Functional compensation of glutathione S-transferase M1 \((\text{GSTM1})\) null by another GST superfamily member, \(\text{GSTM2}\)

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The gene for glutathione-S-transferase (GST) M1 \((\text{GSTM1})\), a member of the GST-superfamily, is widely studied in cancer risk with regard to the homozygous deletion of the gene \((\text{GSTM1} \text{ null})\), leading to a lack of corresponding enzymatic activity. Many of these studies have reported inconsistent findings regarding its association with cancer risk. Therefore, we employed in silico, in vitro, and in vivo approaches to investigate whether the absence of a functional GSTM1 enzyme in a null variant can be compensated for by other family members. Through the in silico approach, we identified maximum structural homology between \(\text{GSTM1}\) and \(\text{GSTM2}\). Total plasma GST enzymatic activity was similar in recruited individuals, irrespective of their \(\text{GSTM1}\) genotype (positive/null). Furthermore, expression profiling using real-time PCR, western blotting, and \(\text{GSTM2}\) overexpression following transient knockdown of \(\text{GSTM1}\) in HeLa cells confirmed that the absence of GSTM1 activity can be compensated for by the overexpression of \(\text{GSTM2}\).
the other members of the GST family, particularly those belonging to the GSTM group, can compensate for the loss of the GSTM1 enzyme due to the absence of GSTM1 under normal physiological conditions.

**Results**

**GSTM1 shares maximum homology with GSTM2.** In our structural homology analysis, the members of the GST superfamily were found to share high sequence homology with each other when examined by ClustalW (http://www.genome.jp/tools/clustalw/) and a domain search using Pfam (http://pfam.sanger.ac.uk). Members of the same class (i.e., other GST\(\mu\) enzymes) share 75–99% sequence identity (maximum homology between GSTM1 and GSTM2), whereas the homology is approximately 25–30% with different classes (GST\(\pi\) and GST\(\varepsilon\)). This finding prompted us to perform a 3D superimposition of the GSTM1 protein structure with other members of the GST\(\mu\) family, GSTT1, and GSTP1 through Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB-PDB). The results distinctly demonstrated that GSTM2 has the highest degree of identical 3D organisation with GSTM1 (root-mean-square deviation [RMSD] value of 0.7 Å). In addition, the enzyme expression pattern from the GeneCard database (http://www.genecards.org/) also suggests a similar pattern of expression among the family members, with maximum similarity in expression patterns in the case of GSTM1 and GSTM2 in different tissues.

**Similar GST enzymatic activities in GSTM1 null and non-null groups.** We recruited 275 healthy individuals for screening the GSTM1-null variant. Among the 275 individuals initially recruited, 68 (24.73%) were found to have a GSTM1-null (homozygous deletion for GSTM1) genotype; the remaining 207 (75.27%) individuals were positive for GSTM1 (had at least one functional GSTM1 allele). The null group was composed of 18 female and 50 male individuals; 36 female and 100 male participants were selected from the 207 GSTM1-positive individuals (matched in terms of age, gender, and tobacco usage to nullify possible confounding factors) for further studies. We measured the total plasma glutathione S-transferase enzymatic activity level in the GSTM1-null and -positive individuals. However, the detection of the actual GSTM1 concentration is difficult due to the limitations of antibody-based detection methods and high cross-reactivity among members of the GST\(\mu\) subfamily; thus, we measured the total plasma GST activity. For this purpose, a non-fluorescent dye, monochlorobimane (MCB), was used. No significant difference was observed in the overall plasma GST activity between the GSTM1-null and -positive individuals (Fig. 1). In other words, the GSTM1-null individuals exhibited the same catalytic efficiency for MCB as the GSTM1-positive individuals.

**Overexpression of GSTM2 in GSTM1-null individuals.** Real-time PCR was performed to explore the contribution of other GST family members compensating for GSTM1 activity in null individuals in vivo. We evaluated the expression pattern of several members of the GST family (GSTM1-GSTM5, GSTT1, and GSTP1) in presence or absence of GSTM1. For this purpose, 15 age, sex, and tobacco usage-matched individuals were selected from both the GSTM1-positive and -null groups, and the gene expression levels of the seven aforementioned GST members were examined. A two-step quantitative real-time polymerase chain reaction (qRT-PCR) approach was implemented using SYBR-Green I. The preliminary results indicated that GSTM2 was expressed at an approximately 2.4-fold higher level in the lymphocytes of GSTM1-null individuals compared to the GSTM1-positive individuals; however, no significant difference was observed in the case of the other GST enzymes (Fig. 2). We also measured the expression pattern of GSTM1 and GSTM2 in the lymphocytes of the GSTM1-positive and -null individuals by western blotting and found that expression of the GSTM2 protein was considerably higher (~2-fold) in the GSTM1-null individuals compared to the GSTM1-positive individuals (Fig. 3).

**Restoration of cellular function by GSTM2 in GSTM1-null individuals.** To verify the observed compensatory role of GSTM2 in the absence of GSTM1, a cell culture-based approach was employed. We used green fluorescent protein (GFP)-tagged plasmid constructs of GSTM1 and/or GSTM2 in HeLa cells and evaluated the capacity of the transfected cells to cleave the glutathione-sulphophorahphate (GSH-SF) conjugate, an isothiocyanate intermediate that is naturally produced in the body during post-digestion, is normally broken down to free sulphorphosphate (SF) by the catalytic action of GSTM1\(^{21}\). Sulforaphane induces cell death, mainly through apoptosis, by acting as a growth inhibitor in such cancer cell lines as HeLa and HCT116 (human colon carcinoma) where the catalytic action of the aforementioned GST members was examined. A two-step quantitative real-time polymerase chain reaction (qRT-PCR) approach was implemented using SYBR-Green I. The preliminary results indicated that GSTM2 was expressed at an approximately 2.4-fold higher level in the lymphocytes of GSTM1-null individuals compared to the GSTM1-positive individuals; however, no significant difference was observed in the case of the other GST enzymes (Fig. 2). We also measured the expression pattern of GSTM1 and GSTM2 in the lymphocytes of the GSTM1-positive and -null individuals by western blotting and found that expression of the GSTM2 protein was considerably higher (~2-fold) in the GSTM1-null individuals compared to the GSTM1-positive individuals (Fig. 3).

**Discussion**

The majority of polymorphisms found to affect genes involved in carcinogenesis are single-nucleotide polymorphisms. In contrast, the complete absence of a function in the form of null allele is relatively rare; thus, the GSTM1 homozygous deletion genotype has attracted much attention of researchers worldwide. Extensive studies have
been attempted to link the GSTM1-null genotype with disease, particularly cancer. However, the results of association studies correlating GSTM1 with disease risk have been inconclusive, and numerous studies, including a large number of meta-analysis reports, have failed to demonstrate a significant association. A meta-analysis of 98 case-control studies was conducted to test the association of GSTM1 null with lung cancer risk, revealing a poor association in both random and fixed effect models; however, no increase risk was seen when only the five largest studies (>500 cases each) were considered. Analysing 130 case-control studies on GSTM1 null with lung cancer risk revealed a similar observation. GSTM1 is highly polymorphic, and the prevalence of the GSTM1-null genotype in different populations ranges from 64% to as high as 100% in Kiribati natives. The frequency also suggests that this gene

Figure 2 | Gene expression profiling of GST enzymes. Normalised gene expression profile (mean ± SEM) for GST family members in 15 GSTM1-null individuals with respect to the 15 age and sex-matched GSTM1-positive individuals. The significantly high GSTM2 expression (2.4 fold) under normal physiological conditions indicates a compensatory mechanism in the individuals completely lacking the GSTM1 enzyme.

Figure 3 | Western blot analysis reveals high GSTM2 expression in the GSTM1-null individuals. Representative figure for (a) β-actin from one GSTM1 positive (Lane 1) and two null individuals (Lanes 2 and 3). (b) A GSTM1-positive individual showing a specific 26-kDa band for GSTM1, whereas no band is observed for the GSTM1-null individuals. (c) GSTM2 is present in all three individuals, though with various intensities. (d) A densitometric analysis (mean ± SEM; pixels/ng) of the target proteins in the western blot. A total of 17 GSTM1 null and 16 GSTM1 positive samples were analyzed. All the blots are representative cropped images and every set have been processed simultaneously, under similar conditions. Representative original blots with cropped demarcations (3b & 3c) are provided in supplementary figure 1.
has not encountered strong environmental selection pressure during evolution and that there might be other enzymes involved in similar chemical detoxification. Based on the results of association studies, it can be clearly understood that GSTM1, a low-penetrant gene, is not a major determinant for cancer association. However, cancer risk can be modulated due to this polymorphism. Therefore, it is important to test the predictive value of the GSTM1-null variant before population-based association studies are conducted. Accordingly, in the present study, we attempted to highlight the role of other family members, particularly in the absence of a functional GSTM1 allele.

Although no enzyme activity is expected in individuals with a null genotype. There are some interesting observations in which GSTM1 activity was identified in GSTM1-null individuals, though the authors failed to present any supportive evidence	extsuperscript{39,40}. These findings support our observation of the total GST activity being similar in individuals, irrespective of the presence or absence of GSTM1. This situation is possible only if another member of the GST family compensates for the loss of GSTM1 in the null individuals. In our study, the enhanced expression of GSTM2, both at the mRNA and protein level, confirmed the role of a compensatory mechanism by a family member in the absence of GSTM1. Moreover, our in vitro functional assays clearly demonstrated a rescue of catalytic activity towards Glutathione-sulforaphane breakdown by GSTM2 over expression in cells where GSTM1 was knocked down. Taken together, these observations, i.e., structural and functional, strengthen our hypothesis of the compensatory role of GSTM2 in the absence of a functional GSTM1 gene. Therefore, a new assessment of the association studies connecting the GSTM1-null phenotype with disease incidence is required, and such studies must be supplemented with functional proof to substantiate their findings. In addition, we might also extrapolate the results of this study to hypothesise that, while studying a disease involving a gene family with high sequence homology and overlapping substrate specificity, the examination of only one gene will not provide the proper insight of the disease in question, as multiple numbers of gene families can function simultaneously.

**Methods**

**Study samples.** Healthy study participants were selected from the East Midnapore district, West Bengal, India. We collected blood samples (approx. 5 ml each) from 275 individuals (ages between 15 and 70 years). All the participants were recruited after a thorough screening by physicians, and each provided informed consent before they were included in the study. The non-physician interviewer examined the participants on the basis of a structured questionnaire that elicited information about their lifetime residential history, occupation, diet, and smoking habit. This study was conducted in accord with the Helsinki II Declaration and approved by the ethics committee of CSIR-Indian Institute of Chemical Biology.

**Isolation of plasma, DNA, RNA, and protein.** Blood samples were centrifuged at 1000 × g for 10 minutes at 4°C to isolate plasma. Nucleic acids and proteins were isolated from blood using the QiaGen DNA, RNA, protein isolation kit following the
manufacturer’s protocol (Qiagen, Hilden, Germany). The concentration and quality of the nucleic acids were measured using a NanoDrop instrument (NanoDrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis. The protein concentration was determined using the standard protocol of Bradford (Amresco, OH, USA) assay using bovine serum albumin (HiMedia, India) as a standard; the specific enzyme activity was expressed in mU/mg "in vitro".

**Screening of GSTM1-null samples.** For identifying and confirming the GSTM1-null variant, two exons (exon 2 and exon 7) were amplified separately35. To ensure that the absence of PCR products for any template was due to the presence of a null mutation and not the result of amplification failure, GSTM2 exon 1 (FP, 5′-CTGTGCCAGAATTCACAGGC-3′; and RP, 5′-CTGGACGTGCCATCAACCT-3′) was amplified as a positive control. Cycling was performed using an Eppendorf Mastercycler (Hamburg, Germany), as follows: a pre-PCR step of a 5-min denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing for 30 sec, and extension at 72°C for 30 sec, and a final 5-min incubation at 72°C; the annealing temperature was 69°C for (M1_exon 2) or 58°C for (both M1_exon 7 and M2_exon 1). All PCR products were separated by polyacrylamide gel (6%) electrophoresis, stained with ethidium bromide, and photographed under UV light.

**Total plasma GST enzyme activity assay.** The total plasma GST enzyme activity was measured using the Bio-Vision Fluorometric activity assay kit (CA, USA) following the manufacturer’s protocol. Monochlorobimane (MCB), a non-fluorescent substrate, was used; MCB fluoresces blue upon reaction with glutathione, and the level of fluorescence is directly proportional to the enzyme activity. The fluorescence was quantified using a micro-plate fluorospectrometer (LS 55, Perkin Elmer) at Ex/Em of 380/460 nm. The plasma protein concentration was estimated using the Bradford assay, as described above, prior to the enzyme activity analysis.

**Expression profile of the GSTM group using real-time PCR.** A two-step qRT-PCR approach was considered using SYBR-Green I (Brilliant SYBR Green QPCR master Mix, Agilent Technologies, CA, USA). Total RNA (1 μg) isolated from each sample was treated with DNase I (Applied Biosystems, Foster City, CA) prior to cDNA synthesis using the MMLV-based reverse transcriptase enzyme (RevertAid H Minus First strand cDNA synthesis kit, Fermentas Life Sciences, USA). Primers were designed for the exon-exon boundary using Primer 3 software, and the β-actin gene was used as an internal control (Table 1). Each sample, in duplicate, was amplified as follows: one cycle of 95°C for 1 minute for pre-incubation, followed by 40 cycles of 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute, with a subsequent melting curve analysis using Mx3000p (Stratagene, Agilent Technologies, CA, USA). In addition, after qRT, the amplified product was further analysed by PAGE. An efficiency correction was performed using Agilent software. The fold change in the target gene expression in the GSTM1-positive versus the GSTM1-null samples was calculated using the formula 2-ΔΔCt following the general guidelines discussed by Schmittgen and Livak.36 The fold difference was calculated after the data were normalised with the internal control. A less than 0.5 fold change was considered to be under-expression, whereas a >2.0-fold increase was considered to be overexpression.

**Western blotting.** Protein lysates were prepared using 1% sodium dodecyl sulphate (SDS) lysis buffer and resolved by 12.5% SDS-PAGE for 2 hours at 150 mA, followed by dry transfer (i-Blot protein transfer apparatus, Invitrogen, USA) and incubation with primary antibodies. Rabbit anti-human GSTM1 antibody (Upstate Biotechnology, Lake Placid, NY, USA), rabbit anti-human GSTM2 antibody (Lifespan Biosciences, Inc., Seattle, WA) and rabbit anti-rabbit IgG (Santa Cruz Biotechnologies, CA, USA) were purchased from OriGene Technologies Inc. (Rockville, MD, USA). The plasmids were propagated in E. coli DH5α and purified using Qiagen plasmid purification kits (Qiagen, Valencia, CA, USA). A 20-mM stock solution of the GSH-SF conjugate (USBio, Swampscott, MA, USA) was stored at a molar concentration-grade with Invitrogen, Carlsbad, CA, USA) and stored at -20°C until use. Water was used as vehicle/control for the GSH-SF treatment. Empty GFP vector and scrambled siRNA were used as the control for the overexpression and knockdown experiments, respectively. For GSTM1 knockdown, HeLa cells were transfected with scrambled or GSTM1 siRNA (Dharmacon Inc., USA) using the Dharmafect-1 transfection reagent according to manufacturer’s instructions. At 72 hours post-RNA GSTM2 transfection (the overexpression of GSTM1 knockdown) was carried out with GSTM2 or control plasmids using Lipofectamine LTX (Invitrogen, Carlsbad, CA, USA) reagent according to the manufacturer’s protocols.36 At 48 hours post-GSTM2 transfection, the expression efficiency was evaluated by ~80%, as based on the GFP reporter expression. The cells were starved overnight and were treated with 15 μM GSH-SF or vehicle for additional 48 hours.

**Post-treatment, cell viability was then assessed by trypan blue exclusion assay using a Neubauer haemocytometer under an inverted bright-field microscope (Leica Microsystems, GmbH, Germany). The data represent the total counts of 200 cells, expressed as percentage, from each group across three independent experiments.

**Statistical analyses.** All the data are expressed as the mean ± S.E. The statistical analyses were performed with the Mann–Whitney test or Student’s t-test, as applicable. GraphPad was used for the analyses.

| Table 1 | Primers used in gene expression profiling |
|---------|-----------------------------------------|
| **Primer** | **Sequence** |
| M1FP | AGCGGCTATGGTGGCAAGA |
| M1RP | TCTCACAAGGCTCTAACAGCCG |
| M2FP | CGAGCAAAGCGCCATCCT |
| M2RP | GTTCGCCAGAGGAGGT |
| M3FP | TCATCATGCTGTCAGGCAGCTG |
| M3RP | TCATACGACAGCTCTGGCCA |
| M4FP | TGGAAAGACCGGCTATGAGGCG |
| M4RP | CACCGGACTGGAAGAAGGGTG |
| M5FP | AACGCAACAGCTGTTGGGAGG |
| M5RP | AGACAGCTGAGCAAGCCG |
| P1FP | TATTTTGACAGCTGACGGCCG |
| P1RP | AACCTGGGAGCTGCTGCTG |
| T1FP | CGTCGCGCCGGCTGTATA |
| T1RP | TCCAAGGAGCAAGCTG |
| β-actin F | CGACGACAGACAGCAGCTG |
| β-actin R | TCTATCATCGATGGACAGGCG |
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**Author contributions**
P.B., S.P. and A.K.G. conceived and designed the experiments. P.B., S.P., D.P. and P.B. conducted the experiments. P.B., N.G., A.B. and A.K.G. analysed and interpreted the results. P.B., M.B. and A.K.G. wrote the manuscript. A.K.G. supervised the project. All the authors contributed to the scientific planning and discussions.

**Additional information**

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