ABSTRACT

Background and Purpose: Heterogeneity in patient’s response to chemotherapy is consistently observed across populations. Pharmacogenomics, the study of inherited differences in drug disposition and effects, is emerging as a tool to predict efficacy and toxicity of drugs. Glutathione S-transferases (GST) are involved in the metabolism and detoxification of environmental carcinogens and some classes of chemotherapeutics. Polymorphism of GSTM1 and GSTT1, in the form of homozygous deletion, is encountered in varying frequencies in normal population. It has been associated with altered response and toxicity from cytotoxic chemotherapy. In this study, we investigated the impact of these polymorphisms on response and side effects of chemotherapy in adult acute myeloid leukaemia (AML) patients. Correlations between these genetic polymorphisms and other prognostic factors were also investigated.

Patients and Methods: We genotyped GSTM1 and GSTT1 in 98 adult AML patients using multiplex PCR. Induction therapy included Doxorubicin and Cytosine arabinoside (3+7) regimen. Treatment outcomes were compared in those with or without GSTM1 and GSTT1 genes.

Results: The frequencies of GSTM1 null and GSTT1 null genotypes were 56% and 14%, respectively. Six percent (6%) were double null. The rate of toxic death during induction was 3/7 (43%) and 17/56 (30%) in GSTT1 null and GSTT1 present patients, respectively, p=0.67. This constituted 75% and 42% of total deaths in each group, respectively, p=0.31. Differences were not statistically significant. On the other hand, the rate of complete remission (CR) in patients with GSTM1 present compared to those with GSTM1 null genotype was 12/27 (48%) versus 23/36 (64%), p=0.21. GSTT1 null genotype was significantly associated with lymphoid marker (mainly CD7) expression (p=0.03), known with its adverse effect on prognosis. Overall survival and disease-free survival were similar in patients with and without the genes. No significant associations were encountered between GST genotypes and treatment outcomes.

Conclusion: Our data suggest possible association, though not significant, between GSTT1 null genotype and toxic death during induction and between GSTM1 present genotype and lower rate of CR. Studies on larger numbers are needed focusing on selection of anticancer agents to avoid adverse reactions and therapeutic failure, with special emphasis on drug toxicity and dose adjustment.

Key Words: Glutathione S-transferase - Polymorphism - Acute myeloid leukaemia - Toxicity.
50% and 15% of most populations have a homozygous deletion of GSTM1 and GSTT1, respectively [6]. Individuals vary in their ability to metabolize several DNA-damaging agents because of polymorphism of such detoxifying enzymes. The GSTM1 and GSTT1 null alleles have been associated with altered response and toxicity from chemotherapy in patients with acute leukemias, breast cancer and metastatic colorectal cancer [7-10].

Acute myeloid leukaemia (AML) is a heterogeneous clonal disorder of haematopoietic progenitor cells [11]. Evidence indicates that GST expression plays an important role in determining cytotoxicity of chemotherapeutic drugs including anthracyclines and alkylating agents used in acute myeloid leukemia [12,13]. Impaired detoxification of chemotherapeutic agents could also contribute to increased DNA damage and subsequent development of second neoplasms. The GSTM1 and GSTT1 null alleles have been associated with increased risk of development of a variety of solid tumours such as smoking-induced lung cancer, bladder, breast and gastrointestinal stinal cancer and malignant haematologic diseases such as MDS and acute leukemias [14-19].

In this study we investigate the impact of genetic polymorphism of GSTM1 and GSTT1 on response and toxicity from chemotherapy in adult AML patients. We also evaluate the prognostic significance of genetic polymorphism of both genes and its correlation with other prognostic factors.

**PATIENTS AND METHODS**

Ninety-eight newly diagnosed adult acute myeloid leukaemia (AML) patients who presented to the Medical Oncology Department of the National Cancer Institute, Cairo University, in the period from November 2003 to June 2005 were enrolled in this study. Written consent was obtained from the patients and the protocol was approved by the Institution Research Board. The diagnosis of AML was established according to the morphologic and cytochemical criteria of the FAB classification and immunophenotyping (IPT). They included 54 males and 44 females. Evaluated clinical data included age, gender, total leucocytic count (TLC) and blast percent at diagnosis, and the presence of previous cancer or myelodysplastic syndrome (MDS). DNA was extracted from the peripheral blood or bone marrow using the salting out technique [20]. A multiplex PCR assay was used for simultaneous amplification of GSTT1 and GSTM1 genes [7]. Coamplification of BCL2 served as internal control. A negative control containing no DNA template was included. The PCR was performed in 50µl reaction mixture, containing 100ng of genomic DNA, 1.5mM MgCl2, 200µM each dNTP, 2.5U Taq polymerase (Promega, Madison WI, USA) and 10pmole of each primer (Pharmacia biotech) (Table 1). The PCR conditions consisted of 35 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute and extension at 72°C for 1 minute using the Gene Amp 2400 thermal cycler (Perkin Elmer, USA). This results in a fragment of 480bp for GSTT1, 219bp for GSTM1 and 154bp for BCL2. The genotype assignment was made by electrophilic analysis of the amplified product and visualization in 2% agarose gel stained with ethidium bromide.

Complete remission (CR) was defined as less than 5% blasts in bone marrow. Toxic death during induction was defined as death occurring after the start of treatment and before bone marrow evaluation on day +28. Disease free survival (DFS) was defined as the time from the end of induction of CR to relapse or death. Overall survival (OS) was calculated from first day of therapy to death.

**Protocol of treatment:**

**Treatment and follow-up:**

Patients were treated according to (3+7) regimen, combining Doxorubicin 45mg/m² IV on days 1 to 3 and Cytosine arabinoside 100mg/m² by continuous infusion from day 1 to day 7 for induction.

Evaluation of response has been done after 2-3 weeks. Complete remission (CR) was defined as cellular marrow with less than 5% blasts, no circulating blasts, no evidence of extramedullary leukaemia and recovery of granulocytes ≥1.5x10⁹/L and platelets ≥100x10⁹/L. If the marrow was not hypocellular and there was unequivocal residual leukaemia, a second course of therapy with similar doses was administered.
After achievement of CR, consolidation chemotherapy was carried out by HAM regimen, consisting of Cytosine arabinoside 3g/m$^2$ from day 1 to day 3 by infusion over 3 hours and Mitoxantrone 12mg/m$^2$ from day 3 to day 5 by short infusion.

**Supportive care:**

Blood component transfusion was given to keep the haemoglobin level at 8g/dl or higher. Therapeutic platelet transfusion was given to patients with bleeding manifestations and thrombocytopenia. Prophylactic platelet transfusion was given when platelet counts $<10 \times 10^9$/L or at a higher level if patients had complications or were planned for invasive procedure.

Evaluation and management of infection was applied according to the rules recommended for infection management in the immunocompromised patients [21] and according to the ongoing institutional protocols.

**Statistical analysis:** Statistical package for social sciences (SPSS) version 12.0 was used for data analysis. Quantitative variables were summarized using median (range). Qualitative data were summarized using frequencies and percentages. Non-parametric t-test compared means of 2 independent groups. Chi-square and Fisher exact tested proportion independence. Kaplan-Meier method was used to estimate survival and Log rank test to compare curves. $p$ value was significant at $\leq 0.05$ level.

**RESULTS**

The frequency of GSTT1 and GSTM1 null genotypes was 56% and 14%, respectively. Six percent were double null and 35.7% were double positive. Fig. (1) shows GST genotypes.

No association between GSTT1 and GSTM1 null genotypes and other clinical prognostic factors for AML such as age, total leucocytic count and blast percentage at diagnosis and FAB subtype was encountered (Table 2). However, the frequency of patients over 60 years in GSTT1 null compared to GSTT1 present patients was 27% versus 13% ($p=0.22$). With regards to immunothenotyping, the GSTT1 null genotype showed significant association with lymphoid marker expression (mainly CD7) ($p=0.03$) and near significant association with DR expression ($p=0.07$), while GSTM1 present genotype showed near significant association with CD34 expression ($p=0.08$). We also analyzed the distribution of GST genotypes according to history of previous cancer; 2 patients with GSTM1 null, 1 with GSTT1 null and 1 with double positive genotypes were secondary AML.

The median duration for follow-up was 4.75 months ranging from 1 week to 35 months. Clinical outcome and follow-up of the 63 evaluable patients are shown in Table (3). The rate of toxic death during induction in GSTT null compared to GSTT1 present patients was 43% versus 30%, $p=0.67$. The rate of toxic death during induction among total deaths in each group was 3/4 (75%) versus 17/41 (42%), $p=0.31$, in GSTT1 null compared to GSTT1 present patients, respectively. The differences were not statistically significant. The main causes of death were infection and bleeding. On the other hand, no difference in the rate of toxic death was observed in GSTM1 null compared to GSTM1 present patients, 31% versus 33%, constituting 46% and 45% of total deaths in each group, respectively. The rate of complete remission (CR) in GSTM1 null compared to GSTM1 present patients was 64% versus 48%, $p=0.21$, yet the difference was not statistically significant. A similar rate of complete remission of 57.1% was observed in GSTT1 null and GSTT1 present patients. The median disease free survival (DFS) was 8 months; survival estimate at first and second years was 31.3% and 14.9%, respectively (Fig. 2). The DFS was comparable in the groups with and without the deletion for both genes (Figs. 3,4). The median overall survival (OS) was 6 months; survival estimate at first and second years was 26.2% and 14.2%, respectively (Fig. 5). No statistically significant difference was encountered between the different groups (Figs. 6,7).

<table>
<thead>
<tr>
<th>Table (1): Sequence of primers.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTM1 5’-GAACCTC CCTGAAAAGCTAAAGC-3’</td>
</tr>
<tr>
<td>5’-GTTGGGCTCAAATATACCGTGG-3’</td>
</tr>
<tr>
<td>GSTT1 5’-TCCTTACTGTCCTCCTACATCTA-3’</td>
</tr>
<tr>
<td>5’-TCACCGGATCATGGCCAGCA-3’</td>
</tr>
<tr>
<td>BCL2 5’-GCAAATTCGCAATTAATTCATGG-3’</td>
</tr>
<tr>
<td>5’-GAAACAGGCCACCGTAAAGCA-3’</td>
</tr>
</tbody>
</table>
Glutathione S-Transferase GSTM1 & GSTT1 Polymorphisms

Table (2): Clinical characteristics of patients according to GSTM1 and GSTT1 genotypes.

<table>
<thead>
<tr>
<th>Age*</th>
<th>Male</th>
<th>Female</th>
<th>N (%)</th>
<th>GSTM1+</th>
<th>GSTT1+</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;60</td>
<td>3/85</td>
<td>1/42</td>
<td>0.88</td>
<td>24/55</td>
<td>31/19</td>
<td>0.22</td>
</tr>
<tr>
<td>&gt;60</td>
<td>2/88</td>
<td>1/42</td>
<td>0.88</td>
<td>8/11</td>
<td>11/8</td>
<td></td>
</tr>
<tr>
<td>Sex:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>3/85</td>
<td>1/42</td>
<td>0.98</td>
<td>8/14</td>
<td>45/83</td>
<td>0.83</td>
</tr>
<tr>
<td>Female</td>
<td>2/87</td>
<td>1/42</td>
<td>0.98</td>
<td>6/14</td>
<td>38/83</td>
<td></td>
</tr>
<tr>
<td>TLC (x10⁹/L)*</td>
<td>8.4 (1.9-608)</td>
<td>19.1 (1.6-340)</td>
<td>0.75</td>
<td>16.8 (1.5-68)</td>
<td>17.2 (1.6340)</td>
<td>0.50</td>
</tr>
<tr>
<td>Blast %*</td>
<td>75 (10-96)</td>
<td>75 (20-98)</td>
<td>0.71</td>
<td>81 (30-85)</td>
<td>72 (10-98)</td>
<td>0.50</td>
</tr>
</tbody>
</table>

**FAB:**

| M1  | 20/41 | 12/34 | 35 | 5/8 | 27/67 | 0.40 |
| M2  | 13/39 | 16/34 | 47 | 2/8  | 27/67 | 0.40 |
| M3  | 5/41  | 3/34  | 9  | 0/8  | 8/67  | 0.12 |
| M4  | 1/41  | 1/34  | 3  | 0/8  | 2/67  | 0.31 |
| M5  | 2/41  | 1/34  | 3  | 1/8  | 2/67  | 0.31 |
| M7  | 0/41  | 1/34  | 3  | 0/8  | 1/67  | 0.31 |

**IPT:**

| MPO | 31/42 | 42/53 | 79 | 9/14 | 64/81 | 0.79 |
| CD13 | 45/54 | 39/42 | 93 | 0.16 | 12/14 | 86 |
| CD33 | 46/53 | 39/42 | 93 | 0.34 | 13/14 | 39 |
| DR  | 37/53 | 32/41 | 78 | 0.37 | 13/14 | 39 |
| CD34 | 9/24 | 15/24 | 63 | 0.08 | 5/9 | 49 |
| Lymphoid markers | 15/54 | 10/43 | 23% | 0.61 | 7/14 | 50% |

*Median (range). **p=0.07, 0.08 and 0.03 for DR, CD34 and lymphoid marker expression, respectively.

Table (3): Outcome of treatment according to GST genotype.

<table>
<thead>
<tr>
<th>Rate of CR</th>
<th>GSTM1+</th>
<th>p-value</th>
<th>GSTT1+</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>23/36 (64%)</td>
<td>13/27 (48%)</td>
<td>0.21</td>
<td>4/7 (57%)</td>
<td>32/56 (57%)</td>
</tr>
<tr>
<td>Toxic Death</td>
<td>11/36 (31%)</td>
<td>9/27 (33%)</td>
<td>0.97</td>
<td>3/7 (43%)</td>
</tr>
</tbody>
</table>

Fig. (1): Agarose gel electrophoresis showing genotype analysis of GSTM1 (219bp) and GSTT1 (480bp) using multiplex PCR. BCL2 was used as internal control (154bp). Lane 1: Molecular Weight marker; Lanes 2,5,8: Show patients with GSTM1/GSTT1 double present genotype; Lane 3: Shows patient with GSTM1/GSTT1 double null genotype; Lanes 4,6,7: Show patients with GSTM1 null/GSTT1 present genotype. Lane 9: Negative control.
DISCUSSION

In AML patients, the occurrence of a sizeable number of deaths related to drug toxicity is an important limitation for the success of treatment [22]. Pharmacogenomic studies aim to elucidate the genetic basis for interindividual differences in drug response with the aim of predicting its safety, toxicity and efficacy [23]. GST enzymes are involved in the conjugation reactions in phase II metabolism of xenobiotics [24]. Several studies have reported associations between GST polymorphisms and the efficacy and toxicity of cancer chemotherapy [8,25,26].

The frequency of GSTM1 null genotype in our patients was 56%, higher than 42% reported in AML cases by Voso et al. [7]. This frequency was also beyond 44% previously reported in normal Egyptian population by Abdel Rahman et al. [27], but within the published range of (42%-57%) [17,28,29]). This suggests that GSTM1 polymorphism is a probable potential risk factor for the development of AML in Egyptians. A study reported positive associations between inherited risk of AML and GSTM1 null genotype [19]. On the other hand, no evidence of association could be detected in our series between GSTT1 null and double null genotypes and the incidence of AML. The frequencies of GSTT1 null and double null genotypes in our study were 14% and 6%, respectively; these frequencies were comparable to those reported in the Egyptian population by Abdel Rahman et al. [27] (14.4% and 8.8%, respectively), lower than those reported by Voso et al. [7] in adult AML (28% and 17.9%, respectively) but within the published range of 10%-21% [30]. Our results are in accordance with Basu et al. [31], Preudhomme et al. [32] and Rollinson et al. [29], who failed to identify an association between GSTT1 null genotype and the development of AML. In contrast, Chen et al. [33] and Naoe et al. [26] reported an increased risk of MDS and acute leukemia associated with the GSTT1 null genotype. On the other, Rollinson et al. [29] reported an association between ALL and GSTT1 null genotype. This difference between studies could indicate differences between populations in the influence of these genetic polymorphisms on genetic susceptibility to leukemia or in exposures involved in leukemogenesis [34].

Examination of GSTM1 null and GSTT1 null genotypes showed no statistically significant association with other prognostic factors such as age, total leucocytic count at diagnosis and FAB subtype. However, age over 60 years was detected in 27% of GSTT1 null patients compared to 13% of patients with GSTT1 present genotype, p=0.22. Voso et al. [7] reported an increased frequency of GSTM1 null genotype in patients with AML over 60 years age. Prolonged exposition of hematopoietic progenitor cells to toxic agents in combination with a reduced capability of detoxification might contribute to the pathogenesis in the elderly [7]. In our series, the FAB subtype M5 constituted 13% and 3% of patients with GSTT1 null and GSTT1 present allele, respectively. M3 subtype was present in GSTT1 present but not in GSTT1 null patients. In contrast, Davies et al. [19] reported a significantly increased frequency of GSTM1 null in FAB M3 and M4 subtypes. These data indicate that these subtypes may be particularly influenced by exposure to environmental agents detoxified by GSTM1 [19]. The discrepancy in frequencies could be due to small number of patients in each FAB subtype.

Regarding IPT, GSTT1 null genotype showed significant association with lymphoid marker expression, mainly CD7 (p=0.03), which is associated in some studies with adverse effects [35]. On the other hand, GSTM1 present genotype showed near significant association with expression of the stem cell marker CD34, reported to have an adverse effect [36].

Secondary AML is a clinical entity of special interest because of its relation to genotoxic exposures and MDS [33]. In our series, no such association could be elucidated due to the small number of patients with previous cancer or MDS.

Glutathione S transferase enzymes play an important role in the detoxification of chemotherapeutics. GSTM1 and GSTT1 enzyme polymorphism in the form of deletion of either or both genes affect pharmacodynamics of chemotherapeutic drugs used in the treatment of leukemia. The importance of a genetic polymorphism in drug metabolism may differ based on the nature and intensity of the treatment regimen [37]. When drugs are being dosed at levels that are near those that produce toxicity (common in AML), the inheritance of deficiency of an enzyme involved in the detoxification of such
drug can lead to a worse outcome because of greater toxicity [37]. Anthracyclines, such as doxorubicin used in the treatment of AML, are known to generate high levels of reactive oxygen species, including hydrogen peroxide and hydroxyl radicals which are destructive to the cells [38]. The occurrence of a substantial number of deaths in remission caused by the toxicity of therapy is an important limitation on success of treatment of AML [24]. GST modulates drug effect through deactivation of drug-generated hydroperoxides or other reactive oxygen species [3,4]. In our study, the rate of early death during induction due to drug toxicity was 43% versus 30% in GSTT1 null compared to GSTT1 present patients, \( p=0.67 \). This constituted (3/4) 75% and (17/41) 42% of deaths in each group, respectively \( (p=0.31) \), yet differences were not statistically significant. This lack of significance could be due to the small sample size. Davies et al. [8] and Naoe et al. [26] reported an increased rate of early death in GSTT1 null compared to GSTT present group. In the study conducted by Davies et al. [8], toxicity of therapy was particularly increased in those receiving the most intensively timed chemotherapy regimen. On the other hand, the South West Oncology group (SWOG) did not report such association in a group of AML patients over 56 years [39]. In our series, infection and bleeding were the common causes of early death, in accordance with Naoe et al. and Riley et al., in adult and childhood AML [24,26].

In our series, the rate of complete remission did not differ significantly in groups with and without the deletion. However, the rate of CR in GSTM1 null compared to GSTM1 present patients was 64% versus 48%. Although the difference was not significant, this was in accordance with the study conducted by the SWOG [39] which reported a lower risk of resistant disease in patients with GSTM1 null compared to those with the GSTM1 present genotype in AML patients. On the other hand, Davies et al. and Naoe et al. [8,26] reported no difference in CR rates between GSTT1 present and GSTT1 null genotypes in adult and childhood AML. In contrast, Voso et al. [7] indicated that AML patients with deletion of M1 or T1 or both had a lower probability to achieve complete remission on induction therapy as compared to patients with intact GST genes [7]. This is in accordance with Zhang Y et al. [40] who reported a significant high rate of CR in patients with GSTT1 present compared to GSTT1 null alleles. This could be explained by the fact that lack of detoxification may contribute to accumulation of genetic changes in the process of leukemogenesis and more aggressive disease [7].

Although absence of GST enzyme may reduce or delay metabolism of the chemotherapeutic drugs used for AML and might be expected to lead to a reduced relapse rate in addition to increased toxicity, in our patients, GST null genotype was not associated with significant difference in DFS, in accordance with Davies et al. and Naoe et al. [8,26] who reported no association between GSTT1 null and reduced rate of relapse in adult AML. It is possible that the GSTT1 genotype influences production or excretion of drug metabolites that contribute to toxicity to a greater degree than it influences metabolites that have an antileukemic effect. On the other hand, Voso et al. [7] demonstrated a lower chemotherapy response rate in adults with AML and at least one GST null genotype. In contrast, in a study on childhood AML, DFS was reduced in GSTM1 present compared with GSTM1 null cases receiving standard but not intensively timed therapy [8]. Children receiving a less intensive regimen who are able to metabolize drugs efficiently may receive an inadequate dose of cytotoxic therapy, leading to higher relapse rate. In childhood ALL reports have been controversial. While Stanulla et al. [41] and Tanaski et al. [42] reported that the deletion of GSTM1 and GSTT1 genes was associated with reduced risk of relapse, Chen et al. [43] reported no impact on the response to therapy. On the other hand, Davies et al. [8] reported a higher frequency of relapse.

In our study, no difference in the OS was encountered between different groups. On the other hand, Atrup et al. [44], in a study on adult AML, noted that patients with GSTM1 null genotypes had a poorer survival compared to those with GSTM1 present alleles. In another study by Naoe et al. [26] on adult AML, GSTT1 null genotype was an independent prognostic factor for OS.

In childhood AML, patients with GSTT1 null genotype had reduced survival compared with those possessing at least one GSTT1 allele.
The prognostic importance of GST genotype was supported by Voso et al. [7], where the GST genotype was an independent predictor for OS in a multivariate analysis. The presence of at least one GST deletion proved to be a poor prognostic factor for survival [7]. In addition, GST genotyping could discriminate between favourable and unfavourable prognosis in patients within the intermediate-risk karyotype group which contains the majority of AML patients [7]. In ALL, no effect on survival was found [41].

The conflicting results in different studies could be due to the use of different therapeutic regimens. The significance of GST enzyme may be different in each malignancy and therapy [26]. In addition, as GST genotype may be associated with leukemogenesis in specific cytogenetically and or molecular genetically defined leukaemia subsets [45,46], the evaluation of GST genotypes in association with leukaemia outcome will have to take in consideration cytogenetic and molecular genetic information [41].

Pharmacogenetic factors can influence the outcome of therapy and particularly dose-intensive therapy. In our series, the rate of early toxic death during induction was 43% in GSTT1 null compared to 30% in GSTT1 present patients. Though the difference was not significant, a more extensive study on a larger group of patients would support the association between null genotype and toxic death. Pharmacogenetic screening prior to chemotherapy may lead to identification of patients predisposed to drug toxicity. GSTT1 genotype might be useful in selecting appropriate chemotherapy regimens for patients with AML. Patients with GSTT1 null genotype would benefit from changing or lowering the dose of chemotherapy to decrease the frequency of toxic death. On the other hand, in our series patients with GSTM1 present genotype, who are able to metabolize drugs efficiently, were associated with a lower frequency of CR. This group of patients would benefit from increasing the dose of chemotherapy without fear of greater toxicity. A larger study on a wider group of patients is needed to confirm such associations.

In AML patients, the occurrence of a sizeable number of deaths related to drug toxicity is an important limitation for the success of treatment. Our data suggest possible association, though not significant, between GSTT1 null genotype and toxic death during induction and between GSTM1 null genotype and higher CR rate. Studies on larger numbers are needed focusing on selection of anticancer agents to avoid adverse reactions and therapeutic failure, with special emphasis on drug toxicity and dose adjustment.

REFERENCES


