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Mu class glutathione S-transferase mRNA isoform expression in acute lymphoblastic leukaemia

Pamela R. Kearns,[1] Zofia M. A. Chrzanowska-Lightowlers,[2] Rob Pieters,[3] Anjo Veerman[4] and Andrew G. Hall[5] [1]*Department of Paediatric Oncology, University of Bristol,* [2]*Department of Neurology, University of Newcastle upon Tyne, UK,* [3]*Department of Paediatric Oncology, Erasmus University Medical Centre, Rotterdam,* [4]*Department of Paediatric Haematology and Oncology, Vrije Universiteit, Amsterdam, The Netherlands, and* [5]*Department of Paediatric Oncology, University of Newcastle upon Tyne, UK*

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Summary. Glutathione S-transferases (GSTs) are implicated in cytotoxic drug resistance in leukaemia. In a previous study, expression of mu class GST (GSTM) was associated with poor prognosis in childhood acute lymphoblastic leukaemia (ALL), however, that study did not differentiate between individual GSTM isoforms. This study, therefore, investigated individual GSTM isoform expression in ALL blasts at the mRNA level. Leukaemic blasts from 21 children with ALL were studied. Interindividual variation in the pattern of *GSTM* mRNA isoform expression was demonstrated. *GSTM2* transcript was expressed in all patients in contradistinction to *GSTM5*, which was not detected in any sample. *GSTM3* and *GSTM4* expression varied between individuals, with *GSTM3* expressed in 62% and *GSTM4* in 24% of patients. Lymphoblast expression of *GSTM3* was positively related to good prognosis whereas expression of *GSTM4* was not related to clinical outcome in this small cohort. No relationship was demonstrated with established indicators of prognosis, including sex, age, immunophenotype and presenting white cell count. The results suggest that expression of *GSTM3* may play a role in determining prognosis in childhood ALL and could provide more information for accurate stratification of treatment. Further studies are required to determine whether there is a causal relationship between *GSTM3* expression and clinical outcome.

Keywords: glutathione S-transferases, mu class isoforms, ALL, prognostic indicators, drug resistance.

Cytosolic glutathione S-transferases (GSTs) form a multigene family of enzymes, currently divided into four classes, α (GSTA), μ (GSTM), π (GSTP) and θ (GSTT), on the basis of sequence homology and immunological cross-reactivity (Mannervik *et al*, 1992; Tsuchida & Sato, 1992). GSTs form an important part of intracellular detoxification pathways. They catalyse the conjugation of glutathione (GSH) with electrophilic compounds, including certain groups of cytotoxic drugs and other xenobiotics. The resulting conjugate is generally less toxic, more soluble and more readily excreted from the cell via energy-dependent protein pumps, including the multidrug resistance protein, MRP (Jedlitschky *et al*, 1996). All GSTs are highly specific with regard to GSH but their electrophile specificity varies considerably between the classes. Furthermore, within each class, the isoforms exhibit significant differences in substrate specificity, in spite of having considerable sequence homology (Commandeur *et al*, 1995; Gulick & Fahl, 1995). For example, the human μ class isoenzyme GSTM1 can catalyse the conjugation of GSH with *trans*-stilbene oxide, a capability not shared by other GST classes or even other isoforms of the μ class family (Commandeur *et al*, 1995).

Correspondence: Pamela R. Kearns, Department of Paediatric Oncology, Royal Hospital for Children, Paul O'Gorman Building, Upper Maudlin Street, BS2 8BJ, UK. E-mail: Pamela.kearns@bristol.ac.uk

The level of gene product expression of the different classes of GST varies between tissues. In human liver, GSTA constitutes 80% of the total GST protein expressed, of which the GSTA1-1 isoform predominates (Van Ommen *et al*, 1990). In contrast, human colonic tissues express GSTP as the major class of the GST family (Singhal *et al*, 1992). Differential expression of GST subclasses can also occur within an organ. For example, in the kidney, GSTA predominates in the proximal tubules, whereas GSTP and GSTM are the major isoforms in the thin loop of Henle, distal tubules and collecting ducts (Campbell *et al*, 1991).

Both GSTM1 and the θ class GSTs (GSTT) exhibit interindividual differences in expression as a consequence

of the following genetic polymorphisms (Zhong *et al*, 1993). In approximately 50% of the Caucasian population, the *GSTM1* gene is deleted from both alleles on chromosome 1 (Pemble *et al*, 1994). Similarly, for the *GSTT* gene, which is localized to chromosome 22 (Tan *et al*, 1995), a *GSTT1* null genotype is described in approximately 16% of Caucasians (Chen *et al*, 1996). A number of studies have emphasized the importance of these genetic polymorphisms in susceptibility to carcinogenesis. For example, an increased frequency of the *GSTT1* null genotype has been described in patients with myelodysplastic syndrome (Chen *et al*, 1996) and the homozygous deletion of the *GSTM1* gene is associated with increased risk of lung carcinoma in smokers (Ketterer *et al*, 1992; Nakajima *et al*, 1995). As yet, however, no correlation with drug resistance has been reported for either polymorphism.

Previous work reported by our group demonstrated a correlation between GSTM expression and poor prognosis in childhood ALL. In this study, we observed that patients whose bone marrow expressed GSTM, as determined by immunohistochemistry, had a significantly increased risk of relapse (Hall *et al*, 1994). The antibody used was a polyclonal anti-GSTM antibody raised against GSTM purified from liver extracts and, therefore, was unable to distinguish between the isoforms within the mu class of GSTs. In order to determine if prognosis was related to the *GSTM1* polymorphism gene, we examined the *GSTM1* genotype in the same study population but no correlation was found between *GSTM1* genotype and clinical outcome (Matheson *et al*, 1997). In the absence of any studies investigating the expression of the remaining four GSTM isoforms in leukaemic blasts, we chose to study the expression of the GSTM isoforms 2–5 at the mRNA level. This was performed using reverse-transcription polymerase chain reaction (RT-PCR) to examine the relationship between individual *GSTM* isoform expression and established indicators of prognosis in childhood ALL.

MATERIALS AND METHODS

Patient samples analysed were derived from patients presenting to major medical centres in the Netherlands, Denmark or Germany. Patients were treated according to their respective national protocols for the Dutch Childhood Leukaemia Study Group (SNWLK), the Danish Nordic Society of Paediatric Haematology and Oncology (NOPHO) and the German Co-operative ALL (COALL) Study Group. The bone marrow and blood samples were sent to the Haematology, Oncology, Immunology (HOI) Laboratory, Vrije University, Amsterdam, where the initial sample preparation was performed. Leukaemic blast pellets were prepared by Ficoll extraction and pellets of 1×10^7 cells were stored at −80°C for subsequent RNA extraction.

Total RNA extraction was performed using caesium chloride ultracentrifugation based on the method of Chirgwin *et al* (1979).

RNA preparation and reverse transcription was performed using the Superscript^{tm} preamplification system (Gibco BRL, Paisley, UK following manufacturer's recommendations). Total RNA (0·1 μg) was used together with 50 pmol/l of an oligo dT primer to programme each reverse transcription reaction. The resulting first strand cDNA was either maintained on ice, if PCR was to be performed immediately, or stored at −20°C.

PCR was performed in a Perkin Elmer (Bucks, UK) thermal cycler (Model 480). Each reaction mixture contained; 1 × Bioline buffer, 2·5 units Taq (Promega, Southampton, UK), 200 μmol/l dNTPs, 1·5 mmol/l $MgCl_2$, 2 μl cDNA and 50 pmol/l of the appropriate primer. Positive controls for *GSTM* isoforms 2–4 and *GADPH* were prepared using cDNA derived from a neuroblastoma cell line, SHSY1, known to express these transcripts. No positive control for *GSTM5*, however, could be obtained.

Primer sequences are shown in Table I. GADPH primers were used as an internal standard as described by Moretti *et al* (1994). Primers were synthesized using an automated DNA/RNA synthesiser (model 292; Applied Biosystems, CA, USA).

Table I. Primer base sequences.

Target mRNA	Name of primer sequence	Primer sequence	*PCR product size (bp)
GSTM2	HGTM2-338	5′ GTA TGC AGC TGG CCA AAC	
	HP2	5′GAG ATG AAG TCC TTC AGA TTT	235
GSTM3	HGTM3-338	5′ GCA CAC AAC TGA TAA GGC	
	HGTM3–607r	5′ TTG CAG AAC TGA TCA GAC	292
GSTM4	HGTM4-338	5′ CCA ATC AGC TGG CCA GAG	
	GSTM4-E7	5′ GGC TCA AAT ATA CGG TGG AG	193
GSTM5	HGTM5-338	5′ACA TGG AGC TGG TCA GAC	
	HGTM5-E7	5′ GGC TCA AAT ATA CGC TTC AT	196
GADPH	GADPH8	5′ CGT GTC CCC CAC TGC CAA C	
	GADPH9	5′ TGT TGC TGT AGC CAA ATT CG	268

*PCR product size generated from a cDNA template.

All primers were targeted to exonic sequences. GAPDH primers were used as an internal standard as described by Moretti *et al* (1994).

The PCR profile consisted of 35 cycles of 94°C for 3 min, 56°C for 1 min and 72°C for 30 s. The products were electrophoresed through 2% agarose and visualized using ethidium bromide, alongside a 123 bp marker (Gibco). PCR products for each of the GSTM isoforms 1–5 are shown in Fig 1. If a band of the appropriate size was visible, the sample was designated as positive for mRNA expression ('1' in Table II), while if no band was visible at the expected base-pair size, this was designated as negative ('0' in Table II). The band was designated positive irrespective of the degree of intensity. The data were analysed without knowledge of the clinical details of the patients.

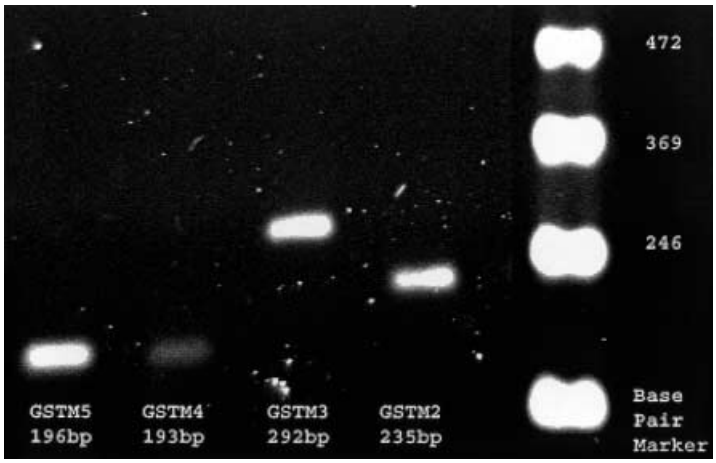

Fig 1. RT-PCR products from each primer pair specific for GSTM isoforms 1–5. PCR products from leukaemic blasts separated on 2% agarose gel, stained with ethidium bromide and visualized under ultra-violet light to demonstrate the products.

Table II. Lymphoblast GSTM mRNA isoform expression in childhood ALL.

	Presence of RT-PCR product				
Patient	M2	M3	M4	M5	GADPH
494	1	0	0	0	1
3446	1	0	1	0	1
3882	1	1	0	0	1
4093	0	1	0	0	1
4483	1	1	1	0	1
4506	1	0	0	0	1
6597	1	0	0	0	1
6920	1	1	1	0	1
7275	1	0	1	0	1
7335	1	1	0	0	1
7346	1	0	1	0	1
7376	1	1	0	0	1
7698	1	1	0	0	1
7792	1	1	0	0	1
7811	1	0	0	0	1
7821	1	1	0	0	1
7874	1	1	0	0	1
7965	1	0	0	0	1
8091	1	1	0	0	1
8096	1	1	0	0	1
8348	1	1	0	0	1
Number of patients positive for GSTM isoform	20	13	5	0	21
% of patients positive for GSTM isoform	95	62	24	0	100

RT-PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. '1' designates the presence of the correct RT-PCR product. '0' designates no detectable RT-PCR product. GADPH was used as a positive control for the reverse transcription.

The identity of PCR products was verified by restriction fragment length polymorphisms (RFLP) using the endonucleases *Dde*1, *Sfa*N1 and *Mbo*1 (NE Biolabs, Herts, UK). PCR products were separated by electrophoresis through 3·5% Genetic Technology Grade™(GTG) Nusieve agarose gels, and the PCR bands visualized and purified (Genelute^{TM}) prior to restriction endonuclease digestion. RFLP analyses for GSTM 2, 3, 4 and 5 all generated the predicted size products.

RESULTS

Ten male and 11 female patients were studied (Table III). The mean age at presentation was 63 months, range 4–135 months. Three samples were T-lineage ALL immunophenotype and 18 were B-lineage immunophenotype.

Representative results are shown in Figs 2 and 3, demonstrating positive expression of mRNAs encoding *GSTM2*, *M3*, *M4* and *GADPH*. In two patients, two separate pellets were available and were used to confirm the reproducibility of the technique (data not shown). The results for all the patients included in this study are shown in Table II. *GSTM2* mRNA was expressed in all but one patient sample while that for *GSTM5* was not expressed in any of the samples included in this study. *GSTM3* transcripts were expressed in 13 samples (62%) and for *GSTM4* in five samples (24%). In only two samples were both

Table III. Clinical details of patients analysed for GSTM mRNA expression.

Patient	Immunophenotype*	Sex	Age at diagnosis (months)	WCC† ($\times 10^9$/l)
494	C	female	39	168
3446	null	male	114	85
3882	T	female	135	46
4093	preB	female	25	37
4483	C	male	86	49
4506	preB	male	25	71
6597	C	female	4	492
6920	C	female	43	356
7275	preB	male	49	20
7335	T	male	131	371
7346	C	female	64	26
7376	C	male	61	81
7698	C	female	68	36
7792	preB	female	36	95
7811	preB	male	42	10
7821	preB	female	52	60
7874	C	male	73	103
7965	C	female	45	5
8091	C	male	88	6
8096	T	male	126	800
8348	preB	female	28	54

*Immunophenotype: C, common ALL; N, null-type ALL; preB, early B-lineage ALL; T, T-lineage ALL.

†Peripheral blood white cell count at initial presentation.

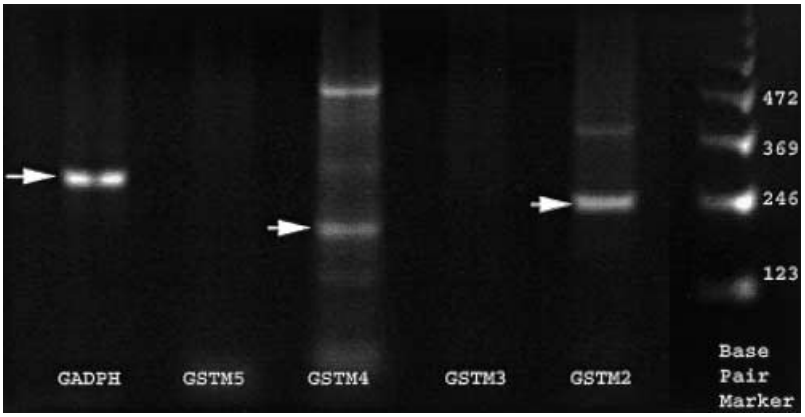

Fig 2. Expression of GSTM isoforms in leukaemic blasts. PCR products from patient 7275 separated on 2% agarose gel and visualized with ethidium bromide. Patient 7275 expressed GSTM2, M4 and GADPH but not GSTM3 and M5.

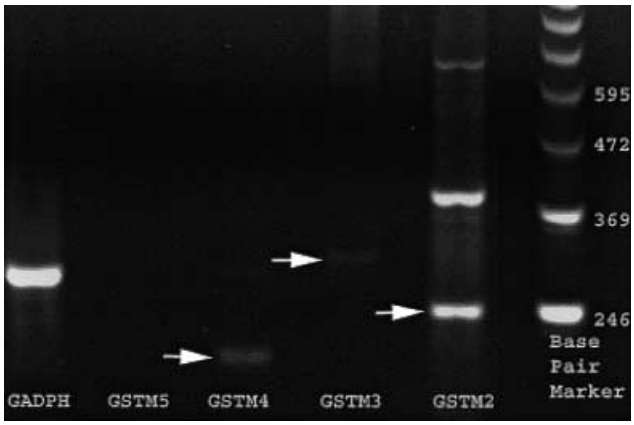

Fig 3. Expression of GSTM isoforms in leukaemic blasts. PCR products from patient 4483 separated on 2% agarose gel and visualized with ethidium bromide. Patient 4483 expressed GST M2, M3, M4 and GADPH but not GSTM5.

GSTM3 and *GSTM4* mRNA species co-expressed. The 'housekeeping' gene *GADPH* was used as a positive control for each cDNA sample and was shown to be present in all samples analysed.

The relationships between expression of *GSTM 3* and *4* and known indicators of prognosis in childhood ALL were analysed. *GSTM2* and *GSTM5* were not considered in this analysis as their ubiquitous presence or absence would preclude any significant contribution to differences in clinical outcome. The expression of *GSTM3* and *GSTM4* was shown to be the same in male and female patients. Similarly, no significant relationships were demonstrated between expression of either *GSTM3* or *GSTM4* transcripts and age (Mann–Whitney *U*-test $P = 0{\cdot}12$ and 0·4 respectively) or white cell count (WCC) at presentation (Mann–Whitney *U*-test $P = 0{\cdot}44$ and 0·76 respectively). All of the three samples immunophenotyped as T-lineage ALL expressed *GSTM3* but none expressed *GSTM4* whereas, of those samples immunophenotyped as B lineage, 10/18 (56%) expressed *GSTM3* and 5/18 (28%) expressed *GSTM4* (Table IV).

The availability of cytogenetic data for the patients included in this study was limited. As historical samples were used, it was not possible to undertake any further molecular analysis. Therefore, any relationship between GSTM isoform expression and cytogenetic abnormalities could not be examined.

Survival data were available on 20 of the 21 patients studied. The overall 5-year survival for the cohort of patients in this study was 55% and the 5-year event-free survival was 35% (Fig 4). Events included one patient whose death occurred during induction therapy, while in all other patients the events were relapses. A significantly better overall survival was observed in patients with blasts expressing *GSTM3* mRNA [Fig 5, log-rank test 5·22, degrees of freedom (d.f.) 1, $P = 0{\cdot}02$]. These patients also showed better event-free survival (log-rank test 6·18, d.f. 1, $P = 0{\cdot}01$). In contrast, clinical outcome was not influenced by expression of the *GSTM4* transcript (Fig 6).

DISCUSSION

Interindividual variation in the expression of *GSTM* isoforms in leukaemic blasts was demonstrated in this cohort of children with ALL. *GSTM2* mRNA transcripts were expressed in 95% of patients analysed whereas no patient expressed *GSTM5*. The expression of *GSTM3* and *GSTM4* displayed the greatest interindividual variation. These two isoform transcripts were expressed simultaneously in only two patients (9·5%). *GSTM3* was expressed in all of the three T-lineage samples analysed and in 10 of 18 (56%) B-lineage samples, whereas *GSTM4* was only expressed in five of 18 (28%) B-lineage samples and not in the T-lineage samples. No relationships were demonstrated between the mRNA expression of either *GSTM3* or *GSTM4* and other established indicators of prognosis in childhood ALL, including sex, age at presentation and presenting WCC.

Table IV. Comparison of the mRNA expression of GSTM3 and GSTM4 with immunophenotype in childhood ALL.

		GSTM3				GSTM4			
		expressed		not expressed		expressed		not expressed	
	Number of samples	*n*	%	*n*	%	*n*	%	*n*	%
B lineage	18	10	56	8	44	5	28	13	72
T lineage	3	3	10	0	0	0	0	3	100

n, number of samples.

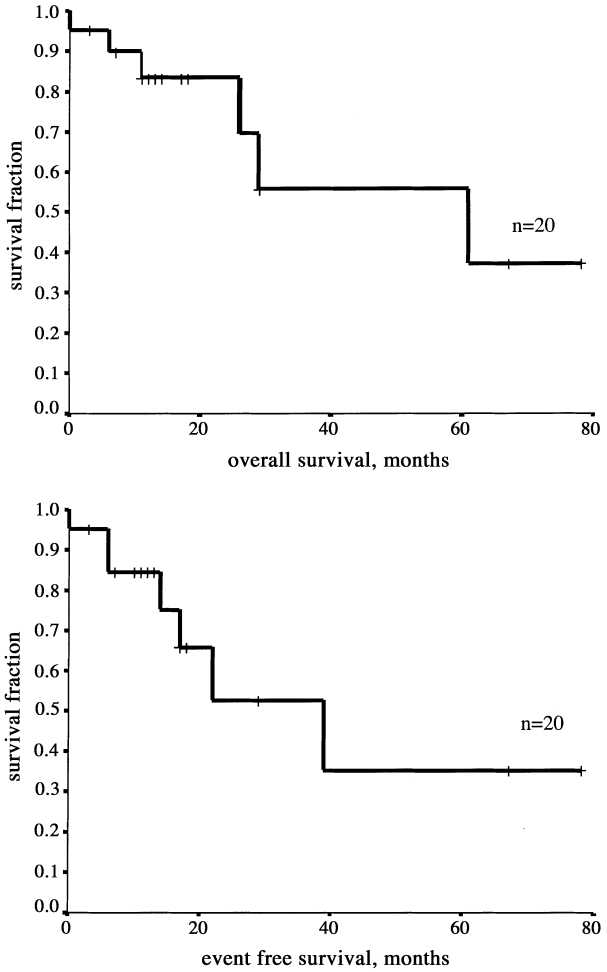

Fig 4. Kaplan–Meier overall and event-free survival curves for childhood ALL. Survival data were available on 20 of the 21 patients analysed. Vertical lines represent censored patients. Five-year overall survival was 55% and 5-year event-free survival was 35%.

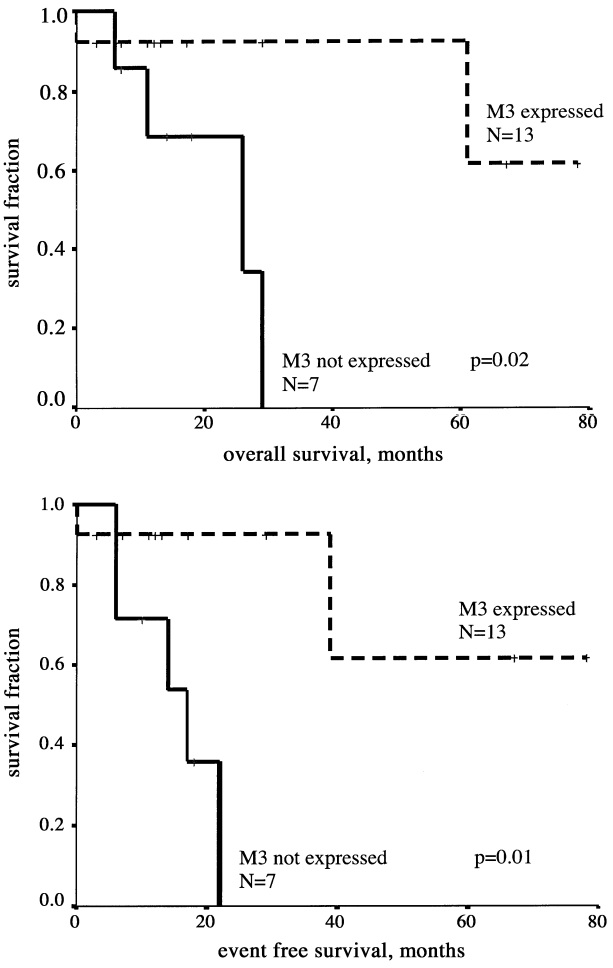

Fig 5. Comparison of overall and event-free survival in children with ALL grouped according to expression of GSTM3 in leukaemic blasts. Event-free survival data were available on 20 of the 21 patients analysed. Vertical lines represent censored patients. Both overall and event-free survival were significantly increased in patients with lymphoblasts positive for GSTM3 (log-rank test 6·18, d.f. 1, $P = 0{\cdot}01$ and log-rank test 5·22, d.f. 1, $P = 0{\cdot}02$ respectively).

GSTM3 expression was positively related to clinical outcome, however, no relation was found between *GSTM4* expression and survival. The Kaplan–Meier survival curves generated in this study should be interpreted with caution because the number of patients included in the study was small and the follow-up period for many patients was short. Nevertheless, the results of this initial analysis suggest that further investigation of the lymphoblast expression of GSTM isoforms with a larger number of patients could prove valuable.

The five-year overall and event-free survival of the patients included in this study (55% and 35% respectively) were considerably less than currently reported survival figures. However, there was an inherent bias in selection of patients for this study. A large number of cells was needed to obtain sufficient mRNA for analysis. Samples from patients presenting with a high WCC were, therefore, more likely to be sent for research purposes and result in a successful RNA extraction. This was reflected in the study cohort which included a high proportion of patients with a high WCC at presentation; 11 from 21 patients (52%) had a presenting WCC greater that 50×10^9/l. A high WCC is a known predictor of poor prognosis and could account for the poor clinical outcome of patients included in this study.

This is the first reported study of individual *GSTM* isoform expression at the mRNA level in childhood leukaemia. Earlier studies of GSTM expression in leukaemia have used immunologically based techniques. We previously reported that expression of GSTM was related to an increased risk of relapse in childhood ALL (Hall *et al*, 1994) but could not differentiate between GSTM isoforms. The rabbit polyclonal antibodies used in that study were raised against GSTM purified from hepatic extracts, which comprise predominantly of GSTM1 (Hall *et al*, 1994). Recently, Den Boer *et al*

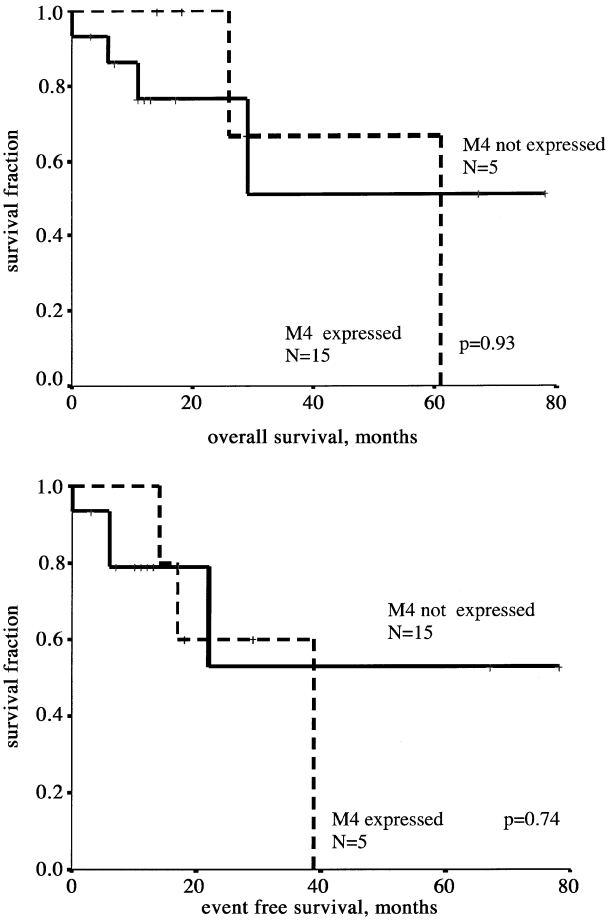

Fig 6. Comparison of overall and event-free survival in children with ALL grouped according to expression of GSTM4 in leukaemic blasts. Survival data were available on 20 of the 21 patients analysed. Vertical lines represent censored patients. There was no significant difference between GSTM4-positive and -negative lymphoblasts for overall survival (log-rank test 0·01, d.f. 1, $P = 0{\cdot}92$) and event-free survival (log-rank test 0·11, d.f. 1, $P = 0{\cdot}74$).

(1999) examined GST expression in childhood leukaemia by flow cytometry using the same antibodies. They also found no relationship between GSTM expression and known indicators of prognosis, however, the follow-up period on their patients was too short to yield survival data. Again, the technique used by Den Boer *et al* (1999) could not differentiate between individual GSTM isoforms. Campbell *et al* (1991) reported that antisera raised against GSTM1 or GSTM2 did not cross-react with GSTM3. It is, therefore, probable that in our previous studies, the immunopositivity reflected either GSTM1 or M2 expression rather than expression of the other GSTM isoforms.

It is known that GST expression is tissue-type specific, as is the expression of each of the GSTM isoforms (Campbell *et al*, 1991; Ramjee *et al*, 1994; Rowe *et al*, 1997). Furthermore, interindividual variation in GSTM1 expression, as a result of genetic polymorphism, is well known Approximately 40–60% of the population are homozygous to the *GSTM1 null* gene (Bell *et al*, 1992; Ketterer *et al*, 1992). More recently, allelic variation in the *GSTM3* gene has been reported but, unlike *GSTM1*, this is not due to a null allele but results from a 3 bp deletion in intron 6 (Inskip *et al*, 1995). Studies examining the association between GSTM3 polymorphism and the risk of lung and colorectal cancer suggest the incidence of the *GSTM3* allelic variant to be around 20% in the normal population (Loktionov *et al*, 2001; Risch *et al*, 2001). Interindividual differences in expression of *GSTM4* in a given tissue type have not been previously reported. Moreover, although *GSTM3* polymorphism has been studied in the context of cancer susceptibility, it has not been studied in the context of leukaemia and there no are previous studies examining the influence of *GSTM3* expression on response to treatment.

The mechanism by which expression of GSTM3 might convey a survival advantage in childhood ALL is presently unclear and warrants further investigation. GSTs are usually described as detoxifying enzymes, therefore, overexpression of an isoform would not be expected, *a priori*, to lead to increased cytotoxicity of a drug. Cytotoxic GST conjugates have been described (Pickett & Lu, 1989; Gulick & Fahl, 1995) but the conjugates were of halogenated hydrocarbons rather than the cytotoxic drugs used in the treatment of leukaemia. There is considerable evidence that the GSTM3 protein is the most distantly related member of the GSTM family. The protein encoded by the *GSTM3* gene shares only 70% homology with other GSTM isoforms, and has atypical catalytic and structural properties, differing significantly from other GSTs. The GSTM 1–5 gene cluster has been mapped to human chromosome 1p13 (Pearson *et al*, 1993; Gough *et al*, 1994). A physical map of the five μ class genes demonstrated that four of these are orientated in a head to tail array, 5′-M4-M2-M1-M5-3′ (Xu *et al*, 1998). More recently, the *GSTM3* gene was identified as having an inverted orientation, situated downstream from GSTM5 in a 5′-M5 3′-3′-M3 5′ orientation (Patskovsky *et al*, 1999). A greater knowledge of the physicochemical properties of human GSTM3 and the factors regulating its expression are required before any mechanism of enhancing survival in childhood leukaemia can be postulated.

It is possible that expression of GSTM3 itself does not directly contribute to the mechanism for a good treatment response but is simply a marker of altered expression of an unrelated gene. It is interesting to note that chromosome 1 is frequently involved in recurrent gene rearrangements in ALL (Nourse *et al*, 1990), however, breakpoint loci in the vicinity of the GSTM gene cluster have not yet been reported.

Improvement in the outlook for children with acute leukaemia might be achieved by directing more intensive therapy to 'high-risk' disease. For patients predicted to have a good prognosis, less intensive treatment may reduce both short- and long-term morbidity without an increase in mortality. This approach assumes that patients with resistant disease can be accurately identified. WCC, immunophenotype, and cytogenetics. Furthermore, in a current

Dutch–German collaboration, *in vitro* drug sensitivity data are being used to stratify patients. However, a recent prospective study analysing the predictive values of the traditionally accepted indicators of prognosis found the prognostic information was contained in four variables: age, sex, WCC and cytogenetic features (Donadieu *et al*, 1998). The predictive power of each variable was low and it was suggested that each factor accounted for no more than 4% of the overall variability in prognosis. To date, there appears to be no single determinant of resistant disease in childhood leukaemia. Our results suggest that mRNA expression of *GSTM3* was associated with good prognosis, however, further studies are indicated. This study was limited by the selection bias in the cohort towards patients with high WCC. Further studies involving a greater number of patients would be required to establish the true predictive power of *GSTM3* expression in childhood ALL. If expression of *GSTM3* proves to have a direct role in determining clinical outcome in childhood ALL, it may be possible to exploit this observation in the development of novel therapeutic agents.

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