

Glutathione S-transferase polymorphisms may be associated with risk of oedematous severe childhood malnutrition

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It has been estimated that more than 50% of deaths before the age of 5 years have undernutrition as an underlying cause. Severe childhood malnutrition, an extreme form of undernutrition, occurs as oedematous and non-oedematous syndromes. The reasons why only some children develop oedematous severe childhood malnutrition (OSCM) have remained elusive, but the heterogeneity of clinical appearances among children from relatively homogeneous backgrounds suggests that interindividual variation in susceptibility to OSCM may exist. We investigated variants of four glutathione S-transferase (GST) genes in a retrospective study among subjects (n 136) previously admitted to the Tropical Metabolism Research Unit, Jamaica, for the treatment of either OSCM (cases) or non-oedematous severe childhood malnutrition (controls). We found that GSTP1 Val¹⁰⁵ homozygotes were significantly more common among the cases (odds ratio (OR) 3.5; 95% CI 1.1, 10.8). We also found an association of borderline significance between non-deletion GSTT1 genotypes (i.e. +/+ or +/0) and OSCM (OR 2.4; 95% CI 1.0, 5.9). There was no significant association between OSCM and any of the other GST variants. These preliminary findings suggest that genetic variation within the GST superfamily may contribute to the risk of OSCM. Additional, larger data sets and studies of variants in other candidate genes are required in order to properly assess the true contribution, if any, of genetic variation to risk of OSCM. Such studies may improve our understanding of the causes of clinical heterogeneity in malnutrition.

Oedematous childhood malnutrition: GSTP1: GSTT1

Undernutrition in childhood is an important public health problem worldwide. It is estimated that more than 5.5 million deaths among children under the age of 5 years have undernutrition as an underlying cause (Caulfield *et al.* 2004; Bryce *et al.* 2005). Severe childhood malnutrition (SCM), with the highest mortality risk, includes clinical presentations that range from marked weight loss only, to marked weight loss plus dramatic hair and skin changes that are often associated with oedema. In many centres, however, only the degree of weight deficit and the presence or absence of oedema are routinely used to categorise these clinical syndromes (Lancet, 1970; World Health Organization, 1999, 2000).

Blood concentrations of GSH are considerably lower in oedematous SCM (OSCM) than in non-oedematous SCM (NOSCM), with little or no overlap between the two groups (Jackson, 1986*a,b*; Golden & Ramdath, 1987). GSH is a tripeptide (glutamic acid–cysteine–glycine) of almost universal tissue distribution and is the major source of cellular low-molecular weight sulfhydryl (–SH) groups, providing protection against attack by peroxides, reactive oxygen species and electrophilic xenobiotics (Wang & Ballatori, 1998).

The observation of a low GSH level in kwashiorkor was important in the development of the free radical theory (Golden & Ramdath, 1987), which proposes that the development of the features of OSCM is due to an imbalance between the generation of free radicals and their safe disposal.

The cause of low GSH levels in OSCM is uncertain. We have recently shown that GSH synthesis rates are lower in OSCM than NOSCM (Reid *et al.* 2000) and that cysteine supplementation increases *in vivo* erythrocyte GSH synthesis rates in OSCM (Badaloo *et al.* 2002). These observations suggest that a limiting availability of precursors such as cysteine may lead to reduced concentrations of GSH and may thus play a primary role in the pathogenesis of OSCM. It is possible that there may be alternative or additional causal explanations, however, as low levels of precursors may be a secondary phenomenon.

The proportion of patients suffering from OSCM varies by locale (Hendrickse, 1991; Waterlow, 1992). Within any particular locale, however, patients with OSCM appear to be similar to patients with NOSCM in terms of socio-economic status, nutritional history, rate of infection and many biochemical variables (Gopalan, 1970; Laditan & Reeds,

Abbreviations: GST, glutathione S-transferase; NOSCM, non-oedematous severe childhood malnutrition; OR, odds ratio; OSCM, oedematous severe childhood malnutrition; SCM, severe childhood malnutrition.

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1976; Christie *et al.* 1992; Waterlow, 1992). The very different clinical outcomes seen among children with similar environmental exposures suggests the possibility that there may be interindividual variation in the ability to handle these exposures. Such variation might explain why some children end up with OSCM (and low GSH levels) despite having very similar backgrounds to children with NOSCM.

There are several different isoforms of glutathione S-transferase (GST); these isoforms catalyse the irreversible conjugation of GSH to a wide variety of electrophilic compounds including organic hydroperoxides, unsaturated hydroxyalkenals and epoxides (Commandeur *et al.* 1995; Wang & Ballatori, 1998). In SCM, in which there are both macronutrient and micronutrient deficiencies, many of the 'normal' anti-oxidant defences are likely to be compromised (Golden & Ramdath, 1987), and there may thus be an increase in the relative importance of GST-mediated anti-oxidant defence. In addition, there are genetic variants, which alter the catalytic activity of several of the GST isoforms (Chen & Board, 1987; Seidegård *et al.* 1988; Zimniak *et al.* 1994; Inskip *et al.* 1995). It is possible, therefore, that an inherited variation in GST activity might play a role in predisposition to oxidant damage and susceptibility to OSCM.

In the present paper, we report on a case-control study among Jamaican children previously admitted to hospital for the treatment of SCM. We explore associations between genetic variants in four enzymes of the GST superfamily and the risk of a diagnosis of OSCM. We also explore the relationships between urinary isoprostanes (a marker of *in vivo* lipid peroxidation), nutritional diagnosis and GST genotypes.

Materials and methods

Participants

The metabolic research ward of the Tropical Metabolism Research Unit, Faculty of Medical Sciences, University of the West Indies, is located at the University Hospital of the West Indies in the parish of St Andrew at the south-eastern end of Jamaica. The Tropical Metabolism Research Unit ward also serves as a tertiary referral centre for children with severe malnutrition from the entire island. Admission records for the ward were reviewed in order to generate a list of potential subjects.

The principal inclusion criterion was a diagnosis of SCM according to the Wellcome Classification (Lancet, 1970), that is, a diagnosis of marasmus (<60% weight-for-age, no oedema), marasmic kwashiorkor (<60% weight-for-age with oedema) or kwashiorkor (60–80% weight-for-age with oedema). Patients who had 'secondary malnutrition' (e.g. HIV seropositivity, congenital malformations, cerebral palsy) were excluded. A total of 691 patients met these criteria. Potential participants were also required to have had an address in Kingston (the capital city, whose limits are within the parish of St Andrew), St Andrew or St Catherine (a neighbouring parish to the west of St Andrew) and to have been discharged from the ward not more than 15 years previously. This additional criterion reduced the number of potentially eligible participants to 320. The study was approved by the University Hospital of the West Indies/University of the West Indies Faculty of Medical

Sciences Ethics Committee, and written, informed consent was given by a parent or other adult guardian for each child.

At the end of the recruitment period, we had attempted to contact 256 former patients; 136 of whom were eventually recruited. Among these former patients, three were excluded after a review of their original admission data indicated that they were ineligible. There were several sets of siblings (including twins) among the former patients; for any set of siblings, only the child with the earliest date of admission was retained in the data set for analysis. If siblings were admitted to the ward on the same day, one child from the sibship was chosen at random. Thus, the final data set contained 124 former patients. Of the 120 former patients who were not recruited, we were unable to trace forty-nine. We contacted fifty-three who were not successfully recruited ('not interested', 'appointment dates/times not convenient'). Eleven were reported to have moved or migrated, and seven were reported to be dead.

Anthropometry and laboratory assays

Height and weight measurements were made on all subjects. All measurements were carried out at the Tropical Metabolism Research Unit using standardised procedures (Cooper *et al.* 1997). Height-for-age and BMI *z* scores were calculated using the 2000 CDC charts option in Epi Info (version 3.3.2; Centers for Disease Control and Prevention, Atlanta, GA, USA).

DNA was extracted from blood samples using a phenol/chloroform protocol (Sambrook *et al.* 1989). Genotypes of biallelic polymorphisms within four GST enzymes (θ 1, GSTT1; μ 1, GSTM1; μ 3, GSTM3; π 1, GSTP1) were determined by agarose gel electrophoresis of either PCR products or restriction enzyme digests of PCR products followed by ethidium staining of the gels. All genotyping was performed according to previously published protocols with only minor modifications (Brockmöller *et al.* 1993; Pemble *et al.* 1994; Inskip *et al.* 1995; Harries *et al.* 1997).

For both GSTT1 and GSTM1, the polymorphism that was typed is caused by a large deletion in which the null genotype (0/0) is associated with absence of enzyme activity. In the present PCR-based assay, 0/+ heterozygotes were not distinguishable from +/+ homozygotes. Thus, in the analysis for these genes, only 'null' (i.e. 0/0) and 'positive' (i.e. 0/+ and +/+ combined) genotypes were considered. For the biallelic polymorphisms in GSTP1 and GSTM3, the three possible genotypes could be detected. The polymorphism in GSTP1 is caused by a point mutation; the variant allele codes for valine rather than isoleucine at amino acid 105 and is associated with changes in enzyme activity towards different substrates (Hayes & Strange, 2000). The polymorphism in GSTM3 results from a 3 bp insertion/deletion in intron 6 and thus does not change the primary sequence of the protein product. The deletion variant, however, harbours a recognition motif for a negative transcription factor and could be a marker of reduced GSTM3 activity (Inskip *et al.* 1995).

A spot urine sample was collected from all subjects. The concentration of urinary isoprostanes (principally 8-iso-prostaglandin F_{2α}), a marker of *in vivo* lipid peroxidation (Halliwell & Gutteridge, 1999; Pratico *et al.* 2004), was estimated using an ELISA method that has been implemented in a commercially available kit (Oxford Biomedical Research, Oxford, MI, USA). Urinary creatinine concentrations were measured

on an Abbott Alcyon 300i autoanalyser (Abbott Laboratories, Abbott Park, IL, USA). Concentrations of isoprostanes are expressed as ng/mmol creatinine. Samples from a large urine pool were included in each analytical run, yielding a CV of 12% for urinary isoprostanes.

Statistical methods

The principal aim was to determine whether there were differences in either genotype or allele frequencies between cases and controls. The strength of this association is expressed in terms of the odds ratio (OR) and 95% CI for a previous diagnosis of OSCM. A secondary aim was to explore whether there were between-group (cases *v.* controls) or between-genotype differences in the level of *in vivo* lipid peroxidation as estimated by concentrations of urinary isoprostanes. Between-group differences were analysed using χ^2 tests or Student's *t* tests as appropriate. Linear regression models were used to determine whether there were significant associations between urinary isoprostanes, diagnosis and genotype. The significance of terms in the regression models was tested using a manual, stepwise, backward elimination procedure; pairs of models were evaluated with likelihood-ratio tests. Differences in $-2 \times \log_e(\text{likelihood})$ are approximated by a χ^2 distribution with degrees of freedom equal to the difference in the number of parameters fitted in each model. All models were fitted using Stata 8 software (Stata Corporation, College Station, TX, USA).

Results

Tests of association between glutathione S-transferase genotypes and OSCM

In the final data set, seventy-four participants had been previously diagnosed as having OSCM (cases) and fifty participants were diagnosed as having NOSCM (controls). The mean age on admission was 15.1 (SD 21.5) months, and the mean elapsed time since admission was 7.6 (SD 4.5) years. The proportion of males among the cases was greater than among the controls (Table 1), but this difference was not significant (χ^2 0.75, 1 df, $P=0.39$).

The relationship between polymorphisms in different GST genes and the risk of having a previous diagnosis of OSCM is shown in Table 2. For GSTP1, there was a significant association between genotype and OSCM: subjects who were homozygous

for the valine¹⁰⁵ allele were more likely to have a previous diagnosis of OSCM (OR 3.48, 95% CI 1.1, 10.8, $P=0.0213$) when compared with isoleucine¹⁰⁵ homozygotes. Heterozygotes were also more likely to have a previous diagnosis of OSCM when compared with isoleucine¹⁰⁵ homozygotes (OR 2.25, 95% CI 0.90, 5.66), but this increased risk was not significant at the 5% level. A score test for trend indicated that the risk of OSCM increased significantly with the number of copies of the valine¹⁰⁵ allele (χ^2 5.89, 1 df, $P=0.015$) and provided evidence of a 'dose-response effect'; unconditional logistic regression under an additive model gave an OR for OSCM of 1.92 (95% CI 1.13, 3.26) for each additional valine¹⁰⁵ allele.

There was an association between GSTT1 genotype and OSCM: there was a larger proportion of subjects with the GSTT1 + genotype (i.e. +/+ or +/0) among the cases than among the controls (33.3% *v.* 17.1%), a difference that was just significant at the 5% level (χ^2 4.12, 1 df, $P=0.042$). The OR for a previous diagnosis of OSCM was 2.44 (95% CI 1.00, 5.88) among subjects with the + genotype compared with subjects whose genotype was 0/0.

For GSTM3, there was no evidence of a significant association between genotype and a previous diagnosis of OSCM. Insertion/deletion heterozygotes appeared to have a lower risk of a previous diagnosis of OSCM (OR 0.81, 95% CI 0.38, 1.75), and the infrequent insertion/insertion homozygotes appeared to have a higher risk of OSCM (OR 2.67, 95% CI 0.50, 14.4) when compared with deletion/deletion homozygotes. In neither case, however, were the risk ratios significantly different from 1, and there was no significant change in the risk of OSCM with increasing numbers of copies of the insertion allele (χ^2 for trend 0.24, 1 df, $P=0.62$).

For GSTM1, there was also no significant association with OSCM. The proportion of subjects with the + genotype among the cases was somewhat higher than that among the controls (36.0% *v.* 25.4%), but this difference was not significant (χ^2 1.59, 1 df, $P=0.207$). The OR for OSCM among subjects with the + genotype was 1.67 (95% CI 0.75, 3.70) when compared with subjects whose genotype was 0/0.

Tests of between-group and between-genotype differences in urinary isoprostane level

We also examined the determinants of urinary isoprostanes among the cases and controls; we were particularly interested

Table 1. Current age, weight, height, BMI and urinary isoprostane levels for subjects with previous diagnoses of oedematous (cases) and non-oedematous (controls) severe childhood malnutrition (Values are means and standard deviations)

	Cases		Controls		P values*
	Mean	SD	Mean	SD	
<i>n</i> (% male)	74 (68%)		50 (60%)		NS†
Age (years)	7.5	4.9	9.6*	3.9	0.010
Weight (kg)	25.3	16.9	28.1	13.6	NS
Height (m)	118.1	30.9	129.4*	22.8	0.023
BMI (kg/m ²)	16.5	3.0	15.7	2.5	NS†
Urinary isoprostanes (ng/mmol creatinine)	266.1	164.2	196.5*	99.6	0.013

* *P* values are two-sided and are derived from the unpaired *t* test or, in the case of the percentage of males in each group, from a χ^2 test with 1 df.

† *P* > 0.05.

For details of subjects and procedures, see p. 244.

Table 2. Risk ratios for a previous diagnosis of oedematous severe childhood malnutrition associated with genetic polymorphisms of genes encoding glutathione S-transferase (GST)

	Cases	Controls	Odds ratio	95 % CI
GSTP1				
Ile/Ile	17	23	1.00	
Ile/Val	25	15	2.25	0.90, 5.66
Val/Val	18	7	3.48	1.12, 10.78
GSTT1				
0/0	12	16	1.00	
+ /+ or +/0	58	32	2.44	1.00, 5.88
GSTM3				
Deletion/deletion	30	20	1.00	
Insertion/deletion	33	27	0.81	0.38, 1.75
Insertion/insertion	8	2	2.67	0.50, 14.36
GSTM1				
0/0	18	18	1.00	
+ /+ or +/0	53	32	1.67	0.75, 3.70

For details of subjects and procedures, see p. 244.

in testing whether there were significant between-group or between-genotype differences in isoprostanes in this group of participants with documented histories of SCM who were not severely malnourished (mean height-for-age z score -0.85 (SD 1.8), mean BMI z score -0.75 (SD 1.1)) at the time they were studied; a significant association with genotype would suggest that inherited variation might contribute to the differences between OSCM and non-OSCM. Table 1 provides data on anthropometric variables and urinary isoprostanes for cases and controls. In a preliminary survey of the differences between the two groups, the level of urinary isoprostanes was found to be lower among controls than the cases, and this difference was significant ($t -2.5$, $P=0.013$). The controls were older, taller and heavier than the cases; the differences between groups for age ($t 2.6$, $P=0.010$) and height ($t 2.2$, $P=0.033$) were significant, but those for weight and BMI were not ($P>0.05$).

In order to determine whether previous SCM status or any of the GST genotypes was associated with the level of urinary isoprostanes after adjustment for the age and anthropometric differences between the case and control groups, we fitted multivariable linear regression models that included terms for OSCM (i.e. either case or control), gender, age, age² and height, and also for the different GST genotypes as predictors. There was significant heteroscedasticity of urinary isoprostanes, so the regression models were fitted to the isoprostanes data after

log transformation. The regression coefficients and associated standard errors for OSCM and for the GST genotypes were estimated from the most general model and are given in Table 3. Each coefficient represents the effect of that variable on urinary isoprostanes after adjustment for the effects of the other variables in the model. In this model, the previous nutritional state of a subject was not a significant predictor of current level of urinary isoprostanes ($B 20.2$, $P>0.05$), nor were any of the GST genotypes ($P>0.05$ for all). The only terms that had a significant influence on model fit were age, age² and height.

As an additional exploration, ANOVA models were fitted to the isoprostane data, including a term for diagnosis that had three levels (kwashiorkor, marasmic kwashiorkor, marasmus), rather than the two levels of 'cases' and 'controls'. This was done to determine whether there were differences between the two types of OSCM. The variance due to diagnosis defined in this way was, however, small and not significant both with and without the inclusion of the covariates included in the linear regression models (data not shown).

Discussion

GSH plays an important role in the body's antioxidant defences, and low GSH levels indicate that these are impaired. It is not entirely certain, however, how GSH becomes depleted, nor is it clear why low GSH levels should be found in OSCM and not NOSCM. Recent data from Malawi, where a double-blind randomised trial of antioxidant supplementation failed to demonstrate a significant reduction in risk of kwashiorkor (Ciliberto *et al.* 2005), further emphasise these uncertainties surrounding the pathogenesis of OSCM. The existence of interindividual variation in the ability to handle the stresses imposed by malnutrition might help to explain heterogeneous clinical outcomes in the face of apparently homogeneous environments. The hypothesis explored in this paper is that persons who had developed OSCM and its associated increased levels of oxidant damage would have different genotype and allele frequencies for genetic variants of different GST isoforms when compared with those who had NOSCM.

In the present retrospective study, we have examined the association between OSCM and the functional or potentially functional variants that have been described (Chen & Board, 1987; Seidegård *et al.* 1988; Zimniak *et al.* 1994; Inskip *et al.* 1995) in four genes of the GST superfamily; the use of functional variants instead of 'anonymous' or

Table 3. Coefficients (B) and standard errors for the regression of urinary isoprostanes on age, age², height, previous nutritional status (oedematous severe childhood malnutrition) and glutathione S-transferase (GST) genotype

Variable	B	SE	P value*
Oedematous severe childhood malnutrition	20.2	23.3	0.39
GSTM1	-51.5	23.7	0.14
GSTT1	-0.45	26.8	0.60
GSTP1	-14.9	14.1	0.12
GSTM3	-12.0	18.0	0.20

* Estimated from the ratio of the likelihood of the general model to that of a restricted model.

For details of subjects and procedures, see p. 244.

non-functional genetic markers potentially makes the task of establishing the biological connection between DNA sequence variation and outcomes simpler. We found that there was a significant association between the valine¹⁰⁵ variant of GSTP1 and risk of OSCM: subjects who were homozygous for the valine¹⁰⁵ allele were almost 3.5-fold more likely to have had a previous diagnosis of OSCM ($P=0.02$). We also found that subjects with the GSTT1 + genotype (i.e. +/0 or +/+) had an approximately 2.4-fold higher risk of OSCM, which was of borderline significance ($P=0.04$). In the case of GSTM1 and GSTM3, no significant associations were found. We did note, however, that GSTM1 +, like GSTT1 +, was associated with a higher risk of OSCM (approximately 1.7-fold), even though the increased risk was not significant ($P=0.21$). These preliminary data provide some support for the idea that genetic variation in GST may contribute to risk of OSCM.

Based on the roles of GST in detoxifying harmful xenobiotics (Commandeur *et al.* 1995; Wang & Ballatori, 1998; Hayes & Strange, 2000; Rinaldi *et al.* 2002), it might have been expected that GST variants with reduced activity would confer an increased risk of OSCM, but this does not appear to be the case in the present study. One possibility is that the variants that appear to be associated with an increased risk of OSCM may actually catalyse the bioactivation or toxification of some endogenous or exogenous ligand; this has been described for substrates of GSTT1 and GSTP1 (Commandeur *et al.* 1995; Sherratt *et al.* 1997). An alternative possibility, which is plausible for the significant association observed for the GSTP1 variant, is that the associated variant does actually have decreased activity against some (unknown) reactive substrate. The valine¹⁰⁵ GSTP1 variant, which has increased activity against *anti*-diol epoxides of benzo(a)pyrene, has reduced activity against chlorodinitrobenzene and *anti*-diol epoxides of benzo[*g*]chrysene compared with isoleucine¹⁰⁵ (Hayes & Strange, 2000). Although either of these proposed explanations would be expected to result in a higher risk of oxidant damage to cell constituents, the biological basis for the observed associations remains to be established.

Erythrocyte GST activity against chlorodinitrobenzene has been reported to be increased in children with OSCM or NOSCM compared with children without (Ramdath & Golden, 1988, 1993). These reports underscore the increased relative importance of GST pathways in SCM but provide little, if any, information about the influence of genetic variation on risk of OSCM. Chlorodinitrobenzene is a relatively non-specific substrate for GST, with only a small difference in activity between the most and least effective isoenzymes (van Ommen *et al.* 1990). Studies using chlorodinitrobenzene would thus be expected to have low power to detect the contribution of genetic variants to between-group differences in erythrocyte GST activity. Of potentially greater importance, in any event, would be measures of the impact of genetic variation in GST activity on metabolic processes in, for example, the vascular endothelium or liver.

It is also possible, even if there are biologically plausible explanations, that the differential survival of patients with low-activity alleles or some other type of selection bias, or type I error, might account for the observed associations. For example, the in-hospital mortality of patients with OSCM has been reported to be higher than that of patients

with NOSCM (Schofield & Ashworth, 1996), but the low overall mortality (approximately 7%) at the Tropical Metabolism Research Unit (Cooper & Smartt, 1996) suggests that the magnitude of this particular bias is likely to be small. We also found that there were no significant between-group or between-genotype differences in the excretion of urinary isoprostanates. This finding is consistent with the view that the effect of variant GST on lipid peroxidation would be expected to be small or negligible among persons who have been restored to normal nutritional status. It seems unlikely, therefore, that there would be an association between GST genotype and survival after discharge from hospital. Ultimately, however, the possible effects of biases and chance will need to be addressed by attempting to replicate the present findings.

Studies of GSH that utilise stable isotope methods in a small number of research participants provide important information about metabolic adaptations in SCM (Reid & Jahoor, 2000, 2001). There remain difficulties, however, in distinguishing primary from secondary phenomena. The study of genetic determinants may be useful in this regard; associations between outcomes and functional genetic polymorphisms, in which the polymorphisms mimic a proposed exposure, may provide important clues about causation and are not likely to be susceptible to 'reverse causation' (Youngman *et al.* 2000; Davey Smith & Ebrahim, 2003). Furthermore, the inferences derived from genetic studies are not restricted to easily available cells – coding information about isoforms expressed in inaccessible tissues is available from any DNA-containing cell.

It has only recently been demonstrated that outcomes in infectious (i.e. 'environmental') diseases such as *Plasmodium falciparum* malaria, tuberculosis and HIV/AIDS are influenced by variants in a range of genes (Segal & Hill, 2003). The results of the present small study provide some support for the idea that genetic variation might contribute to the clinical picture of OSCM. Larger, retrospective and prospective studies of genetic variants of GST and other candidate genes, in Jamaica and elsewhere, will be required in order to confirm our preliminary findings. Such studies may provide insights that could complement other approaches to the understanding of OSCM.

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