Impact of glutathione S-transferase gene deletion on early relapse in childhood B-precursor acute lymphoblastic leukemia

MAMI TAKANASHI, AKIRA MORIMOTO, TOMOHITO YAGI, KIKUKO KURIYAMA, GEN KANO, TOSHIHIKO IMAMURA, SHIGEYOSHI HIBI, SHINJIRO TODO, SHINSAKU IMASHUKU

Background and Objectives. The glutathione-S-transferase (GST) polymorphism may affect the outcome of treatment of leukemia because GSTs play an important role in detoxifying the chemotherapeutic agents used to kill leukemia cells. However, results of previous reports have been controversial. This study was undertaken to clarify the influence of GST polymorphism on the outcome of childhood B-precursor acute lymphoblastic leukemia (ALL).

Design and Methods. Eighty-two patients with childhood B-precursor ALL treated during 1988-1999 with our ALL protocol (median follow-up time 89.5 months, range 31 –169 months) were examined for GST gene patterns. The effect of GSTM1 and GSTT1 deletion genotypes on the clinical features and therapeutic results was analyzed.

Results. All patients attained complete remission but 12 had an early relapse (within 30 months of the initiation of treatment). In univariate analysis, early relapse of ALL was correlated significantly with the presence of the t(9;22)(q34;q11) cytogenetic abnormality (p=0.0003), high white blood cell counts (p=0.015) and double null genotype (p=0.027). Multivariate analysis revealed that the GST double null genotype was the only significant independent predictor of early relapse (p=0.018).

Interpretation and Conclusions. The simultaneous deletion of both the GSTM1 and GSTT1 genes is more predictive than any other parameter of early relapse of childhood B-precursor ALL.

Key words: glutathione S-transferase gene deletion, acute lymphoblastic leukemia, relapse, prognosis.

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From the Department of Pediatrics, Kyoto Prefectural University of Medicine, (MT, AM, TY, KK, GK, TI, SH, ST); Kyoto City Institute of Health and Environmental Sciences (SI).

Correspondence: Mami Takanashi, M.D., Department of Pediatrics, Kyoto Prefectural University of Medicine, Kajiicho 465 Hirokoji, Kamigyoku, Kyoto, 602-8566, Japan. E-mail: mamikiti@koto.kpu-m.ac.jp

A variety of genes are involved in metabolizing carcinogens. Polymorphism in these genes can result in less or more efficient metabolic processes which may contribute to the susceptibility of an individual to develop cancer, depending on the substrate they are metabolizing.[1] The glutathione-S-transferase (GST) genes are crucially involved in the detoxification of important environmental carcinogens (e.g. benzopyrene and other polyaromatic hydrocarbons). It is believed that polymorphism in the GST genes may play a role in susceptibility to leukemogenesis.[1,2] Furthermore, GST polymorphism may also affect the treatment of leukemia as the GSTs play an important role in detoxifying the active metabolites of cytotoxic chemotherapeutic agents used to kill tumor cells.

The GST genes in humans are divided into four major subfamilies designated as GSTα, GSTμ or M, GSTθ or T, and GSTp. Two of these subfamilies show genetic polymorphism in that GSTM1 and/or GSTT1 can be deleted. These deletions can be tested for by a method using a polymerase chain reaction (PCR) on genomic DNA.[3] The incidence of these deletions in healthy subjects differs depending on the ethnic origin of the population tested[4] but it has been suggested that they occur with higher frequencies in patients with several malignancies, including smoking-related lung cancer and gastrointestinal cancer.[5,6] Loss of GSTM1 and/or GSTT1 has also been associated with the development of leukemia and myelodysplastic syndrome.[7-12]

In contrast to the putative role of GST deletions in carcinogenesis, it has been reported that the GSTM1 and GSTT1 null genotypes may actually confer a reduced risk of relapse in childhood acute lymphoblastic leukemia (ALL).[13,14] However, other studies were unable to find a convincing correlation between the GST genotype and the outcome of childhood ALL.[7,15-17] To resolve this issue we examined GST gene patterns of 82 patients with childhood B-precursor ALL. We report here that in our study population, the simultaneous deletion of both the GSTM1 and GSTT1 (double null) genes is more predictive than any other parameter of early relapse (within 30 months of the initiation of treatment).

Design and Methods

Patients

A total of 108 Japanese patients (aged 1.5 to 15 years) with childhood B-precursor ALL were treated during

1988-1999 with our ALL protocol (median follow-up time 89.5 months, range 31-169 months). Of these, 86 patients (57 boys and 29 girls) were eligible for this study because their DNA specimens taken at diagnosis were available. The median age and WBC count at diagnosis of these 86 patients were 5.3 (range 1.8-15.8) years and 9,900 (range 600-667,500)/μL, respectively. The diagnosis of ALL was made from the results of a combination of morphologic tests (Wright-Giemsa and myeloperoxidase staining) and flow cytometric immunophenotyping of bone marrow or peripheral blood.[18] Chromosome analysis was performed by using a routine trypsin-Giemsa banding procedure and analyzed according to the ISCN. Phenotypically, all samples were compatible with B-precursor ALL. Four patients had t (9;22)(q34;q11), 6 had a hyperdiploid karyotype, 14 had other cytogenetic abnormalities and the remaining had a normal karyotypes.

Risk groups and treatment protocols

The treatment differed according to the patients' risk stratification. Three risk groups were identified. Standard risk patients were defined as being between 2 to 10 years old and having WBC counts ≤ 20,000/μL. High risk patients were younger than 2 or older than 10 and had WBC counts < 50,000/μL, or were aged between 2 and 10 and had WBC counts between 20,000/μL to 50,000/μL. Very high-risk patients included all cases with WBC counts ≥ 50,000/μL. Although this study covers 12-year span, the key therapeutic strategies for the treatment of B-precursor ALL remained the same in our institute. Briefly, induction therapy consisted of doxorubicin (ADR), vincristine (VCR) and prednisolone (PSL) with or without cyclophosphamide (CPM). The intensification included a combination of L-asparaginase (L-asp)/ VCR/ PSL, associated with central nervous system prophylaxis including cranial radiotherapy. The maintenance therapy was continuous 6-mercaptopurine with alternating cycles of CPM/VCR/PSL and methotrexate/L-asp/ PSL every 2 weeks. Etoposide and cytarabine were also included for patients in the very high-risk group. Patients in the standard and high-risk groups were treated for a total of 36 months, those in the very high-risk group were treated for 42 months.

Survival and relapse

Bone marrow was taken from all patients 14 days after the initiation of therapy to determine the number of residual blasts (day 14 blasts). Event-free survival (EFS) was defined as the time from the initiation of treatment to relapse or death from any cause, while overall survival (OS) was defined as the time from diagnosis to death. Hematologic and extramedullary relapses were both included as events. Patients who relapsed within 30 months of the initiation of treatment were defined as having an *early relapse*[19] while the remaining patients who did not relapse or relapsed later than 30 months were defined as *non-early relapse*.

Samples for GST determination

Specimens of bone marrow (n=70; median proportion of leukemic blasts, 93.3%; range 41-99.5%) or leukemic peripheral blood (n=16; median proportion of leukemic blasts, 80.5%; range 8-98%), taken at diagnosis and stored as cell suspensions or as glass slide smears, were subjected to GSTM1 and GSTT1 genotype analysis. In three cases, the GSTM1 and GSTT1 genotype of the bone marrow cells (leukemic blasts, 92%, 86.5% and 81.3%), was compared with that of somatic cells collected from buccal epithelial cells. ALL samples were collected, with informed consent, at our institute or affiliated hospitals. For control samples, peripheral blood was obtained from 47 Japanese healthy adult volunteers.

DNA extraction

Genomic DNA at diagnosis was extracted from bone marrow or peripheral blood cells stored at –20°C (n=79) or buccal epithelial cells (n=3) by using a PCR template isolation system (DnaQuick DNA extraction kit, Dainippon-pharmacy, Co., Osaka, Japan), or from scrapings from bone marrow or peripheral blood smears (n=7) by using a QIAamp, DNA Mini Kit (QIAGEN K.K., Tokyo, Japan).

Genotyping

Multiplex PCR methods originally described by Pemble *et al*. were employed to detect GSTM1 and GSTT1 in genomic DNA. The primer sets used were sense 5'- GAACTCCCTGAAAAGCTAAAGC-3' and antisense primers 5'-GTTGGGCTCAAATATACG-GTGG-3' for GSTM1, and sense 5'-TTCCTTACTG-GTCCTCACATCTC-3' and antisense primers 5'-TCACCGGATCATGGCCAGCA for GSTT1.[20,21] As an internal reference for each specimen, β-globin was amplified in the same PCR mixture. PCR was performed for 35 cycles consisting of 30 seconds at 94°C, 60 seconds at 58°C, and 90 seconds at 72°C, with a final extension step of 7 minutes at 72°C. The PCR products were fractionated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. The product sizes were 219 bp for GSTM1, 480 bp for GSTT1, and 268 bp for β-globin. To avoid false null positive results, we accepted only gels in which the β-globin band was clearly detected. The genotype was classified as either GSTM1 null, GSTT1 null, double null or double positive.

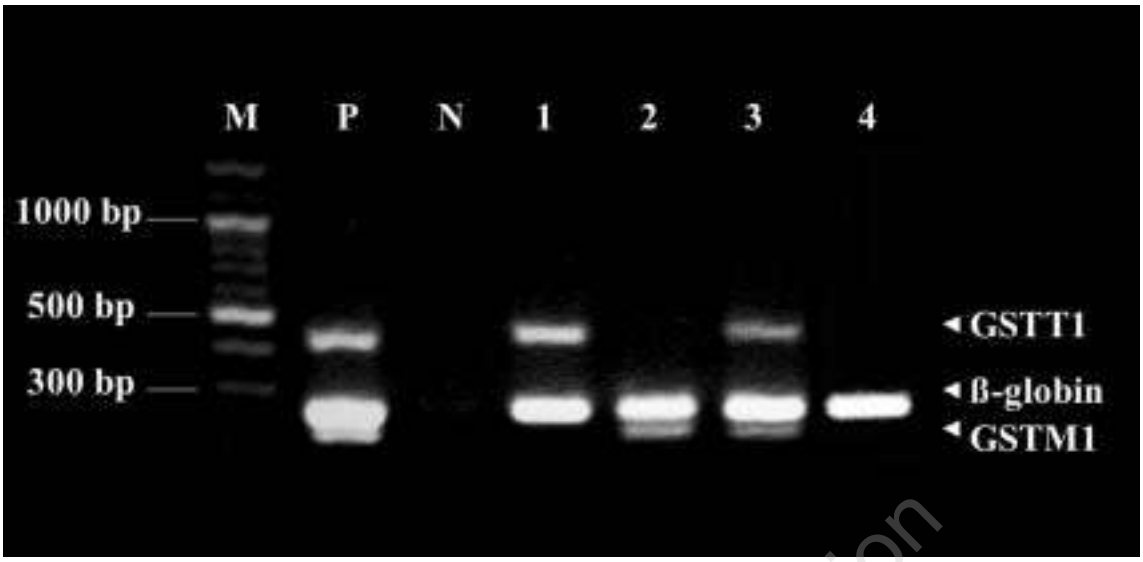

Figure 1. Multiplex PCR for GSTT1, GSTM1, and β-globin using genomic DNA samples from representative cases. The PCR product sizes were 480 bp for GSTT1, 219 bp for GSTM1 and 268 bp for β-globin. Lane M: molecular marker; Lane P: positive control for GSTT1 and GSTM1; Lane N: negative control; Lane 1: case 43, Lane 2: case 10, Lane 3: case 76, and Lane 4: case 4.

Statistical analysis

Patients with particular GST genotypes were first tested for various clinical parameters in univariate analysis. To further determine factors predicting an early relapse, univariate analysis and multivariate analysis with the logistic regression method as well as Cox regression analysis were employed. Only factors with *p* values less than 0.2 in univariate analysis were introduced into the multivariate analysis as explanatory variables. Event-free survival (EFS) and overall survival (OS) were estimated using Kaplan-Meier analysis at certain time-points (3 years, 7 years) with the t-test and for the entire period with the log-rank test. Values of $p<0.05$ were considered statistically significant.

Results

Eighty-two of the 86 samples were eligible for further analysis of GST genotypes. Four genotypes of GST were observed (Figure 1). The prevalence of particular GST genotypes in the B-precursor ALL patients and in the control subjects was 40.2% vs. 59.6% for GSTM1 null (p=0.044), 41.5% vs. 36.2% for GSTT1 null (p=0.58), 23.2% vs. 25.5% for double null (p=0.83) and 41.5% vs. 29.8% for double positive (p=0.19). Thus, in this study, the GSTM1 null genotype was found to occur significantly less frequently in the ALL group. In the 3 cases in which both leukemic blasts and somatic cells were examined, GST genotypes were identical in both cell sources; two of them were double null and the other was T1 positive and M1 null. In terms of therapeutic response, all patients attained complete remission. No death in remission was observed. Relapse (20 hematologic and 4 extramedullary) occurred in 24 patients. Of these 24 patients, 12 relapsed early: 3 within six months, 5 between 6-18 months and 4 between 19-30 months after initiation of therapy. Twelve of the 24 patients who relapsed were successfully rescued by chemotherapy and/or allogeneic hematopoietic stem cell transplantation (n=14). Correlating GST genotypes and relapse pattern, we found that 6 early relapse patients had the GST double null genotype, clearly demonstrating that the GST double null genotype had a statistically significant impact on early relapse (Table 1). Thus, the 82 ALL patients were then analyzed by dividing into the GST double null group (19 patients) and the non-double null group (63 patients) as shown in Table 2.

Table 1. Impact of GST null genotypes on ALL early relapse.

Variables	*Early relapse*[a]	*Non-early relapse*	*p*[b]
N.	12	70	
GST genotypes			
M1 null	1	13	0.68
T1 null	4	11	0.22
Double null	6	13	0.027

[a]*relapse less than 30 months;* [b]*p Fisher's exact test.*

Table 2. Correlation of GST genotypes and clinical and therapeutic variables of childhood B precursor ALL.

Variables	*Double null*	*Non-double null*	*p*[b]
Number	19	63	
Clinical features			
Sex			
M/F	14/5	39/24	0.42
Age (yrs)			
median	4	5.8	0.34[c]
WBC			
<50,000	12	53	
≥50,000	7	10	0.06
Risk group			
standard	7	34	
high	5	19	
very high	7	10	0.13
Day 14 blasts (%)			
<5	11	34	
≥5	7	28	
unknown	1	1	0.79
Cytogenetics			
t(9;22) (q34;q11)	1	3	
others	18	60	0.99
Therapeutic results			
Initial response			
CR attained	19	63	0.99
Total relapse	7	17	0.41
Time of Relapse			
Early (<30 mo.)	6	6	
Late (≥30 mo.)	1	11	0.024
Alive after relapse by chemo and/or SCT	1/7	11/17	0.069
Death			
Early (<30 mo.)	6	2	
Late (≥30 mo.)	0	4	0.061
Survival (mean±SE)[a]			
EFS at 3 yrs	63.2±11.1 %	90.5±3.7 %	0.010[d]
EFS at 7 yrs	63.2±11.1 %	65.7±6.8 %	0.425[d]
OS at 7 yrs	64.4±11.9 %	89.2±4.7 %	0.026[d]
EFS (entire period)			0.321[e]
OS (entire period)			0.015[e]

M: male; F: female; CR: complete remission; SCT: stem cell transplantation; EFS: event-free survival; OS: overall survival; SE: standard error; [a]Kaplan-Meier analysis; [b]χ^2 test or Fisher's exact test; [c]Mann-Whitney's U test; [d]t test; [e]log-rank test.

Table 3. Correlation of early relapse or non-early relapse and clinical variables of childhood B precursor ALL.

Variables	*Early relapse*[a]	*Non-early relapse*	*p*[b]
Number	12	70	
Clinical features			
Sex			
Male/Female	9/3	44/26	0.53
Age (yrs)			
median	6	5.3	0.86[c]
WBC			
<50,000	6	59	
≥50,000	6	11	0.015
Risk group			
standard	2	39	
high	4	20	
very high	6	11	0.011
Day 14 blast (%)			
<5	4	40	
≥5	7	28	
unknown	1	2	0.20
Cytogenetics			
t(9;22) (q34;q11)	4	0	
others	8	70	0.0003
GST genotypes			
double null	6	13	
non-double null	6	57	0.027

[a]relapse less than 30 months; [b]p: χ^2 test or Fisher's exact test; [c]Mann-Whitney's U test.

Univariate analysis of the GST genotype with respect to the clinical and therapeutic variables considered showed that, as described above, the double null genotype occurred significantly more frequently in early relapse patients (p=0.024) and also in patients with high WBC counts (p=0.06) (Table 2). When we examined the survival of the patients with particular GST genotypes by Kaplan-Meier analysis, we found that there was a significant difference in OS (p=0.015, log-rank test) but not in EFS (p=0.321, log-rank test) between the double null and non-double null groups (Figure 2). However, when we evaluated EFS at 3 years, we found a significantly worse outcome for the double null group (63.2±11.1% vs. 90.5±3.7%, p=0.010, t-test). The high number of patients who had an early relapse and could not be rescued was thought to be responsible for the poor OS in the double null genotype group, as shown in Table 2 and Figure 2. Similarly, Cox regression analysis showed that double null

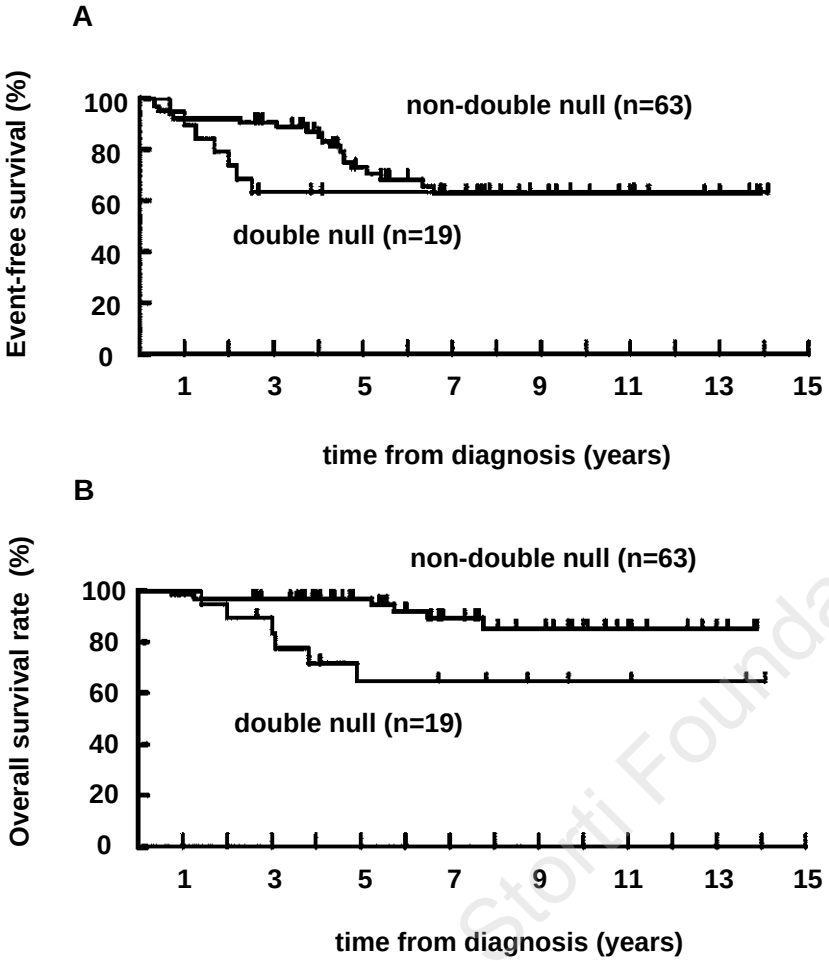

Figure 2. Kaplan-Meier analysis of the event-free (A) and overall (B) survival of the non-double null (n=63) and double null (n=19) groups of patients with childhood B-precursor ALL. The *p* value by log-rank test was 0.321 and 0.015, respectively.

genotype had a significant impact on OS, but not on EFS. The presence of GSTT1 null alone or GSTM1 null alone was not significantly associated with any clinical or therapeutic variables (*data not shown*).

The early relapse group (n=12) was compared to the non-early relapse group (n=70, 58 of whom did not relapse at all) (Table 3): t(9;22)(q34;q11) cytogenetic abnormality and WBC counts higher than 50,000/μL occurred significantly more frequently in the early relapse group (p=0.0003 and 0.015, respectively). The number of blasts in the bone marrow samples taken from the patients at day 14 did not differ between the two relapse groups (p=0.2).

Multivariate analysis with the logistic regression method revealed that the double null genotype was the only significant risk factor for early relapse (p=0.018, OR=7.94 with 95% CI=1.42-45.45). In contrast, poor treatment response (day 14 blasts ≥5%) (p=0.12), high WBC counts (≥50,000/μL) (p=0.83), poor risk group (non-standard treatment group) (p=0.21) and the presence of t(9;22)(q34;q11) (p=0.97) were not significant risk factors for early relapse in the multivariate analysis.

Discussion

The role of GST genotypes in disease susceptibility in leukemia has been long debated. This study was designed to examine the effect, if any, of GST genotypes on leukemic relapse, rather than to focus on their role in disease susceptibility. Among several possible types of ALL, we chose B-precursor ALL for this purpose, because it is the major leukemia in children. As well known, GST genotypes are not the same in various ethnic groups.[4,22] The frequencies we found in our control subjects are characteristic for the Japanese population and compatible with previous reports.[9,10] Several research groups have found that certain GST genotypes, such as GSTM1 or GSTT1 null genotypes, occur more frequently in ALL patients than in control subjects, and have suggested that the presence of these genotypes may render both adults and children more susceptible to developing ALL.[8,11] However, our data show no significant differences in genotype frequencies between the patients with ALL and the controls except for the GSTM1 null

genotype, which occurred less frequently in childhood B-precursor ALL. Thus, our study seems not to support the notion that GST deletions are closely linked with the development of ALL.

In our study, all the patients' samples for GST genotyping were obtained at diagnosis of ALL, indicating that the majority of cells tested were not normal cells but leukemic blasts. Previously reported studies of GST genotypes in hematologic malignancies examined various types of material such as bone marrow cells at diagnosis,[23,24] peripheral blood cells in remission,[7,13] or normal buccal epithelial cells;[8] some reports did not mention what materials were actually studied.[12,15,22] We also studied bone marrow or peripheral cells taken at diagnosis; these were mixtures of leukemic blasts and normal hematopoietic cells at various ratios. We assumed that GST genotype is the same in leukemic blasts and normal cells in a given leukemic patient. However, one could argue that an acquired loss of a GST allele(s) in the leukaemic clone might have affected the genotype analysis in our study. We consider this possibility unlikely for two reasons; (a) we found no reports of acquired loss of GST genes in ALL in our literature survey and (b) when we simultaneously examined GST genotypes in leukemic blasts and somatic cells in three cases, the genotype patterns were identical in all cases.

It has been reported that the GSTM1 and GSTT1 null genotypes confer a reduced risk of relapse in childhood ALL,[13,14] although this correlation was not observed in four other studies.[7,15-17] The studies that found a lower rate of relapse in patients with GST null genotypes proposed that this may be due to the better response by these patients to certain chemotherapeutic agents because of lower rates of drug metabolism by the patients' GST enzymes. However, in contrast, in our study we found that the presence of the GST double null genotype was actually associated with a higher risk of developing early relapse in childhood B-precursor ALL. Indeed, multivariate analysis identified the double null genotype as the only significant risk factor for early relapse.

Supporting our observations, other studies have shown that the GSTM1 null and/or GSTT1 null genotypes are linked to resistance to chemotherapy or shorter survival in adult acute myeloid leukemia[24] and ovarian cancer.[25] The suggested hypothesis to explain this was that the lack of GST enzymes may reduce the consumption of glutathione (GSH) in GST-catalyzed reactions, thereby leading to higher levels of GSH which may block apoptosis and promote the proliferation of neoplastic cells. Supporting this notion, Kearns *et al*. reported an association between elevated GSH levels in leukemic cells and an increased risk of relapse in childhood ALL.[23]

We found that our double null patients had a poorer EFS at 3 years and OS at 7 years than did the non-null patients (Table 2). Davies *et al*. reported that patients with a GSTT1 null genotype have a lower OS and that this may be caused by the greater toxicity of the chemotherapy in these patients.[12] By contrast, the low EFS and OS in our patients were thought to be due to early relapse rather than to treatment failures such as toxicity and death.

Identifying the patients with childhood B-precursor ALL who probably to have a poor prognosis is vital, as these patients are more likely to benefit from more prompt or aggressive therapies or alternative treatment regimens. To date, various factors have been found to be useful in predicting poor prognosis in childhood ALL, such as high WBC counts at diagnosis, certain chromosomal abnormalities or initial therapy response.[26] Indeed, in univariate analysis, we found that high WBC counts and Philadelphia chromosomes were associated with the development of early relapses (p=0.015, p=0.0003, respectively) (Table 3). However, when multivariate analysis was performed, only the GST double null genotype was significantly linked with early relapse, which in turn was responsible for the poor OS, as assessed by several methods of statistical analysis. These observations suggest that a larger multi-institutional study involving more patients is warranted to investigate this issue further.

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Pre-publication Report & Outcomes of Peer Review

Contributions

MT, AM, TY, KK, GK, TI, SH, ST, SI: conception and design, or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content and final approval of the version to be published. We thank Professor Tohru Sugimoto for promoting our clinical and research activities and Yasuko Hashimoto for assistance in preparing this manuscript.

Disclosures

Conflict of interest: none.

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Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Maja Krajinovic who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Krajinovic and the Editors. Manuscript received May 5, 2003; accepted September 20, 2003.

In the following paragraphs, Dr. Krajinovic summarizes the peer-review process and its outcomes.

What is already known on this topic

The wide range of drug substrates renders glutathione S-transferases (GSTs) interesting candidates for therapeutic response including that of childhood acute lymphoblastic leukemia (ALL). It is not thus surprising that several research groups addressed this issue. The reported results are however conflicting. Whereas Stanulla *et al.*[13] and Anderer *et al.*[14] observed the protective role of GSTs, and particularly that of GSTT1, absence of any GST-related effect on ALL treatment outcome was noted in several other studies.[7,15,16]

What this study adds

In contrast, Takanashi *et al.* here provide the evidence for an increased risk of an early relapse associated with GST double null genotype, which resulted in shorter overall survival. The similar finding was reported for acute myeloid leukemia patients.[12,24] The underlying hypothesis is that the lack of GST activity would result in higher glutathione (GSH) levels, which in turn might inhibit the apoptosis. This is in agreement with the previous finding reporting the association between elevated GSH leukemia cells levels and increased risk of relapse of childhood ALL.[23] The report of Takanashi and coworkers is interesting and certainly different. However, if we are about to understand which group of patients is likely to benefit from GST genotyping, further analysis are required to understand the discrepancies across studies, as well as to determine the importance of GST genotypes relative to those of other relevant loci.