Glutathione *S*-transferase homozygous deletions and relapse in childhood acute lymphoblastic leukemia: a novel study design in a large Italian AIEOP cohort

Aim: In the AIEOP-BFM 2000 trial, 15% of pediatric patients treated according to risk-adapted polychemotherapeutic regimens relapsed. The present study aimed to investigate the influence of *GST-M1* and *GST-T1* deletions on clinical outcome of children with acute lymphoblastic leukemia treated according to the AIEOP-BFM ALL 2000 study protocol. **Materials & methods:** A novel-design, two-phase study was applied to select a subsample of 614 children to be genotyped for the deletions of *GST* genes. Cumulative incidence of relapse was then estimated by weighted Kaplan–Meier analysis, and the Cox model was applied to evaluate the effect of *GST-M1* and *GST-T1* isoenzyme deletions on relapse. **Results:** No overall effect was found, but the *GST-M1* deletion was associated with better clinical outcome within prednisone poor-responder patients (hazard ratio [HR]: 0.45; 95% CI: 0.23–0.91; p = 0.026), whereas the *GST-T1* deletion was associated with worse outcome in the standard-risk group (HR: 4.62; 95% CI: 1.04–20.6; p = 0.045) and within prednisone good responders (HR: 1.62; 95% CI: 1.02–2.58; p = 0.041). **Conclusion:** Our results show that *GST-M1* and *GST-T1* homozygous deletions have opposite correlation with relapse, the former being protective and the latter unfavourable in specific subsets of acute lymphoblastic leukemia patients.

Original submitted 1 August 2012; Revision submitted 27 September 2012

KEYWORDS: acute lymphoblastic leukemia ▪ glutathione *S*-transferase ▪ polymorphisms ▪ prognostic genetic factors ▪ relapse ▪ risk-adapted polychemotherapy ▪ two-phase design

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Nowadays, more than 80% of pediatric patients affected by acute lymphoblastic leukemia (ALL) become long-term cancer survivors and healthy adults [1–3]. Despite continuous advances in survival rates during the past 50 years, due to the successful risk-adapted polychemotherapy, relapse remains the main cause of treatment failure, with salvage therapy being still associated with unsatisfactory outcome [4]. Choice of optimal intensity of front-line therapy, by promptly employing more aggressive regimens in patients with less-responsive disease, and by reducing intensity of treatment and the related toxicity in responsive patients, is considered the best approach to optimize a patient's outcome. ALL is a heterogeneous disease, having many subtypes characterized by genetic alterations in the leukemic cells of patients' whose prognostic values are generally known (i.e., unfavorable chromosomal translocations t[9;22] and t[4;11]) and considered for treatment risk stratification. On the contrary, patients' polymorphisms in genes influencing drug disposition, metabolism and mechanisms of action are generally not taken into account in current clinical protocols, with the exception of thiopurine *S*-methyltransferase variants used for modulating 6-mercaptopurine dosage. Such genetic features might affect treatment efficacy and could therefore be promising candidates to better assess the risk of each patient and to personalize therapy [5,6].

Glutathione *S*-transferases (GSTs) are phase II enzymes that catalyze the conjugation of electrophilic compounds to glutathione (GSH), thus producing water-soluble compounds that are more easily excreted via the kidneys. As metabolizing enzymes, they detoxify a wide range of drugs, including those normally employed in chemotherapy regimens [7–13]. Furthermore, GSTs sequester alkylating agents and steroids by direct binding [14] and also act as cell-signaling modulators by protein–protein interactions. In particular, GST-P1 and GST-M1 are negative regulators of proapoptotic signaling pathways. Overexpression of GST proteins and high levels of GSH have been associated with the development of drug resistance in a variety of cell lines and tumor tissues, [15,16] probably as a consequence of enhanced metabolism and decreased bioavailability of anticancer agents, as well as of impairment of the apoptotic process in malignant cells.

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GST-M1 and *GST-T1* exhibit a genetic polymorphism in Caucasians, with 42–60 and 13–26% of individuals displaying a homozygous deletion of the gene (null genotype), respectively. Subjects carrying the null variants fail to express the corresponding proteins [17,18]. *GST-M1* and *GST-T1* deletions were previously proposed as candidates for disease susceptibility and as variables influencing clinical outcome in childhood ALL, although controversial/conflicting results have been reported [19–24].

Pharmacogenomic research is challenged by the need to investigate large cohorts but, when precious biological samples from data banks need to be analyzed, resources might be limited for data ascertainment on all subjects. For this reason, we propose a two-phase design that allows the study of information available from large cohorts (known as the first phase) and also information available only on a selected subset (the second phase) of the same cohort at the same time. The design optimizes the choice of the subcohort to be genotyped in order to acquire efficient estimates [25].

In this study, we propose a two-phase pharmacogenetic design to investigate the influence of *GST-M1* and *GST-T1* deletions on clinical outcome in the large cohort of children with ALL treated according to the AIEOP-BFM ALL 2000 study protocol.

Materials & methods

Study population

Patients

This study was based on 1999 consecutive patients (mainly European Caucasians, aged between 1 and 18 years, median age: 5 years) who were newly diagnosed with Philadelphia chromosome–negative ALL in Italian AIEOP centers between September 2000 and July 2006. Within this cohort, 1113 patients were males (55.7%). The majority of patients were preschool children (age less than 6 years; 59.6%), while 402 (20.1%) were older than 10 years of age. Patients were treated according to the AIEOP-BFM ALL 2000 study protocol (ClinicalTrials.gov identifier NCT00613457 [101]). Based on minimal residual disease (MRD) levels, response to the first week of steroids, resistance to induction therapy and presence of chromosomal translocations t(4;11) and t(9;22) [26], patients were divided into standard- (515; 25.76%), medium- (1173; 58.68%) and high-risk groups (311; 15.56%), and treated with risk-adjusted polychemotherapeutic regimens [27]. Overall, 306 patients relapsed (15.3%), 28 in the standard-, 186 in the medium- and 92 in the high-risk group (Table 1). Written informed consent was obtained from the parents or legal guardians before patient enrolment, while the protocol was approved by the ethics committee of each participating institution.

Study design

This work adopts a novel approach, known as two-phase design, for both the selection of the subsample to be genotyped and the statistical analysis for the correlation between genotype and clinical outcome [25,28,29].

In principle, the design selects for genotyping patients of the AIEOP-BFM ALL 2000 trial, along the following optimal sampling fractions: first, 100% of the patients who relapsed by April 2008 (306 cases); and second, 13, 26 and 64% of nonrelapsed patients randomly chosen within the standard-, medium- and high-risk groups, respectively, for a total subset of 460 patients in remission. These optimal sampling fractions have been set on the basis of a pilot study on 164 genotyped patients and vary proportionally to the genetic variability reported within each of the risk groups, thus maximizing the precision of the estimate of the genotype effect on outcome. Because of this optimization, the subcohort is not representative of the entire cohort and thus

Table 1. Sampled genotyped subgroup with respect to the whole cohort of 1999 acute lymphoblastic leukemia patients enrolled in the AIEOP-BFM ALL 2000 study protocol, classified according to relapse and risk group.

Relapse	Risk group: genotyped subgroup (n)/ whole cohort (n), (%)			Total cohort: genotyped subgroup (n)/whole cohort (n), (%)
	Standard	*Medium*	*High*	
No	56/487 (11.5)	196/987 (19.9)	111/219 (50.7)	363/1693 (21.4)
Yes	22/28 (78.6)	149/186 (80.1)	80/92 (87.0)	251/306 (82.0)
Total	78/515 (15.1)	345/1173 (29.4)	191/311 (61.4)	614/1999 (30.7)

The percentage of effective sampling fraction in each stratum is reported in brackets.

the method subsequently adopted for statistical analysis introduces appropriate weights in order to recover the representativeness of the subsample [25,28,29]. The optimal sampling fractions have been computed using the software developed by Reilly and Salim available from the Karolinska Institutet, Stockholm, Sweden [102]. Software for mean score analysis and optimal design of two-phase studies was written in R (R cran, R Foundation for Statistical Computing, Vienna, Austria), SPLUS (Insightful Corp., WA, USA) and STATA (StataCorp LP, TX, USA).

Overall, out of the 766 children for whom genotyping was required, stored DNA material was available for 251 relapsed individuals and for 363 patients in remission. Table 1 reports the distribution of genotyped patients in comparison with the whole cohort and the effective sampling fractions used in each stratum.

ALL pediatric patients have been considered in subgroups defined according to the risk-adapted polychemotherapeutic protocols they were treated with (Table 2). Furthermore, they have been also been analyzed by subgroups defined according to the early (steroid prephase) *in vivo* response (Table 3). This major subdivision has been introduced in order to reach more informative results for ALL patients carrying detailed features. Since we are referring to a large cohort of patients, the defined subgroups still remain large in size.

Statistical analysis

The influence of the GST genotypes on hazard of relapse was evaluated. The analysis takes into account the different sampling probabilities by risk group (standard, medium and high) and status by April 2008 (relapse or no relapse), with a median follow-up of 4 years [28]. In practice, each genotyped individual is considered to represent the similar nongenotyped individuals in the same strata of the whole cohort. This is performed using weights equal to the inverse of the probability to be sampled. For example, the 56 genotyped children in the standard-risk group (Table 1) who did not relapse are weighted by the inverse of the sampling fraction (by 1/0.115) so that they contribute to statistical analysis for the 487 patients not relapsed in the standard risk group. Similarly, each of the 22 relapsed children genotyped in the standard-risk group are weighted (by 1/0.786) to represent all relapsed children in the same risk group. These weights were used in the evaluation of the distribution of *GST-M1* and *GST-T1* deletions within the ALL cohort by the adjusted χ^2 statistics [28], as well as in estimating the relapse cumulative incidence by Kaplan–Meier estimator, censoring toxic deaths. The comparison was based on the Wald test in a Cox model with genotype deletion as covariate. The weighted Cox model for two-phase design was applied to evaluate the effect of deletion on hazard of relapse, adjusting for risk group, age and gender. A secondary analysis evaluated the effect of deletion on the *in vivo* response to the prednisone prephase by means of a weighted logistic model, adjusted by age group and gender. Two-sided p-values <0.05 were defined as statistically significant. Analyses were performed with the survey package of the software R [28].

Genetic analysis

DNA of the patients was provided by the AIEOP Biobank (Clinical and Experimental Hematology, Department of Paediatrics, University of Padua, Italy). Patients' DNA for genotyping analysis was extracted from immature blasts of bone marrow aspirates at diagnosis or, when not available, at relapse by use of a commercial kit (Qiagen, Milan, Italy) according to the manufacturer's protocol. To rule out the possibility that genetic mutations were acquired upon blasts proliferation, genotyping analyses were performed on matched bone marrow DNA (tumoral, AIEOP Biobank) and DNA extracted from peripheral blood of the patient in remission, any time it was possible. Genotypes were identical between samples of the same individual.

Genotype analysis for both *GST-M1* and *GST-T1* stratifies individuals into two genetic categories, one being homozygous or heterozygous (norm) and the other having a homozygous deletion (null). Genotyping was performed by multiplex PCR as previously described [30]. Only samples in which the β-globin band (used as an internal quality control of successful PCR reaction) was clearly detected were accepted. To avoid false null results due to failure in primer annealing, patient samples were mass-amplified (at least 20 at once) and randomly replicated. Overall, deletion frequencies were those expected for Caucasians.

Results

Table 4 summarizes the polymorphism distribution among the 614 patients, with 304 null *GST-M1* (51.4%) and 107 null *GST-T1* (16.3%) patients. Cumulative incidence of relapse, estimated with proper weighting to account for study design, is reported in Figure 1 for

Table 2. Impact of glutathione *S*-transferase genotypes (analyzed singly or in combination) on the hazard of relapse in acute lymphoblastic leukemia patients stratified by risk group.

GST genotype		TOT	Relapses (n)	Weighted 5-year cumulative incidence of relapse (%)	SE	Hazard ratio (95% CI)	p-value
Standard risk (75 patients)							
GST-M1	Norm	36	9	4.3	1.7	1 (–)	–
	Null	39	12	7.4	2.3	1.85 (0.51–6.67)	0.350
GST-T1	Norm	64	16	4.8	1.2	1 (–)	–
	Null	11	5	18.0	12.3	4.62 (1.04–20.6)	0.045*
GST-M1/ GST-T1	Norm/norm	34	9	4.5	1.8	1 (–)	–
	Null/norm	30	7	4.9	2.0	1.08 (0.30–3.85)	–
	Norm/null	2	0	25.7	8.9	–[†]	–
	Null/null	9	5	18.0	12.3	4.78 (0.99–23.05)	NA[‡]
Medium risk (340 patients)							
GST-M1	Norm	163	75	21.3	3.2	1 (–)	–
	Null	177	72	17.0	2.5	0.84 (0.56–1.26)	0.400
GST-T1	Norm	281	122	18.6	1.9	1 (–)	–
	Null	59	25	21.0	5.9	1.16 (0.68–1.99)	0.580
GST-M1/ GST-T1	Norm/norm	139	64	20.7	3.4	1 (–)	–
	Null/norm	142	58	16.6	2.7	0.85 (0.54–1.32)	–
	Norm/null	24	11	25.8	12.3	1.26 (0.56–2.82)	–
	Null/null	35	14	18.1	6.6	0.96 (0.46–1.97)	0.847[§]
High risk (188 patients)							
GST-M1	Norm	105	47	35.4	6.6	1 (–)	–
	Null	83	31	29.0	6.4	0.62 (0.36–1.06)	0.080
GST-T1	Norm	149	59	30.6	4.6	1 (–)	–
	Null	37	18	41.0	14.1	1.39 (0.76–2.55)	0.290
GST-M1/ GST-T1	Norm/norm	82	36	34.4	7.4	1 (–)	–
	Null/norm	67	23	25.9	6.5	0.56 (0.30–1.03)	–
	Norm/null	21	10	38.3	17.0	1.15 (0.52–2.54)	–
	Null/null	16	8	42.8	22.4	1.00 (0.42–2.36)	0.463[§]

Analysis by weighed Cox model. Hazard ratio adjusted by sex and age class.
**p < 0.05.*
[†]Because of the small number of patients and relapses in GST-M1/GST-T1 *norm–null and null–null subjects, they were pooled together.*
[‡]No interaction term was assessed because of small number of patients and relapses in GST-M1/GST-T1 *norm–null and null–null subjects, which were pooled together.*
[§]p-value for genotype interaction.
NA: Not assessed; SE: Standard error; TOT: Total.

both GST isoforms. The incidence of relapse was similar for patients with and without *GST-M1* deletion (5 years cumulative incidence ± standard error: 16.1% ± 1.9 vs 19.1% ± 2.2, respectively; p = 0.25; Figure 1A). Table 5 reports the results of the Cox model adjusted by risk group, age and sex. Subjects with deleted *GST-M1* had hazard ratio (HR) of 0.82 with respect to nondeleted subjects (p = 0.21; 95% CI: 0.61–1.12). In univariate analysis, the deletion of *GST-T1* was associated with a higher incidence of relapse (p = 0.05; Figure 1B). This association loses statistical significance when evaluated adjusting by risk group, sex and age in the Cox model

(p = 0.14; HR: 1.34, 95% CI: 0.90–2.00). To assess whether the combination of the two deletions had an increased risk of relapse, we included an interaction term in the Cox model. Results were not statistically significant (overall p = 0.64; Table 5 & Figure 1C).

Another test for interaction was applied in the Cox model to assess the effect of each polymorphism within subgroups defined by risk stratification (Table 2) and *in vivo* prednisone response (Table 3). Although none of these interaction tests reached statistical significance, explorative analysis in Table 2 shows that in the standard-risk group, the *GST-T1*-null genotype had a significant impact on relapse with more than fourfold increase in risk when compared with nonmutated subjects (p = 0.045; HR: 4.62; 95% CI: 1.04–20.6). An adverse role of the deletion of *GST-T1* was similarly observed in the prednisone good responder (PGR) group (p = 0.041; HR: 1.62; 95% CI: 1.02–2.58), whereas a protective role of *GST-M1* deletion was seen in the prednisone poor responder (PPR) group (p = 0.026; HR: 0.45; 95% CI: 0.23–0.91; Table 3). Analysis of combined genotypes did not provide significant results, possibly due to small numbers.

Noticeably, there was no difference in *GST-M1* and *GST-T1* genotype frequencies or in the combined genotype distribution between PGR and PPR (logistic model: *GST-M1*, p = 0.36; and *GST-T1*, p = 0.55, data not shown), suggesting that *GST-M1* and *GST-T1* are not decisive to predict prednisone response itself.

Discussion

Pharmacogenetic studies aimed at investigating the impact of homozygous deletions of *GST-M1* and *GST-T1* on clinical outcome have provided conflicting results [19–22,31–33]; small numbers of patients, insufficient statistical power, disease complex phenotypes, differences in therapeutic procedures and differences in race may explain the substantial heterogeneity

Table 3. Impact of glutathione *S*-transferase genotypes (analyzed singly or in combination) on relapse in acute lymphoblastic leukemia subjects stratified by *in vivo* prednisone response.

GST genotype		TOT	Relapses (n)	Weighted 5-year cumulative incidence of relapse (%)	SE	Hazard risk (95% CI)	p-value
Prednisone good responders (473 patients)							
GST-M1	Norm	233	99	17.0	2.2	1 (–)	–
	Null	240	96	15.3	1.9	0.96 (0.66–1.38)	0.820
GST-T1	Norm	384	154	14.9	1.3	1 (–)	–
	Null	88	40	23.4	5.6	1.62 (1.02–2.58)	0.041*
GST-M1/ GST-T1	Norm/norm	193	82	16.0	2.3	1 (–)	–
	Null/norm	191	72	13.7	2.0	0.92 (0.61–1.38)	–
	Norm/null	39	16	23.5	9.2	1.51 (0.73–3.13)	–
	Null/null	49	24	22.9	7.1	1.58 (0.86–2.93)	0.778†
Prednisone poor responders (129 patients)							
GST-M1	Norm	70	31	34.2	7.9	1 (–)	–
	Null	59	19	24.0	6.6	0.45 (0.23–0.91)	0.026*
GST-T1	Norm	109	42	29.4	5.4	1 (–)	–
	Null	19	8	31.8	14.5	1.14 (0.51–2.55)	0.750
GST-M1/ GST-T1	Norm/norm	61	26	33.2	8.4	1 (–)	–
	Null/norm	48	16	24.5	7.4	0.48 (0.22–1.05)	–
	Norm/null	8	5	47.4	31.2	2.06 (0.73–5.82)	–
	Null/null	11	3	20.0	13.3	0.41 (0.13–1.32)	0.290†

Analysis by weighed Cox model. Hazard ratio adjusted by sex and age.
**p < 0.05.*
†p-value for genotype interaction.
Norm: Normal; SE: Standard error; TOT: Total.

Table 4. Frequency of *GST-M1* and *GST-T1* polymorphisms in acute lymphoblastic leukemia patients genotyped subgroup and weighted percentage of deletions.

Characteristics		Total (n)	*GST-M1*			*GST-T1*		
			Norm (n)	*Null, n (%)*	*NA*	*Norm (n)*	*Null, n (%)*	*NA*
Total		614	299	304 (51.4)	11	494	107 (16.3)	13
Gender	Female	251	125	120 (50.9)	6	213	31 (13.2)	7
	Male	363	179	179 (51.8)	5	281	76 (18.6)	6
Age (years)	1–5	355	170	178 (55.9)	7	280	68 (18.4)	7
	6–9	117	65	49 (37.6)	3	96	16 (9.6)	5
	10–17	142	69	72 (50.0)	1	118	23 (15.7)	1
Risk	Standard	78	36	39 (50.4)	3	64	11 (11.8)	3
	Medium	345	163	177 (53.5)	5	281	59 (17.5)	5
	High	191	105	83 (45.0)	3	149	37 (19.2)	5

NA: Not assessed.

across published results and do not allow a clear conclusion to be drawn. In the present study, the overall population was large and substantially homogenous for ethnicity and risk-adapted chemotherapeutic guidelines. Since the final aim of a pharmacogenomic approach is to move towards personalized medicine, subset analyses have been performed to explore the role of *GST* polymorphisms in specific subjects carrying the same features. In fact, ALL itself is a heterogeneous disease characterized by several immunophenotypes and the therapeutic protocols differ in intensity, particularly if we consider the high- versus standard-risk arms. The effect of potential predictors of response should be assessed within the already consolidated risk stratification.

Within the whole AIEOP-BFM ALL 2000 cohort, 306 children relapsed. Although relapse rate increased across risk groups, 214 recurrences occurred in patients treated with the lower-intensive protocols (28 in the standard- and 186 in the medium-risk group), indicating that the actual reliable risk group classification, based mainly on MRD, can still be improved. The subsample of ALL subjects to be genotyped was selected according to a novel two-phase design. The design and statistical analysis of two-phase studies presents several advantages over the traditional case–control design, which usually has limitations in sample size and in matching patients for demographic and clinical characteristics [34]. Most importantly, this approach is based on already available genetic data of a pilot subsample and it takes into account the clinical information available on the risk group and relapse rate in the whole cohort [28]. The selection of the subcohort to be genotyped depends on the pilot data available and this may be a limitation if such data are sparse or affected by selection bias. In this type of design, genotyping is not performed when the biospecimens are collected, but relies on the quality of stored samples. The statistical analysis of a two-phase design makes use also of the clinical information available on the whole cohort. With proper weights, it mimics the full cohort and derives an unbiased estimate of the incidence of event and of the role of genetic polymorphisms, adjusting by relevant factors through multivariate regression models [28]. This design is meant for studies planned retrospectively with already collected biological samples.

Our analysis did not correlate *GST* genotype with outcome in the overall population (Table 5 & Figure 1), similarly to a previous report on large cohort of ALL children treated within the AIEOP ALL 1995 study protocol [33]. Although similar to the risk-adapted protocols employed, the risk-assessment criteria used in this former AIEOP trial have been overcome by the modern prognostic MRD used in the AIEOP-BFM ALL 2000. In our study, *GST-M1* and *GST-T1* deletions were also not predictive of prednisone response. Similarly, in a case–control study of 45 PPR and 90 PGR patients, Anderer and coworkers did not find any association with relapse and initial prednisone response [19].

The demonstrated regulatory role of GST-M1 in glucocorticoid-induced apoptosis in cellular models let us suppose that it was meaningful to focus on the prednisone response while analyzing the role of *GST* genetic polymorphisms [35]. Deletion of *GST-M1* was associated with a better clinical outcome within PPR patients (p = 0.026; Table 3), but not in all high-risk patients (p = 0.08;

Table 2) according to multivariate analysis. Similarly, Rocha and coworkers have shown a significant correlation between the presence of a normal *GST-M1* gene and an increased incidence of hematologic relapse in 130 ALL patients treated within the high-risk arm of the St. Jude Total XIIIB study protocol, although, in this latter study, risk was assessed by different criteria [32]. Stanulla and coworkers also found that the null genotype for *GST-M1* conferred a protective trend on the risk of relapse (p = 0.078) in standard- and medium-risk patients with B-cell precursor ALL [20]. PPR represents a special subclass of high-risk patients, treated

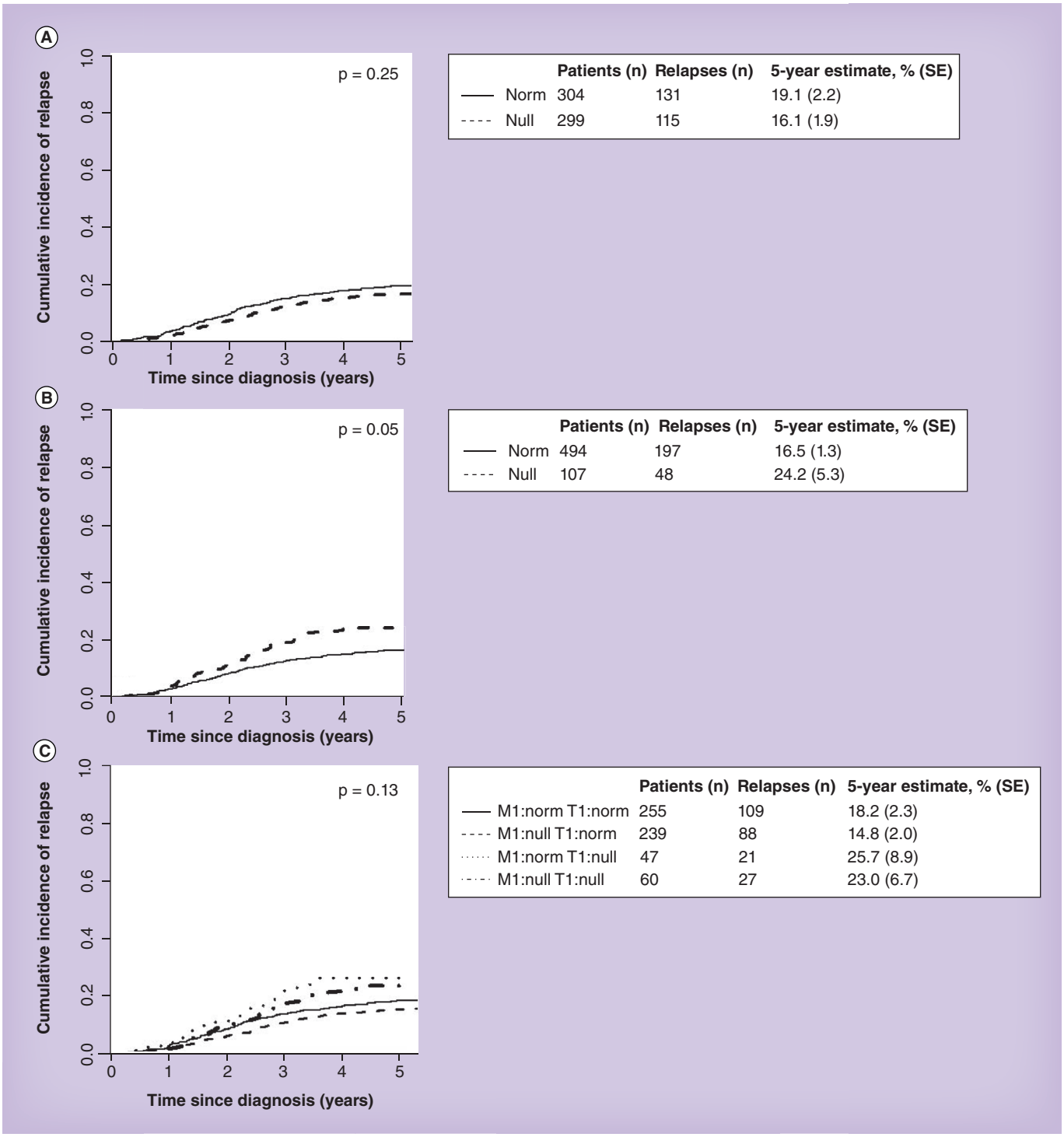

Figure 1. Weighted cumulative incidence of relapse according to glutathione *S*-transferase genotypes. (A) *GST-M1*; **(B)** *GST-T1*; **(C)** *GST-M1/GST-T1*. p-value of the Wald test in Cox model with genotype only as covariate.
M1: *GST-M1*; SE: Standard error; T1: *GST-T1*.

Table 5. Impact of glutathione *S*-transferase genotypes (analyzed singly or in combination) on the hazard of relapse in acute lymphoblastic leukemia patients.

GST genotype		TOT	Relapses (n)	Weighted 5-year cumulative incidence of relapse, n (%)	SE	Hazard ratio (95% CI)	p-value
GST-M1	Norm	304	131	19.1	2.2	1 (–)	–
	Null	299	115	16.1	1.9	0.82 (0.61–1.12)	0.212
GST-T1	Norm	494	197	16.5	1.3	1 (–)	–
	Null	107	48	24.2	5.3	1.34 (0.90–2.00)	0.144
GST-M1/ *GST-T1*	Norm/ norm	255	109	18.2	2.3	1 (–)	–
	Null/ norm	239	88	14.8	2.0	0.78 (0.56–1.10)	–
	Norm/ null	47	21	25.7	8.9	1.24 (0.70–2.20)	–
	Null/ null	60	27	23.0	6.7	1.16 (0.68–1.99)	0.643†

Overall analysis by weighted Cox model. Hazard ratio adjusted by risk group, age class and sex.
†p-value for genotype interaction.
Norm: Normal; SE: Standard error; TOT: Total.

with an early aggressive therapeutic approach since the very beginning of the therapy (day +8). This is equivalent to saying that, among patients treated with the most aggressive protocols (high-risk arm), deletion of *GST-M1* confers a protective advantage but reduces the risk of relapse significantly only in those who respond poorly to steroid prephase. More studies are needed to clarify if *GST-M1* genotyping could be introduced for modulating the front-line treatment in PPR patients, with the *GST-M1* normal PPR carriers eventually being treated with a more aggressive therapeutic regimen and undergoing a closer monitoring in comparison with *GST-M1* null PPR subjects.

Deletion of *GST-T1* showed a tendency towards a worse clinical outcome in patients belonging to the PGR group (p = 0.041; Table 3), according to multivariate analysis. Such association remained borderline significant only for PGR patients falling within the standard-risk arm and was lost for those in the medium-risk group (p = 0.045 and p = 0.58, respectively; Table 2). Although these subgroup results are somehow borderline and thus need further confirmation, they suggest that the adverse effect of the *GST-T1* null genotype could be of predictive interest mainly in patients with the best prognostic factors. In a case–control study comparing relapsed and successfully treated patients, Stanulla and coworkers have found that *GST-T1* deletion reduced the risk of relapse (p = 0.048; OR: 0.36; CI: 0.13–0.99) [20]. The conflicting results are most likely linked to sample size, patient heterogeneity and, eventually, to differences in treatment protocols. Indeed, Stanulla and coworkers investigated 64 matched case–control pairs of standard- and medium-risk patients with B-cell precursor ALL [20]. The relative prevalence of *GST-T1* null genotype was similar in both our and Stanulla's studies (~15 vs 17.8%, respectively), but our analyses have been performed on a larger group of subjects (614 vs 128 subjects, respectively), treated with the most recent protocol, which used a new definition of risk group based on MRD, and with all immunophenotypes represented (82% B-cell precursor ALL and 18% T-cell ALL). Stanulla *et al.* group then assessed the role of *GST-T1* three genotypes (zero, one and two allele genes) in a larger BFM cohort of 420 patients treated with the AIEOP-BFM ALL 2000, finding a nonsignificant tendency toward a better prednisone response in children with the null genotype compared with the normal homozygous genotype (p = 0.12; RR: 0.41; CI: 0.14–1.24). Only after a stratified analysis using those characteristics significantly associated with early steroid prephase, a fourfold decrease in risk of prednisone poor response arose for children with initial blast counts ≥ 3600/µl in comparison with the normal (two gene alleles) genotype (p = 0.04; RR: 0.25; CI: 0.07–0.92; p trend = 0.03) [31].

Since both GST-M1 and GST-T1 are phase II biotransformation enzymes known for their conjugation reaction with GSH, a similar effect on relapse would be expected for both null variants. On the contrary, our analyses show repeatedly an

opposite trend of the two genotypes. It should be noticed that GST-M1 and GST-T1 differ in amino acid sequence and 3D structure, as well as in substrate, organ and cell specificity [36]. Particularly, due to a difference in a conserved key residue for binding and activation of GSH, GST-T1 has a different catalytic and kinetic mechanism for GSH binding and activation compared with *GST-M1*, and a smaller substrate binding domain that is consistent with the selectivity of θ class GSTs for small xenobiotics [37]. Recent findings focused on the regulatory role of GSTs in the apoptotic process, particularly the case of GST-M1 and GST-P1. T-ALL-derived cell lines have shown that overexpression of GST-M1 inhibits the dexamethasone-induced apoptosis by downregulating the p38-MAPK pathway and the consequent activation of the proapoptotic protein Bim [35]. We hypothesize that in subjects carrying the *GST-M1* gene homozygous deletion, such negative regulation does not occur, meaning that that their blasts can more easily reach a permissive apoptotic environment: the null genotype becomes a more favorable condition for patients with otherwise adverse clinical condition, such as those who respond poorly to glucocorticoids. On the contrary, there is no evidence indicating that GST-T1 participates in the apoptotic process. A recent PharmGKB summary on *GST-T1* reports on correlation between the null genotype and antineoplastic-treatment-related toxicities [38]. These toxicities can cause interruption or discontinuation of chemotherapy, enhancing the relapse risk and leading to poor prognosis. In this report, the impact of *GST-M1* and *GST-T1* genotypes on toxicities has not been analyzed but represents an interesting point for further investigation.

The genotyping method adopted in the present study does not discriminate between normal/wild-type (two alleles) and heterozygous (one allele) functional genotypes. A trimodal phenotype pattern (slow, intermediate and fast enzymatic activity measured in erythrocytes) corresponding to genotypes (null, heterozygous and wild-type subjects, respectively) has been described [39–42]. No gene dose effect on prednisone response [31] or ALL treatment outcome [43] has been found so far, when *GST-M1* and *GST-T1* were analyzed separately. However, when the latter authors considered combined *GST-M1*×*GST-T1* gene dose effect, they found that poor metabolizers (patients with zero or one allele copy, independent of the genes considered) showed a better event-free survival and lower risk of relapse than good metabolizers (carriers of at least two allele copies, independent of the genes considered) [43]. In our study, combined *GST-M1*×*GST-T1* genotypes distinguished among more than three total allele carriers (norm–norm), one or two total allele carriers (norm–null and null–norm) and zero allele carriers (null–null). Neither significant correlation with outcome (overall p = 0.643; Table 5) nor trend proportional to the total allele numbers (Figure 1C) have been observed.

In conclusion, our results suggest that *GST-M1* and *GST-T1* genotypes could be relevant in terms of risk of relapse in specific pediatric patients' subsets. If our data are confirmed, *GST-M1* and *GST-T1* polymorphisms could be considered as additional prognostic factors useful to further refine the risk criteria adopted in the AIEOP-BFM ALL 2000 study. These results should be considered preliminary data and need to be further confirmed in a comparable validation cohort before drawing firm conclusions. Contribution of other pharmacogenetic determinants should also be taken into account.

Financial & competing interests disclosure

R Franca is recipient of a postdoctoral fellowship from IRCCS Burlo Garofolo, Trieste. P Rebora was partially supported by a studentship from FIRC (Fondazione Italiana per la Ricerca sul Cancro). This research was partially supported by a grant from the European Commission (FP7-HEALTH-F2-2011 261474). S Crovella is recipient of a fellowship grant from European Project 'Talents for an International House' within the framework of the 7th Research & Development Framework Programme PEOPLE – Marie Curie Actions – COFUND (Co-Funding of Regional, National and International Programmes). This study was partly supported by the special grant '5 per mille' of AIRC (Associazione Italiana per la Ricerca sul Cancro) to Franco Locatelli and Andrea Biondi. Giovanni Cazzaniga is supported by AIRC (Associazione Italiana per la Ricerca sul Cancro). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- More than 80% of pediatric patients affected by acute lymphoblastic leukemia (ALL) become long-term cancer survivors and healthy adults.
- Within risk-adapted polychemotherapeutic groups, there is still wide interpatient variability in clinical response, likely due to patients' genetic features.
- Pediatric ALL treatment success rates can be improved. Patients polymorphisms in genes involved in drug disposition, metabolism and mechanisms of action might affect outcome and could be predictor markers of prognosis.
- Deletions in the *GST-M1* and *GST-T1* genes (leading to missing proteins) have been already studied for their influence on ALL outcome with controversial results.

Material & methods

- Patients were treated according to the Italian AIEOP-BFM ALL 2000 clinical trial.
- Genotype analysis for both *GST-M1* and *GST-T1* stratifies individuals into two genetic categories, one being homozygous or heterozygous (norm) and the other having a homozygous deletion (null).
- Study design and statistical analysis were based on an innovative approach for pharmacogenetic retrospective study. The novel two-phase design allows a considerable gain in precision of the estimate of genotype effect on relapse because it introduces sampling fractions optimized on the basis of a previous correlation between genotypes and outcome in a pilot study on the same AIEOP-BFM ALL 2000 whole cohort.

Results

- No significant association was found in the whole cohort.
- *GST-M1* deletion was associated with better clinical outcome within prednisone poor-responder patients (p = 0.026; hazard ratio [HR]: 0.45; 95% CI: 0.23–0.91).
- *GST-T1* deletion was associated with worse outcome in the standard-risk group (p = 0.045; HR: 4.62; 95% CI: 1.04–20.6) and within prednisone good responders (p = 0.041; HR: 1.62; 95% CI: 1.02–2.58).

Conclusion

- With 614 patients genotyped, this study is probably one of the largest surveys performed on European pediatric ALL protocols.
- In specific subsets of ALL patients, whose risk has been assessed by already-in-use criteria, assessment of *GST-M1* and *GST-T1* deletions could be a candidate genetic tool to improve relapse prediction, probably together with other pharmacogenetic determinants.
- Our results need confirmation in a validation cohort in order to understand their clinical application.
- A deeper knowledge on the regulatory role of glutathione *S*-transferases on cell cycle is required to finally clarify the contradictory results published previously on the clinical effect of glutathione *S*-transferase variants.

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