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GSTM1 and GSTT1 double null genotypes determining cell fate and proliferation as potential risk factors of relapse in children with hematological malignancies after hematopoietic stem cell transplantation

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Abstract

Purpose This study aimed to retrospectively evaluate the genetic association of null variants of glutathione S-transferases GSTM1 and GSTT1 with relapse incidence in children with hematological malignancies (HMs) undergoing busulfan (BU)-containing allogeneic hematopoietic stem cell transplantation (HSCT) and to assess the impact of these variants on BU-induced cytotoxicity on the immortalized lymphoblastoid cell lines (LCLs) and tumor THP1 GST gene-edited cell models.

Methods GSTM1- and GSTT1-null alleles were genotyped using germline DNA from whole blood prior to a conditioning BU-based regimen. Association of GSTM1- and GSTT1-null variants with relapse incidence was analyzed using multivariable competing risk analysis. BU-induced cell death studies were conducted in GSTs-null and non-null LCLs and CRISPR–Cas9 gene-edited THP1 leukemia cell lines.

Results Carrying GSTM1/GSTT1 double null genotype was found to be an independent risk factor for post-HSCT relapse in 86 children (adjusted HR: 6.52 [95% CI, 2.76–15.42; \( p = 1.9 \times 10^{-5} \))). BU-induced cell death preferentially in THP1 GSTM1(non−null) and LCLs GSTM1(non−null) as shown by decreased viability, increased necrosis and levels of the oxidized form of glutathione compared to null cells, while GSTT1 non-null cells showed increased baseline proliferation.

Conclusion The clinical association suggests that GSTM1/GSTT1 double null genotype could serve as genetic stratification biomarker for the high risk of post-HSCT relapse. Functional studies have indicated that GSTM1 status modulates BU-induced cell death. On the other hand, GSTT1 is proposed to be involved in baseline cell proliferation.

Keywords Null genotypes of glutathione S-transferases · Acute leukemia · Hematological malignancies · Hematopoietic stem cell transplantation · Post-transplant relapse · Busulfan resistance

Background

Survival rates of children with hematological malignancies (HMs) undergoing allogeneic hematopoietic stem cell transplantation (HSCT) have improved over the years achieving 91% estimated 2-year overall survival. The improvement is mainly attributed to reduced HSCT-related toxicity and mortality. The incidence of post-HSCT relapse remains a significant complication and varies from 12 to 33% after 2 years (Peters et al. 2021). Risk factors that influence transplant success are on the one hand host- and disease related, such as disease genetics and remission status before HSCT, and on the other hand, transplant related, such as conditioning regimen and treatment-related toxicities including for example severe graft-versus-host disease (GvHD), sinusoidal obstruction syndrome (SOS), and infections (Barrett and

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A bifunctional alkylating agent busulfan (BU) is still often used in conditioning regimens prior to HSCT in children and adolescents (Philippe et al. 2016) and is commonly administered along with other chemotherapeutics, e.g., cyclophosphamide (CY) and fludarabine (FLU) (Ciurea and Andersson 2009; Hao et al. 2020). At least in acute myeloid leukemia (AML), BU has shown lower long-term adverse effects, consequently replacing total body irradiation (TBI) in the conditioning regimen (Lee et al. 2020). In acute lymphoblastic leukemia (ALL), although the recently published results showed lower rates of relapse after TBI-containing conditioning, the results obtained with BU in association with FLU and thiopeta were encouraging and indicate an opportunity to find genetic subgroups of patients who might benefit from the TBI-free conditioning (Peters et al. 2021).

BU is metabolized via conjugation with glutathione (GSH) in the liver, which is predominantly catalyzed by glutathione S-transferase alpha1 (GSTA1) (Czerwinski et al. 1996). In hematopoietic cells (HCs), where GSTA1 is not expressed (Czerwinski et al. 1997), other GST isoenzymes, particularly Mu1 (GSTM1, 46% of the BU conjugating activity of GSTA1 (Czerwinski et al. 1996)) might play the most important role. The role of GSTT1 in BU conjugation is not yet known, but has been mostly reported to have combined effects with GSTM1 on clinical outcomes (Kim et al. 2011; Myers et al. 2017). In addition to their protective role of the conjugation of BU in HCs, they might contribute to multiple cellular processes such as regulation of cell proliferation and apoptosis through the interaction with protein kinases such as apoptosis signal-regulating kinase 1 (ASK1). Under stress conditions, the interaction of the GSTM1:ASK1 complex is dissociated and results in activation of ASK1 that activates the c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinase p38 (MAPK p38) pathways, leading to upstream cytokine- and stress-induced apoptosis (Board and Menon 2013; Tew and Townsend 2012). However, the impact of apoptosis through kinases on BU-dependent cytotoxicity is poorly understood and even less whether those GST genes naturally knocked down might interfere in the post-HSCT relapse potential.

GSTM1 and GSTT1 genes can be homozygously deleted (presented as GSTM1-null and GSTT1-null) and thus completely deprived of the enzyme activities in a high percentage of individuals (the average % in Europe are 51 and 19, respectively) (Saitou and Ishida 2015). In AML adult patients, Weiss et al. (Weiss et al. 2007) showed a perfect concordance of those variants in malignant and germline DNA, which suggests that the germline genotype drives protein expression in malignant cells. Although these variants have been associated with a higher risk of leukemia development (Li et al. 2018), there are conflicting reports on the association of the GSTM1-null and GSTT1-null variants with relapse in patients with HMs (Balta et al. 2003; Franca et al. 2012; Stanulla et al. 2000; Takanashi et al. 2003). To date, there is no evidence available for the association of germline GSTM1-null and GSTT1-null variants with post-HSCT relapse in children with HMs.

Because GSTM1 and GSTT1 are the main remaining GSTs in HCs, we hypothesized that the absence of either or both proteins should affect BU cytotoxicity through conjugation-dependent or -independent ways, interfering in the HSCT outcomes. Hence, a genetic association study based on germline GSTT1- and GSTM1-null variants was undertaken. Further, we conducted in vitro functional analyses to understand the role of these variants in survival and BU-induced apoptosis and necrosis of the immortalized and tumor lymphoblastoid cell lines (LCLs).

**Materials and methods**

**Clinical association study**

**Patients and treatment**

Pediatric patients with ALL, AML or myelodysplastic syndrome (MDS) who had undergone allogeneic HSCT between 2000 and 2013 were enrolled in the study. The Institutional Review Board or ethics committees approved the study and all patients and/or parents provided informed consent. The present study is a subset of the multicentric study under the umbrella of the European Society for Blood and Marrow Transplantation (EBMT) (Clinicaltrials.gov identifier: NCT01257854) (Ansari et al. 2017).

I.v. BU (Busulfex, Otsuka Pharmaceuticals, Saint-Laurant, Montreal, QC, Canada or Busilvex, Pierre Fabre Laboratory, Paris, France) administration was given as a 2 h infusion to the patients, every 6 h for a total of 16 doses. The first BU dose was age and weight based and pharmacokinetic (PK)-guided dose adjustment was performed to obtain a cumulative area under the curve (CumAUC) between 59.2 and 98.56 mg*h/L as reported previously (Ansari et al. 2017).

The primary diagnosis of HMs was made at the referring institution. Patients were considered to be in remission after chemotherapy if they presented < 5% blasts in the normal cellular bone marrow. Relapse in MDS was defined as > 5% and ≤ 20% of blasts at the bone marrow examination after engraftment and/or reappearance of major dysplastic features associated with cytopenias and/or mixed chimerism > 5% and/or detection of the same cytogenetic abnormality present at diagnosis. Relapse in AML and ALL was defined as the presence of blasts in the...
bone marrow > 5%, confirmed by flow cytometry; detection of the gene fusion present at diagnosis; or according to minimal residual disease (MRD) results after the transplantation if available. Disease remission status was defined by the number of bone marrow remission or relapse events before HSCT.

Cumulative relapse incidence, event-free survival (EFS), and overall survival (OS) were defined according to the standard guidelines of EBMT and as detailed in our recent report (Ansari et al. 2017). EFS was calculated from the time of transplant until death, relapse, or graft failure, whichever occurred first. OS was calculated from the time of transplant until death from any cause.

Genotyping and statistical analysis
Genotyping of GSTM1-null and GSTT1-null variants was performed on germline DNA, extracted from whole blood or peripheral mononuclear cells of all patients before the first HSCT as described by Lin et al. (1998).

Pearson Chi-square test was used to analyze the differences in demographics between groups with and without GST-null variants. Estimated cumulative relapse incidence by competing risk analysis with non-relapse mortality as a competing event and the difference among groups were estimated by Gray’s test (Gray 1988). The Fine–Gray model was used for competing risk regression in multivariable analysis to obtain adjusted model was used for competing risk regression in multivariable analysis to obtain adjusted hazard ratios.

Analyses for Windows and Mac, version 3.1.9.2 (RRID: SCR_013726; Dusseldorf, Germany).

In vitro functional studies of the associated variants

Cell models design and cell characterization
A set of 56 immortalized non-malignant lymphoblastoid cell lines (LCLs), acquired in 2012 from International HapMap Consortium’s CEPH Families Reference Panel 142,011/147712 (Coriell Cell Repository, Camden, NJ, USA), and a human monocytic leukemia cell line (THP1; acquired in 2018 from ATCC, Cat# TIB-202, RRID: CVCL_0006; Manassas, Virginia, USA), derived from a 1-year-old patient, were used for baseline and BU-induced functional assessment of GSTM1-null and GSTT1-null variants. The cells were immediately stored at −196 °C and were not used prior to the start of experiments. The cell lines were thawed and cultured in Roswell Park Memorial Institute Medium (RPMI) 1640 medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT) and 1% penicillin–streptomycin (Gibco) and incubated at 37 °C, 5% CO₂-humidified atmosphere according to the manufacturer’s recommendations. The number of passages between thawing and use in each in vitro experiment achieved the range between 5 and 15 times. The IDs of investigated LCLs used for each particular in vitro experiment are listed in Supplementary Table 1.

THP1 GSTM1- and GSTT1-knockout cell lines (THP1\textsuperscript{GSTM1(−/−)} and THP1\textsuperscript{GSTM1(−/−)}GSTM1(−/−) were prepared from parental THP1 representing non-null genotype for GSTM1 (THP1\textsuperscript{GSTM1(+/+)} and THP1\textsuperscript{GSTM1(+/+)}GSTM1(−/−)) using CRISPR/Cas9 gene-editing method. Plasmid PX458 containing 5’-TGATAGTGTGCGATGTGAC-3’ gRNA (GSTM1) or 5’-TAAAGGAGCTGTGGGTCC-3’ gRNA (GSTM1) (prepared by GeneScript. The Netherlands) was transfected into THP1 cells. 10,000 cells were fluorescence-activated cell sorted (FACS) in 24-well plates based on the presence of green fluorescence protein (GFP) 48 h post-transfection. After 48–72 h of recovery, THP1 cells were single-cell cloned in 96-well plates using FACS. Gene-modified clones were genotyped for the presence of deleterious mutations using Sanger sequencing and confirmed by Western blot for the success of gene knockout. Five clones of the same genotype were pooled in a population.

DNA and proteins of the selected cell lines (LCLs, THP1\textsuperscript{GSTM1(−/−)} and THP1\textsuperscript{GSTM1(−/−)} cell models) were extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and standard protein extraction protocol for western blot using RIPA lysis buffer (Sigma-Aldrich, Germany), respectively. The intracellular concentration of extracted proteins was measured using Bradford assay from Bio-Rad (Hercules, CA) according to the manufacturer’s
recommendations. Aliquots containing 20 μg of proteins, sample reducing agent and LDS sample buffer (Thermo Fisher Scientific, USA) were subjected to electrophoresis by using Invitrogen Novex Tris–Glycine Gels (Thermo Fisher Scientific, USA). Dry transfer to a nitrocellulose membrane was performed with the iBlot dry blotting system (ThermoFisher Scientific, USA). A membrane was blocked using 5% milk in PBS and 0.05% Tween 20. The following primary antibodies were used for protein labeling: β-Actin Mouse monoclonal antibody (Abcam Cat# ab6276, RRID: AB_2223210); GSTP1 Monoclonal Antibody Rabbit (DSHB Cat# CPTC-GSTP1-1, RRID: AB_2617266); GSTM1 Monoclonal Antibody Mouse (Thermo FisherScientific Cat# MA5-17,085, RRID: AB_2538556) and GSTT1 Polyclonal antibody Rabbit (Thermo FisherScientific Cat# PA5-22,011, RRID: AB_11154445). Lumii-Light WB Substrate (Roche, CH) was used for the detection of the secondary antibody linked with horseradish peroxidase (HRP). Band intensities were identified using Syngene G-Box System (Syngene, Frederick, MD, USA).

The glutathione transferase activity (Glutathione S-transferase [GST] Assay Kit, Sigma-Aldrich, USA) was measured on cell lysates obtained from one million cells (THP1\(^{GSTM1(-/-)}\) and THP1\(^{GSTM1(+/+)}\)) by measuring absorbance at 340 nm every minute for 10 min in a 200 μl well of a 96-well plate using Spectramax ID3 Multi-Mode microplate reader (Molecular Devices, USA) according to the manufacturer’s recommendations. GST activity was calculated as the following: \(\Delta A_{340nm} \text{ (min)} \times \text{ total volume of the reaction (ml)} / [5.3 \text{ mM}^{-1} \times \text{ volume of enzyme}]\) corrected according to the protein concentration in mg/ml obtained by using a Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, USA) according to the manufacturer’s guidelines.

The results of the characterization of CRISPR–Cas9 THP1 gene-edited cell models with target proteins (GSTM1, GSTT1 and GSTP1) and GST activity are presented in Supplementary Table 1) treated with 500 μM BU using RealTime-Glo MT Cell Viability Assay (Promega, USA). Concentrations of the total (GSH\(_2\)) (GSH-Glo Glutathione Assays, Promega, USA) and oxidized intracellular glutathione (GSSG) (GSH/GSSG-Glo Glutathione Assays, Promega, USA) were measured according to the manufacturers’ recommendations. Prior to measurement, cells from five \(GSTM1(+)\) and five \(GSTM1(-/-)\) LCLs and CRISPR–Cas9 gene-edited THP1 cell models were incubated for 48 h at 500 μM BU and 1% DMSO (control). Results are expressed as the relative proportion of GSSG to GSH\(_2\).

Concentrations of the total (GSH\(_2\)) (GSH-Glo Glutathione Assays, Promega, USA) and oxidized intracellular glutathione (GSSG) (GSH/GSSG-Glo Glutathione Assays, Promega, USA) were measured according to the manufacturers’ recommendations. Prior to measurement, cells from five \(GSTM1(+)\) and five \(GSTM1(-/-)\) LCLs and CRISPR–Cas9 gene-edited THP1 cell models were incubated for 48 h at 500 μM BU and 1% DMSO (control). Results are expressed as the relative proportion of GSSG to GSH\(_2\).

Caspase-Glo 3/7 assay (Promega) was used to measure the total activity of caspases-3 and -7 in a subset of 12 LCLs (Supplementary Table 1) and CRISPR–Cas9 gene-edited THP1 cell models at 48 h BU post-treatment (250, 500 and 1000 μM).

Chemiluminescent signals were measured using Victor3 (Perkin Elmer, Inc., USA). All BU-based data were normalized relative to the negative controls with 1% DMSO.

**Statistical analyses in vitro functional studies**

The cell-based experiments (IC\(_{50}\) distribution, end-point apoptosis and necrosis, real-time monitoring of viability,
apoptosis and necrosis, Caspase3/7 activities; and [GSSG/GSH\textsubscript{T}] ratios) were performed at least in duplicate and results are reported as observed means ± SD stratified by GST-null and GST-non-null variants. Statistical differences between genotypes were assessed using Mann–Whitney, t tests, or two-way ANOVA according to the normality of the distribution and compared to untreated controls using GraphPad Prism 7 software (RRID: SCR_002798). We considered \( p < 0.05 \) to be statistically significant in all analyses.

Results

**GSTM1- and GSTT1-double null genotypes are associated with higher relapse incidence**

Eighty-six children with malignancies aged 5 months–18 years (female/male, 44/42), who received myeloablative conditioning containing four-times-daily i.v. BU followed by HSCT, were enrolled in this study. The patients’ baseline characteristics at the time of their HSCT are summarized in Table 1. The number of patients who had experienced relapse was 16 (18.6% of included patients) with the median time to onset 203 days (range 35 to 817) and 12 (14.0%) patients died with the median time to onset 221 days (range 15 to 979). The median CumAUC of BU achieved 56.96 mg*h/L (concentration range 30.50–115.23 mg*h/L).

Regarding the genotype frequency, 49 patients were GSTM1-null (57.0%), 24 GSTT1-null (27.9%) and 9 had null genotypes in both GSTM1 and GSTT1 genes (10.5%). Characteristics of these patients according to GSTM1-null and GSTT1-null variants are shown in Supplementary Table 2.

Relapse was associated with GSTT1-null compared to GSTT1-non-null subgroups (42.1% vs 16.1%) in the univari-able analysis (\( p = 0.04 \), Fig. 1A, Table 2A). The GSTM1-null was not associated with relapse (Fig. 1B, Table 2A). However, patients carrying null genotypes in both GSTM1 and GSTT1 genes showed significantly increased risk of relapse compared to other genotype subgroups ([GSTM1-non-null/GSTT1-null and GSTM1-null/GSTT1-non-null]; \( p = 0.012 \), Fig. 1C, Table 2A) and this risk remained significant when other genotype subgroups were grouped together (using a gene–gene interaction model; 77.8% vs. 19.0%; \( p = 0.002 \), Fig. 1D, Table 2B). Significantly lower EFS was observed in the group of patients carrying (−/−) alleles in both GSTM1 and GSTT1 genes in comparison to others (54.2% vs. 11.1%, \( p < 0.001 \), Supplementary Fig. 2A). When gene variants were analyzed independently, none of them affected the EFS (data not presented). OS was not significantly associated with GST-null variants (using gene–gene interaction model; 67.1% vs. 37.0%, \( p = 0.401 \), Supplementary Fig. 2B).

**Table 1** Demographic and transplantation characteristics of pediatric patients at the time of HSCT and events follow-up

| Variables | N (%) |
|-----------|-------|
| Sex       |       |
| Male      | 42 (48.8) |
| Female    | 44 (51.2) |
| Ethnicity |       |
| Caucasian | 68 (79.1) |
| Other     | 18 (20.9) |
| Diagnosis |       |
| ALL       | 12 (14.0) |
| AML       | 43 (50.0) |
| MDS       | 31 (36.0) |
| Stem cell source |   |
| BM        | 35 (40.7) |
| CB        | 47 (54.7) |
| PBSCs     | 4 (4.7)  |
| Regimen conditioning | |
| Busulfan/cyclophosphamide | 64 (74.4) |
| Busulfan/melphalan | 2 (2.3) |
| Busulfan/cyclophosphamide/melphalan | 13 (15.1) |
| Busulfan/cyclophosphamide/etoposide | 7 (8.1) |
| Serotherapy |       |
| No        | 31 (36.0) |
| ATG       | 55 (64.0) |
| HLA match compatibility | |
| MRD       | 28 (32.6) |
| MUD       | 19 (22.1) |
| MMRD      | 3 (3.5)  |
| MMUD      | 36 (41.9) |
| Disease phase |       |
| CR1       | 38 (44.2) |
| CR2       | 10 (11.6) |
| CR3 or more* | 9 (10.5) |
| Never treated | 24 (27.5) |
| ND        | 5 (5.8)  |
| Intensity of conditioning* |   |
| 2         | 66 (76.7) |
| 3 or more | 20 (23.3) |
| Median (range) | |
| Age at HSCT (years) | 6.5 (0.5–18.2) |
| Weight (kg) | 24.5 (6.0–87.9) |
| Height (cm) | 122.5 (51.0–183.0) |

*All acute lymphoblastic leukemia; AML acute myeloid leukemia; BM, bone marrow; BU, busulfan; CB cord blood; CR1 first complete remission; CR2 second complete remission; CR3 third complete remission; HLA identical sibling; MDS myelodysplastic syndrome; MMUD non-identical unrelated; MMRD non-identical related; MUD identical unrelated; MRD identical related; ND no data; PBSCs peripheral blood stem cells

*Disease phase “CR3 or more” included all patients either in CR3 or more or in partial remission or those with > 10% of circulating myeloblasts before conditioning

2 alkylating agents (busulfan with cyclophosphamide or melphalan) and 3 agents (busulfan/cyclophosphamide with melphalan or etoposide) The enrolled patients were from CHU St Justine (Montreal, Quebec,
Relapse was compared between GSTM1-null and GSTT1-null variants combined with other possible risk factors (Table 2B). GSTM1/GSTT1 double null status was independently associated with relapse with an HR of 6.52 [95% CI, 2.76 – 15.42; \( p = 1.9 \times 10^{-5} \)]. 25%, 9.7% and 23.3% of patients with ALL, MDS and AML, respectively, were relapsed. Among them, all relapsed patients with the GSTT1-null genotype had ALL (Supplementary Table 3).
Table 2 Relationship between GSTM1-null and GSTT1-null variants and other risk factors with a cumulative incidence of relapse in univariable and multivariable competing risk analyses (Fine–Gray’s test)

### A)

| Covariable | Univariable analysis | Multivariable analysis |
|------------|----------------------|------------------------|
|            | HR  | 95% CI  | \( p \) value (C Risk)  | HR  | 95% CI  | \( p \) value (CRRisk)  |
| GSTM1 genotype |  |  |  |  |  |
| GSTM1(+) \((N=37)\) (Reference) | 1 |  | 0.372 |  |  |
| GSTM1(−/−) \((N=49)\) | 1.627 | (0.586–4.514) | 0.040 |  |  |
| GSTT1 genotype |  |  |  |  |  |
| GSTT1(+/−) \((N=62)\) (Reference) | 1 |  | 0.012 |  |  |
| GSTT1(−/−) \((N=24)\) | 2.822 | (1.058–7.528) |  |  |  |
| GSTM1 and GSTT1 genotypes (4 groups) |  |  |  |  |  |
| GSTM1(+) and GSTT1(+/−) \((N=22)\) (Reference) | 1 |  | 1.9 × 10–5 |  |  |
| GSTM1(−/−) and GSTT1(−/−) \((N=77)\) | 7.190 | (2.165–23.874) | 6.521 | (2.758–15.420) |  |  |

### B)

| Covariable | Univariable analysis | Multivariable analysis |
|------------|----------------------|------------------------|
|            | HR  | 95% CI  | \( p \) value (CRRisk)  | HR  | 95% CI  | \( p \) value (CRRisk)  |
| GSTM1 and GSTT1 genotypes (2 groups) |  |  |  |  |  |
| GSTM1(+) and GSTT1(+/−) \((N=22)\) | 7.190 | (2.165–23.874) | 6.521 | (2.758–15.420) |  |  |
| GSTM1(−/−) and GSTT1(−/−) \((N=77)\) | 7.190 | (2.165–23.874) | 6.521 | (2.758–15.420) |  |  |

Diagnosis

- ALL (Reference) \((N=63)\)
  - HR: 1.077 \( p = 0.232 \)
- AML
  - HR: 0.384 \( p = 0.503 \)
- MDS
  - HR: 0.384 \( p = 0.503 \)

Cum_AUC (mg*h/L)#

- < 59.2
  - HR: 3.891 \( p = 0.062 \)
- 59.2–98.6
  - HR: 1.741 \( p = 0.432–36.040 \)
- > 98.6
  - HR: 1.741 \( p = 0.201–15.055 \)

AUC_1stDose (mg*h/L)

- < 3.7
  - HR: 0.729 \( p = 0.090 \)
- 3.7–6.16
  - HR: 2.230 \( p = 0.276–17.991 \)
- > 6.16
  - HR: 2.230 \( p = 0.276–17.991 \)

Intensity of conditioning

- 2
  - HR: 0.382 \( p = 0.086–1.687 \)
- 3 or more
  - HR: 0.382 \( p = 0.086–1.687 \)

Disease phase

- CR1 (Reference) \((N=62)\)
  - HR: 1.088 \( p = 0.166 \)
- CR2
  - HR: 2.042 \( p = 0.651–6.786 \)
- CR3 or more
  - HR: 2.042 \( p = 0.651–6.786 \)
- Never treated
  - HR: 2.042 \( p = 0.651–6.786 \)

\textit{adj} adjusted; ALL acute lymphoblastic leukemia; AML acute myeloid leukemia; AUC\_day1 area under the curve for BU after the first dose of BU; 95% CI 95% confidence interval; CRRisk multivariate competing risk regression analysis that is presented with Fine–Gray proportional hazard ratios (HR); CR1, first complete remission; CR2, second complete remission; CR3, third complete remission; HR, hazard ratio; disease status; CumAUC, cumulative area under the curve for BU; MDS myelodysplastic syndrome.
LCL sensitivity to BU is associated with GSTM1, but not with GSTT1 genotypes

Significantly higher cell viability after treatment with BU was observed in LCLs with GSTM1-null genotype (1.8-fold, \(p = 0.013\)) and THP1 GSTM1(−/−) cells (1.5-fold, \(p = 0.0006\)) compared to GSTM1-non-null by 48 h end point (Fig. 2A, B, respectively) and the results were confirmed by 72-h kinetic measurements in LCLs (Supplementary Fig. 3). GSTT1-null, alone or in combination with GSTM1-null, did not significantly affect cell viability.

Table 2 (continued)

“CR3 or more” included all patients either in CR3 or more or in partial remission or those with >10% of circulating myeloblasts before conditioning.

*Intensity of conditioning, two alkylating agents (busulfan with cyclophosphamide or melphalan) and three agents (busulfan/cyclophosphamide with melphalan or etoposide).

\(^*\) CumAUC was calculated after 16 doses administered in 6 h intervals and is presented in mg*h/L of which one dose 3.7 mg*h/L is equivalent to 900 \(\mu\text{M} \times \text{min}\) and 6.16 mg*h/L is equivalent to 1500 \(\mu\text{M} \times \text{min}\).

Bold: significant \(p\) values below 0.05

GSTM1-null and GSTT1-null are presented as GSTM1(−/−) and GSTT1(−/−), respectively. GSTM1 non-null and GSTT1 non-null genotypes are presented as GSTM1(+) and GSTT1(+), respectively.
not show a significant association with BU-IC$_{50}$ in LCLs and THP1$_{GSTT1(−/−)}$ cell lines (Fig. 2C, D, respectively). No difference in baseline cell proliferation was seen between GSTM1-null and GSTM1-non-null cells (Fig. 3A), while the proliferation of GSTT1-null cells was significantly decreased in comparison to GSTT1-non-null carriers in LCLs carrying GSTT1-null genotype and THP1$_{GSTT1(−/−)}$ [\(p = 0.03\) (LCL, 48 h end-point measurement, Fig. 3B) and \(p < 0.05\) (THP1$_{GSTT1(−/−)}$, 72 h kinetic plot, Fig. 3C)].

**GSTM1-null genotype is associated with increased apoptosis and decreased primary necrosis after BU treatment**

In a subgroup of ten GSTM1-null LCLs, we observed increased early apoptosis and a decreased primary necrosis (\(p = 0.026\) and 0.006, respectively) at 48 h post-treatment with 250, 500 and 1000 µM of BU in comparison to ten GSTM1-non-null LCLs (Fig. 4A–D). No significant differences between both GSTM1 genotype groups were observed for the number of live cells and apoptotic cells at a later stage (the mix of necrotic and real apoptotic cells). Apoptosis was further assessed through measurement of caspase 3/7 activity according to GSTT1(−/−) variants showing significantly higher activation in GSTM1-null LCLs and THP1$_{GSTT1(−/−)}$ at 250, 500 and 1000 µM BU in comparison to GSTM1-non-null cells (\(p < 0.05\); Fig. 4E), while no differences were observed at baseline. BU-induced activation of caspase 3/7 was not significant in GSTT1-null LCLs (\(p = 0.21\)), while in THP1$_{GSTT1(−/−)}$ was significantly decreased (\(p = 0.002\); Fig. 4E) in comparison to GSTT1-non-null cells.

Cell death mechanisms were further followed by kinetic plots. We observed higher apoptotic rates in GSTM1-null cells (THP1$_{GSTM1(−/−)}$, LCLs) through the whole 72 h of follow-up after BU treatment when compared to GSTM1-non-null cells from unrelated individuals (Supplementary Figs. 4A and 4C, \(p < 0.0001\) and \(p = 2.6E-05\), respectively). In contrast, we observed lower necrotic rates in GSTM1-null cells (THP1$_{GSTM1(−/−)}$, LCLs) that were increasing after 26 h of BU treatment when compared to GSTM1-non-null cells (Supplementary Figs. 4B, D, \(p < 0.001\) and 1.4E-05, respectively). Apoptosis at the same time points was lower
(a) PI+ 
(b) PI- 
(c) V- 
(d) V+ 

| Phenotype | t   | p-value |
|-----------|-----|---------|
| Alive (C.)| 0.188 | 0.666 |
| Early apoptosis (D.) | 5.236 | 0.026 |
| Late apoptosis (B.) | 2.560 | 0.116 |
| Necrosis (A.) | 8.303 | 0.006 |

**; p < 0.05

(e) GSTM1 genotype

GSTM1 +/+(N=10)
GSTM1 -/- (N=10)

(e) GSTT1 genotype

GSTM1 +/+(N=8)
GSTM1 -/- (N=4)

LCL

P = 0.0715 (genotype factor)
P < 0.0001 (treatment factor)

BU dose (µM)

P = 0.212 (genotype factor)
P < 0.0001 (treatment factor)

BU dose (µM)

P = 0.0015 (genotype factor)
P = 0.0036 (treatment factor)

***; p < 0.001
**; p < 0.01
*; p < 0.05
in these cells accounting for the faster cell death, mainly as a result of primary necrosis.

\[ \text{[GSSG/GSH]} \text{~ratios are higher in \textit{GSTM1-non-null} LCLs and THP1 \textit{GSTM1(+/−)} cells after BU treatment compared to \textit{GSTM1-null} while total GSH levels remain unchanged} \]

At baseline, no differences in [GSSG/GSH\textsubscript{2}5] ratios were observed between \textit{null} and \textit{non-null} LCLs and THP1 cells for the \textit{GSTM1} gene. However, 48 h after 500 μM BU treatment, [GSSG/GSH\textsubscript{2}5] ratios were increased 1.6- (\(p = 0.02\), LCLs) and 1.3-fold (\(p = 0.005\), THP1) in \textit{GSTM1 non-null} compared to \textit{GSTM1-null} (Fig. 5A, C, respectively). A similar trend was observed after the 250 μM BU treatment (48 h) in THP1 \textit{GSTM1(−/−)} in comparison to THP1 \textit{GSTM1(+/−)} cells (Fig. 5C). In THP1, no significant difference was observed according to \textit{GSTT1} genotype after the treatment with BU or at baseline (Fig. 5D). In addition, we observed a significant increase in total GSH levels after the 500 μM BU treatment, irrespective of the \textit{GSTM1} genotype (Fig. 5B, \(p = 0.001\)), thus indicating the potential for BU-related induction of GSH synthesis.

\section*{Discussion}

Our clinical association study performed in 86 children with HMs undergoing HSCT following BU-based conditioning regimens demonstrated that patients harboring homozygous deletions in both \textit{GSTM1} and \textit{GSTT1} genes presented a high risk of relapse (HR\textsubscript{relapse} 7.2 [95% CI, 2.2–23.9; \(p = 0.002\)]) and a lower EFS (HR\textsubscript{EFS} 4.092 [95% CI, 1.829–9.152; \(p = 0.001\)]). After adjustment for known risk factors (diagnosis, disease status, the intensity of conditioning regimen and BU exposure), the association remained significant demonstrating that the deletion of both \textit{GST} genes is an independent risk factor for relapse (adjusted HR 6.52 [95% CI, 2.8–15.4; \(p = 1.9 \times 10^{-5}\))). Although it is a small cohort, this is the first report on the risk of post-HSCT relapse in relation to the germline \textit{GSTM1-} and \textit{GSTT1-null} variants in children with HMs. Until now, only one study conducted in BU/CY-based HSCT settings although in adults showed increased relapse rates in patients carrying \textit{GSTM1-null} genotype, while no association was identified with \textit{GSTT1-null} genotype (Terakura et al. 2020). Concerning non-transplant-based studies in pediatric or adult patients, a similar association between \textit{GSTM1/GSTT1 double null} carriers and increased risk of relapse (Barragan et al. 2007; Borst et al. 2012; Takano et al. 2003, Xiao Q. et al. 2014), lower complete remission rate (Xiao Z. et al. 2008) and lower EFS were demonstrated (Chen et al. 1997; Hall et al. 1994; Leonardi et al. 2017; Rocha et al. 2005; Stanulla et al. 2000; Takano et al. 2003; Teachey and Hunger 2013; Woo et al. 2000; Zhang et al. 2017). There are nevertheless a few studies showing no such association (Franca et al. 2012; Zariefar et al. 2013), in which the small number of patients or the different treatment regimens may have mainly precluded defining a relationship between GST variants.

Based on the known detoxifying role of GSTs, our results from the clinical association are contradictory. Although GSTA1 is the main enzyme involved in BU detoxification, \textit{GSTM1} is also highly expressed in the liver and recognized as involved in BU conjugation (Ansari et al. 2017; Bremer et al. 2015; Czerwinski et al. 1996; Kim et al. 2011), precluding the \textit{BU} to cross-link with the DNA strands. Functional variants of the genes coding for GSTs may then interfere in HSCT by affecting \textit{BU} metabolism. It is known that low \textit{BU} exposure (Cum\textsubscript{AUC} < 59 mg × h/L) is associated with graft failure and relapse (Ansari et al. 2017; Bartelink et al. 2016; Philippe et al. 2016), whereas high \textit{BU} exposure (Cum\textsubscript{AUC} > 98.6 mg × h/L) could reduce post-HSCT relapse in leukemia at the cost of an increase in organ toxicities, and therefore transplantation-related mortality (Ansari et al. 2017; Bartelink et al. 2016; McCune et al. 2002; McCune and Holmberg 2009; Philippe et al. 2016). However, at the level of HCs, less is known about the direct effect of \textit{BU}.

We compared \textit{BU}-related cell death mechanisms in LCLs and THP1 with and without \textit{GSTM1} and/or \textit{GSTT1} genes after exposure to BU. LCLs were chosen as the in vitro model to resemble heterogeneity seen in a clinical cohort. Since each LCL is derived from a different individual genetic background, the studied effect due to \textit{BU} is specifically related to the \textit{GSTM1-} and \textit{GSTT1-null} and \textit{−non-null} genotypes. The acute monocytic leukemia (THP1) cell model was selected for the production of the CRISPR–Cas9 \textit{GSTM1-} and/or \textit{GSTT1} knockout model, as we have the most patients with a relapsed AML (Supplementary Table 3). Selection of these two cell models aids in evaluating \textit{BU}-dependent and -independent \textit{GSTT1} and \textit{GSTM1} effects. We demonstrated
that only \textbf{GSTM1-null} (but not \textbf{GSTT1-null}) is associated with higher resistance to BU as determined by higher BU-IC$_{50}$ values of \textbf{GSTM1-null} LCLs and \textbf{THP1}($\text{GSTM1}^{(-/-)}$) cells. This could be due to a change in the redox equilibrium as demonstrated by lower levels of oxidized GSH, lower primary necrosis and higher early apoptosis. An increase of \textbf{GSTM1-null} LCL’s viability was confirmed either by continuous follow-up of redox potential within 72 h. Apoptosis/necrosis kinetic results demonstrate that BU-induced apoptotic processes are more pronounced in \textbf{GSTM1-null} LCLs. In contrast, primary necrotic cell death was more pronounced in \textbf{GSTM1-non-null} cells when comparing with the \textbf{GSTM1-null} cells. In addition, primary necrosis was significantly induced at an earlier stage in \textbf{GSTM1-non-null} cells. These results show that \textbf{GSTM1-null} variants can modulate BU-induced cell death, which were supplemented further by increased activation of known apoptotic markers caspase-3 or -7 in \textbf{GSTM1-null} LCLs and \textbf{THP1} in comparison to \textbf{GSTM1-non-null} cells. Importantly, observed reduced rates of \textbf{GSTM1}-dependent cell death cannot be attributed to the increased baseline cell proliferation.

The findings of higher primary necrosis, lower early apoptosis and lower cell viability in \textbf{GSTM1-non-null} HCs compared to \textbf{GSTM1-null} cells treated with BU were unexpected. Contrary to our observations, many studies showed associations between increased expression or activity of GSTs and resistance mechanisms against a range of cytotoxic drugs (Hoban et al. 1992; Smith et al. 1989). These results could potentially be explained by not only direct detoxification with GSH, but also through negative regulation of pro-apoptotic protein kinases, such as apoptosis signal-regulating kinase 1 (ASK1) (Board and Menon 2013; Tew and Townsend 2012). For instance, stress conditions cause the release of ASK1 from GSTM1, thereby leading to induction of apoptosis, which was shown in our experiments.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig_5.png}
\caption{Glutathione levels in LCLs stratified according to \textit{GSTM1-null} variant, \textbf{THP1}($\text{GSTM1}^{(-/-)}$) and \textbf{THP1}($\text{GSTT1}^{(-/-)}$) cells. [GSSG/GSH$_{\text{t}}$] ratio (A) and GSH$_{\text{t}}$ (B) in LCLs were calculated stratified according to \textit{GSTM1-null} variant; and [GSSG/GSH$_{\text{t}}$] ratios in CRISPR–Cas9 edited \textbf{THP1}($\text{GSTM1}^{(-/-)}$) (C) and \textbf{THP1}($\text{GSTT1}^{(-/-)}$) (D) cell models after the treatment with 500 μM BU. Statistical analysis was performed by the two-way ANOVA considering 250 or 500 μM BU concentration (genotype factor); \textit{t} tests between \textit{GST(−/−)} variants in each condition separately were used; no statistically significant differences were observed between \textit{GST(−/−)} variants in LCLs and THP-CRISPR–Cas9 models in either 1% DMSO or medium; \textit{p} values below 0.05 were considered statistically significant. \textbf{GSTM1-null} and \textbf{GSTT1-null} are presented as \textit{GSTM1}($^{(-/-)}$) and \textit{GSTT1}($^{(-/-)}$), respectively. \textbf{GSTM1 non-null} and \textbf{GSTT1 non-null} genotypes are presented as \textit{GSTM1}($^{(+)}$) and \textit{GSTT1}($^{(+)}$), respectively.}
\end{figure}
after induction with BU. In addition, *GSTM1-null* cells carrying more free ASK1 for phosphorylation activation are expected to have more apoptosis upon BU-induced stress in comparison to *GSTM1-null* cells which is in accordance with our in vitro results.

However, the observed paradox in increased cell death of *GSTM1* well-expressed cells upon BU treatment could additionally be explained by findings of the study of DeLeve et al. (2000), demonstrating that in murine hepatocytes BU is cytotoxic also through oxidative stress caused by BU metabolites (BU glutathione S-conjugate thiophenium ion, GS*HTh*) and by the depletion of GSH in addition to DNA alkylation. The toxic metabolites of BU/GSH metabolism are mainly oxidized by flavin-containing monoxygenases (FMOs, e.g., FMO3) and cytochromes (CYPs, e.g., CYP3A4) (El-Serafi et al. 2017) to water-soluble non-toxic metabolites [e.g., sulfolane (Uppugunduri et al. 2017)].

However, CYP3A4 and FMO3 are mainly expressed in the liver (accounting for 54% of overall tetrahydrothiophene [HTH] disappearance, the metabolite of BU), and less in LCLs, as observed in our laboratory (data not shown) and by others (https://www.proteinatlas.org). After RNA sequencing in LCLs, very low or no gene expressions of *CYP 2D6, 2C19, 2C9, 2B6, 2C8, 4A11, 3A4, FMO1 and FMO3* were identified. In this context, the oxidative burst caused by electrophilic molecules from BU–GSH conjugation (Udensi and Tchounwou 2014; Zmorzynski et al. 2015) in addition to the absence of CYP3A4 and FMO3 could be a reason for the lower sensitivity of *GSTM1-null* HCs to BU, as observed in LCLs and THP1. In contrast, higher total expressions of CYPs and FMOs in hepatocytes (El-Serafi et al. 2017) could explain why GSTA1-slow BU metabolizing individuals in addition to the absence of GSTM1 activity show potentially more treatment-related toxicities [e.g., SOS (Srivastava et al. 2004) and aGvHD (Elhasid et al. 2010)] than carriers with normal GST’s enzyme activities. A hypothetical comparative model of the difference in BU fate between hepatocytes and lymphocytes is suggested in Supplementary Fig. 5 and warrants further investigation.

Another relevant observation is the significantly increased post-HSCT relapse in *GSTT1-null* when combined with *GSTM1-null* genotype in children with HMs. The link between GSTT1 and post-HSCT relapse is not clear yet. Our in vitro observations cannot be attributed to the BU-related differences in IC₅₀ values or [GSSG/GSH₄] ratios. Other pharmacogenomics studies also demonstrated that genetic variations in *GSTT1* are not associated with BU clearance or liver toxicity (Gaziev et al. 2010; Goekkurt et al. 2007; Kim et al. 2011; Srivastava et al. 2004). Nevertheless, we observed faster baseline proliferation in *GSTT1-non-null* LCLs/THP1 and a slightly higher baseline increase of caspase 3/7 activation compared to those with *GSTT1-null* genotype, indicating GSTT1 potential involvement of BU-independent mechanisms in the relapse development.

The results of the present clinical study are limited by the retrospective study design and relatively small pediatric sample size with no clinical validation cohort. However, the sample size of 86 patients has at least 80% power with 10% of observed combined *GSTM1/GSTT1 double null* variants’ frequency and relapse incidence with the estimated observed effect size of ≥ 7.0 and alpha value of 0.05. The primary diagnosis of HMs was made at the referring institution and was not centrally reviewed. Well-known risk factors such as somatic genetic/cytogenetics abnormalities, the donor DNA and the initial response to the treatment (e.g., MRD) were not available. However, as described in Supplementary Table 2, similar characteristics were present between the GST genetic subgroups (*p* values > 0.05). The *GST-null* variants were not associated with the status of the disease before HSCT and we assume that the germline genotype impact on protein expression was present in malignant cells as shown by Weiss et al. (2007). The majority of cases in our study underwent a BU–CY conditioning regimen; however, it is not known if this association is specific to a BU–CY conditioning regimen only or unspecific to other chemotherapeutics used in the HSCT setting (e.g., Thio or Mel) (Hao et al. 2020). For instance, active metabolites of CY (e.g., acrolein) are also eliminated by GSH conjugation catalyzed by GSTs (Uppugunduri et al. 2017). This needs to be evaluated in the future with a focus on whether GSTs play a major role in determining clinical outcomes. This aspect is currently being evaluated by our group using a cohort from multiple centers with the usage of multiple conditioning regimens. Furthermore, the transplant-related mortality or combined toxicities were not associated with the *GSTM1- and GSTT1-null* variants (data are not shown), suggesting compensation of BU conjugation by other GSTs, especially GSTA1, which is mainly expressed in hepatocytes and other somatic cells.
Conclusions

In summary, we report that GSTM1/GSTT1 double null genotypes could serve as genetic biomarkers for identifying pediatric patients with HMs at higher risk of relapse after an allogeneic HSCT following BU-containing conditioning. On the other hand, the absence of those markers might predict the patients who more likely will respond to the chemotherapy-based conditioning. Functional studies indicated different mechanisms of cell death upon exposure to BU based on the presence or absence of GST-null alleles and the in vivo impact of those findings must be further explored.

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Authors’ contributions SJM, CRSU, MA designed the study; SJM, VM, CRSU executed cell viability experiments; SJM, CRSU isolated DNA/mRNA and genotyping from cell lines; SJM, VM contributed to apoptosis and necrosis-based experiments and produced CRISPR–Cas9 THP1 knockout cell models; SJM executed real-time viability and apoptosis/necrosis, caspase 3/7 and GSH–based experiments and western blotting; SJM, CRSU, SR: GST activity; MA, MAR, YC, JJB, RGMB, JHD, CP, SC, HB, MK contributed to patients’ sample and clinical data processing; SJM executed computational and statistical analysis of clinical data that were verified by MA, TN, CRSU, HB; SJM, MA, VM, TN, CRSU contributed to interpretation of results; SJM drafted the manuscript; all authors revised the study critically. MA had final responsibility for the decision to submit for publication. All the authors read and approved the final manuscript.

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Data availability All data presented are provided freely in this manuscript including any supplementary data. The raw datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no competing interests related to the submitted work.

Ethics approval The Institutional Review Board or ethics committees approved the study.

Consent to participate All patients and/or parents provided informed consent.

Consent for publication All authors are aware of the study and approve of this submission.

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