies of GSTM1 null and GSTT1 null genotypes in hematological malignancies such as MDS (28, 31) and acute leukemia (50, 30, 51), but no consistent conclusions have been established. In a systematic review and meta-analysis of 30 published case-control studies, it was suggested that GSTM1 and GSTT1 polymorphisms, appear to be associated with a modest increase in the risk of ALL (52). In another study, it was also suggested that GSTM1 null genotype might be a risk genotype of childhood ALL (53).

The objectives of this study explained here were, to investigate the frequency of GST null genotypes in AML patients and to calculate the prognostic value of these null genotypes. The hypothesis tested was that individuals with an inherited homozygous deletion of the GSTT1 or GSTM1 gene are at increased risk of having prognostically worse AML. For this investigation, the rates of GSTM1 null, GSTT1 null and both genes null genotypes were tested in 180 patients. All patients were white Caucasians. No controls from normal individuals were chosen because it was too difficult to match exactly the ethnic groups of controls with those of the patients. Furthermore, to determine whether a GST null genotype is related to the prognosis of the patients, a study was conducted in which the median overall survival and DFS was compared among different groups of AML patients. If specific genetic polymorphisms can identify those patients with AML who have a shorter survival, it is possible that therapy could be tailored to improve the prognoses of such patients.

In the total of 180 patients studied, the rate of the ‘double genes null’ genotype was 12.8% (23/180), while in 28.9% of patients (52/180) both genes were wild types. GSTM1 null genotype was detected in 63.3% of patients (114/180), while the null genotype rate of GSTT1 gene was 20.6% (37/180). These rates are within the upper limit of the rates detected in the normal European population (39-62% for GSTM1 and 10-21% for GSTT1) (45). There was no significant difference in the rate of different GST null genotypes between de novo and s-AML patients. In the total of 155 de novo AML patients, 21 (13.5%) were double GST null genotype, while in 25 s-AML patients this rate was 8% (2/25). The rate of both gene wild-type in de novo and s-AML patients was 27.1% (42/155) and 40% (10/25) respectively. GSTM1 gene was deleted in 64.5% (100/155) of de novo and 56% (14/25) of s-AML patients while GSTT1 null genotype was detected in 21.9% (34/155) of de novo and 12% (3/25) of s-AML patients.
Furthermore we decided to assess if the deletions detected in this study are complete or partial. The GSTT1 primers used in the multiplex PCR amplified parts of exon 4 and 5, the last exons of its transcript. Therefore, another PCR protocol was designed to amplify the upstream region that covers part of exons 1 and 2 of the GSTT1 gene, to distinguish between possible partial deletion and complete deletion of GSTT1. This study showed that all of the detected GST null genotypes were complete deletions.

Of the total of 180 patients, 74 were male and 106 were female. The rate of patients with no deletion detected (both genes were of wild type) was higher (34%) in females (36/106) than in males [21.6% (16/74)]. Similarly, the rate of the GSTT1 null genotype was higher (22.6%) in females (24/106) than in males [17.6% (13/74)]. While, the GSTM1 null genotype was detected more (73%) in males (53/74) than in females [56.6% (60/106)]. Finally, the rate of the ‘double null’ genotype was similar in males [12.2% (9/74)] and in females [13.2% (14/106)]. Whether these findings indicate that males and females differ in the mechanisms underlying their risk of myeloid malignancies, or is due to multiple testing, selection bias, or the relatively small number of patients, is not clear. However, these data suggest that caution should be used before males and females are pooled for analyses of GST null genotypes as a predictor of disease risk.

The AML patients who were ‘GSTM1 null-GSTT1 wild-type’ showed the shortest overall survival [15.3 months (0.1-193.5)] when compared with the other groups, but this was not statistically significant. The overall survival in the other groups was 24 months (0.1-176.5) for patients with both genes of the wild-types, 22.2 months (8.4-171.1) for ‘GSTM1 wild-type-GSTT1 null’ and 26.6 months (0.1-165) for ‘double null genotype’ patients. The same results were observed for D.F.S. in these patients. D.F.S. was 9.9 months (0.4-192.5) for the ‘GSTM1 null-GSTT1 wild-type’ group, while this was 21.4 months (0.4-175.5) for the ‘GSTM1 and GSTT1 wild-type’ group, 11.6 months (1.5-170.1) for the ‘GSTM1 wild-type-GSTT1 null’ group and 14.6 months (0.4-164) in the both null, group (Table 2). These observations of no significant differences in the prognoses of different GST null genotype groups, suggest that the inherited absence of the GSTT1 and GSTM1 carcinogen detoxification pathway is not an important determinant of prognosis in AML.

In this study the information on specific environmental exposure was not obtainable and, therefore, interaction between GSTT1 or GSTM1 and known or potential GST substrates, present in the environment, could not be assessed. If a true association exists between GST and AML, it would be detected more easily by examining sub-populations that are known to have been exposed to GST substrates. Finally, to our knowledge, there is no study on the rate of GST deletion in Iranian population, therefore it would be interesting to conduct such a study and compare the results with the other studies such as the present study on Caucasian population.

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