SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
FUNCTIONAL GENOMICS
GENE EXPRESSION PROFILING
RNAI
GENETICS

Received
8 January 2013

Accepted
6 August 2013

Published
19 September 2013

Correspondence and requests for materials should be addressed to A.K.G. (akgiri15@yahoo.com; akgiri@iicb.res.in)

* Current address: Department of Environmental Sciences, University of Calcutta, Kolkata-700 019, India

Functional compensation of glutathione S-transferase M1 (*GSTM1*) null by another GST superfamily member, *GSTM2*

Pritha Bhattacharjee[1]*, Somnath Paul[1], Mayukh Banerjee[1], Deblina Patra[1], Priyam Banerjee[2], Nanda Ghoshal[3], Arun Bandyopadhyay[2] & Ashok K. Giri[1]

[1]Molecular and Human Genetics Division, [2]Cell Biology and Physiology Division, [3]Structural Biology and Bio-Informatics Division, CSIR-Indian Institute of Chemical Biology, Kolkata – 700 032, India.

The gene for glutathione-S-transferase (GST) M1 (*GSTM1*), a member of the GST-superfamily, is widely studied in cancer risk with regard to the homozygous deletion of the gene (*GSTM1* 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 *GSTM1* and *GSTM2*. Total plasma GST enzymatic activity was similar in recruited individuals, irrespective of their *GSTM1* genotype (positive/null). Furthermore, expression profiling using real-time PCR, western blotting, and GSTM2 overexpression following transient knockdown of *GSTM1* in HeLa cells confirmed that the absence of GSTM1 activity can be compensated for by the overexpression of GSTM2.

Glutathione-S-transferases (GSTs) belong to a superfamily of ubiquitous, multifunctional dimeric cytosolic enzymes that play a very important role in the Phase II detoxification (or biotransformation) pathway in humans and confer protection against a wide array of toxic insults[1,2]. Several GST isoforms have been identified and characterised, forming seven distinct classes: α, μ, π, *o*, τ, κ, and ζ[3,4]. Functionally, most GSTs catalyse the conjugation of the nucleophilic tripeptide glutathione to a wide range of electrophilic substrates for detoxification. However, the conjugation reaction can occasionally lead to the formation of compounds that are far more toxic than the initial substrate, thereby leading to disease outcomes[1,5,6]. Interestingly, a null variant is encountered for two members, *GSTT1* and *GSTM1*, whereby the entire gene is homozygously deleted in a considerable proportion of different populations, resulting in the complete absence of the corresponding enzyme activity[7,8]. The *GSTM1* gene is highly polymorphic and is located on chromosome 1p13.3. A wide range of variation in *GSTM1* homozygous deletion polymorphism (approximately 20–67%) has been observed globally with regard to various ethnicities[9–12]. It is often hypothesised that, due to the lack of functional *GSTT1* and/or *GSTM1*, the null phenotype is unable to efficiently perform the conjugation reaction (biotransformation) and the subsequent elimination of toxic products via urine and bile. The null variant of *GSTM1* is of particular interest, as a plethora of studies have demonstrated the difference in susceptibility, exposure to environmental toxicants, resistance to chemotherapy treatment, variability in drug response, manifestation of several diseases, and, most importantly, cancerous outcomes.

The four other members of the GSTμ subfamily, *i.e.*, *GSTM2*, *GSTM3*, *GSTM4*, and *GSTM5*, exhibit high levels of sequence homology and substrate specificity with *GSTM1*[13]. Among these genes, *GSTM1* has largely been studied due to its null genotype. Although a large number of studies have attempted to associate the *GSTM1*-null genotype with cancer risk, the results are inconclusive. Several studies have attempted to identify the association of *GSTM1* null with cancer risk through meta-analysis using the existing literature; however, these analyses failed to show a significant association of *GSTM1* with cancer[14–20]. These observations prompted us to search for the functional relevance of this "well known gene" with other family members that are relatively less studied. A possible explanation of the apparently inconsistent results could be that other members of the GST family compensate for the absence of a functional GSTM1 enzyme. In this study, we attempted to ascertain whether

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 GSTμ enzymes) share 75–99% sequence identity (maximum homology between *GSTM1* and *GSTM2*), whereas the homology is approximately 25–30% with different classes (*GSTθ* and *GSTπ*). This finding prompted us to perform a 3D superimposition of the GSTM1 protein structure with other members of the GSTμ 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 GSTμ 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.

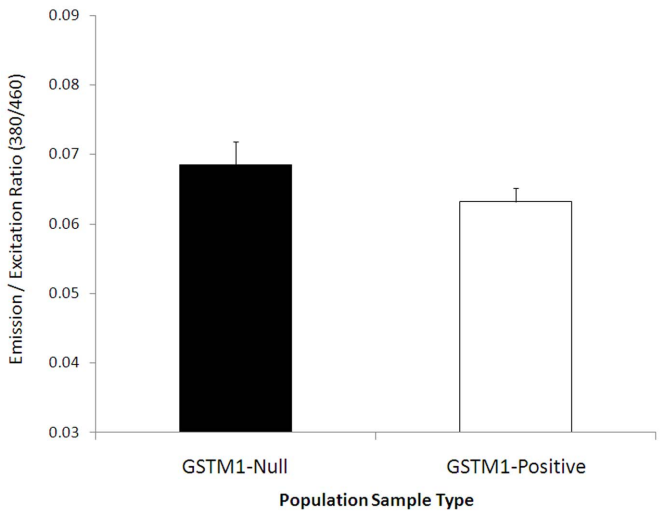

Figure 1 | Total plasma GST activity in the *GSTM1*-positive and -null groups. The total plasma enzyme activity (mean ± SEM, in $mU.mg^{-1}.min^{-1}$) was found to be similar in both groups, though GSTM1 isoenzyme activity should be undetectable in the *GSTM1*-null individuals. The similar level of total plasma GST activity indicates the presence of a counter-balance mechanism.

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-sulphoraphane (GSH-SF) conjugate, an isothiocyanate intermediate that is naturally produced in the body during post-digestion, is normally broken down to free sulphoraphane (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 HT29[22–24]. However, it is not known, whether the GSTM2 enzyme can perform a similar function. Indeed, if the GSTM2 enzyme catalyses the breakdown of this conjugate in a manner comparable to that of GSTM1, the functional similarity between these two isozymes would be established. It is known that HeLa cells express only basal levels of GSTM1, but not GSTM2[25]. We over-expressed *GSTM1* and *GSTM2* in HeLa cells following the transient knockdown of *GSTM1* by siRNA. The breakdown of the GSH-SF conjugate and subsequent release of free SF in each case was estimated from the percentage of cell death, as measured by a trypan blue exclusion assay[26]. The level of expression of GSTM1 and GSTM2 in each case was confirmed by western blotting of whole-cell lysates. The death rate of the cells in the presence of the GSH-SF conjugate either with GSTM1 or GSTM2 (over the background of *GSTM1* knockdown) was similar (Fig. 4). This result clearly demonstrated that both GSTM1 and GSTM2 had the same functional efficiency. In other words, GSTM2 could effectively compensate for the loss of *GSTM1* under physiological conditions.

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

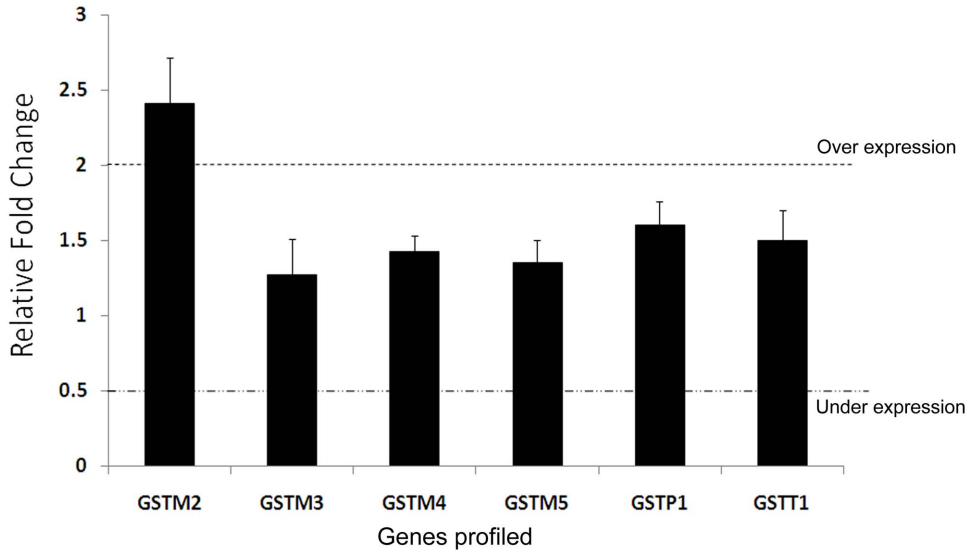

Figure 2 | Gene expression profiling of GST enzymes. Normalised gene expression profile (mean ± SEM) for GST family members in 15 *GSTM1*-null individuals with respect to the 15 age and sex-matched *GSTM1*-positive individuals. The significantly high *GSTM2* expression (2.4 fold) under normal physiological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

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[14,16,17]. 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[27]. Analysing 130 case-control studies on *GSTM1* null with lung cancer risk revealed a similar observation[15]. *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[28]. The frequency also suggests that this gene

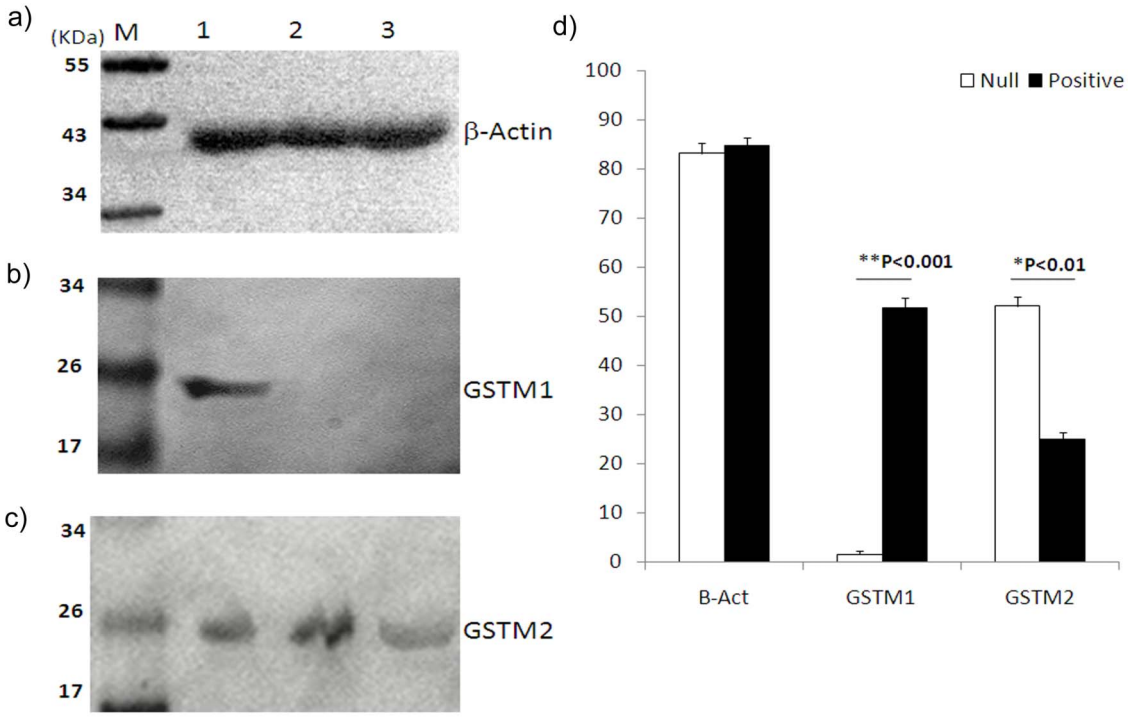

Figure 3 | Western blot analysis reveals high GSTM2 expression in the *GSTM1*-null individuals. Representative figure for (a) β-actin from one *GSTM1* positive (Lane 1) and two null individuals (Lanes 2 and 3). (b) A *GSTM1*-positive individual showing a specific 26-kDa band for GSTM1, whereas no band 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 simultaenously, under similar conditions. Representative original blots with cropped demarcations (3b & 3c) are provided in supplementary figure 1.

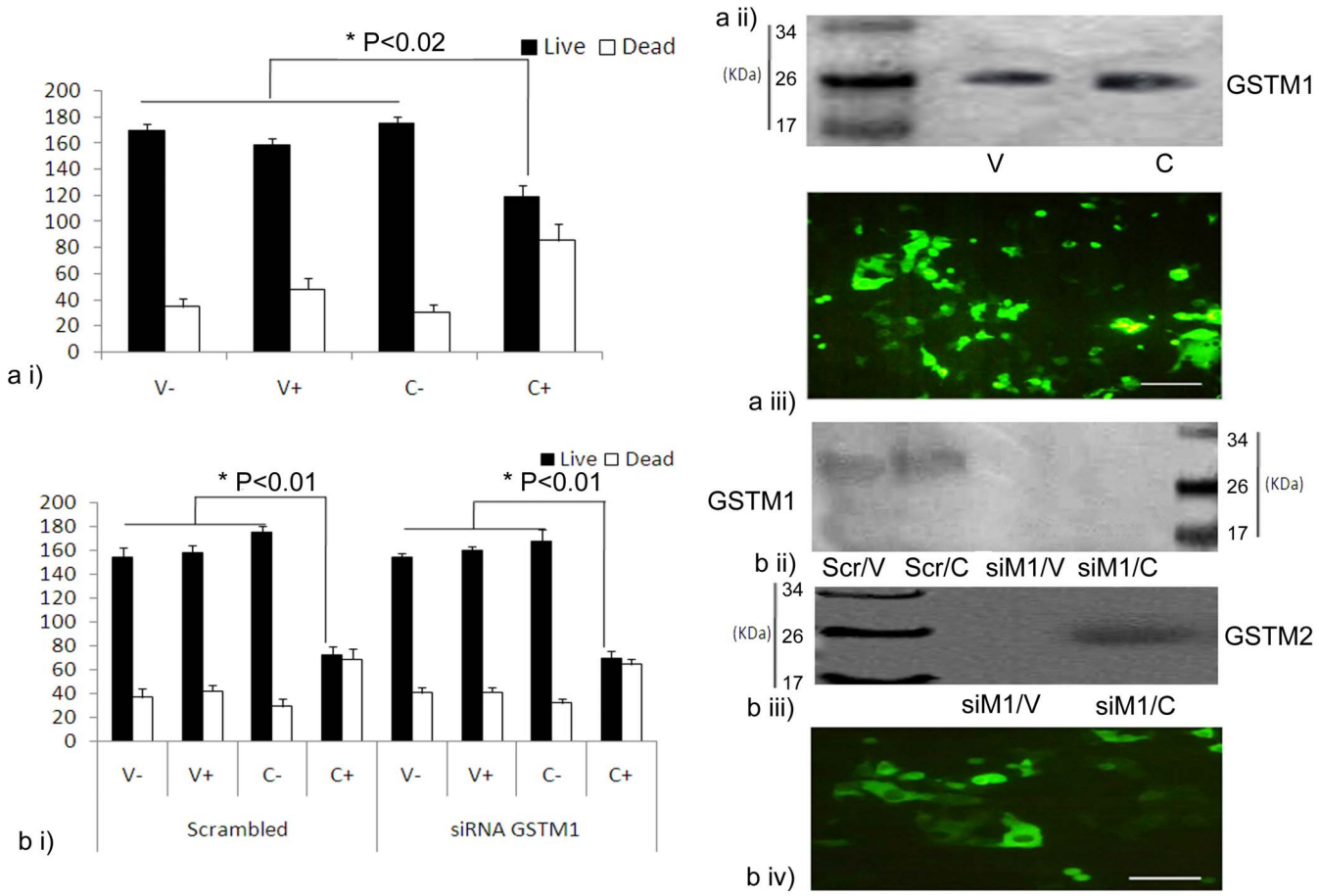

Figure 4 | Evidence for the functional similarity of the isozymes *GSTM1* and *GSTM2*. Group (a): i) Trypan blue cell viability assay for *GSTM1* in HeLa cells. ii) Western blotting analysis of GSTM1 overexpression in transfected (C) and control cells (V). iii) GFP profile in transfected HeLa cells. Group (b): i) Trypan blue cell viability assay of HeLa cells transfected with *GSTM2* and treated either with scrambled or *GSTM1*-targeted si-RNA (V-, empty vector without GSH-SF treatment, V+, empty vector with GSH-SF treatment, C-, plasmid construct with either *GSTM1* (in figure a) or *GSTM2* (in figure b) without GSH-SF treatment, C+, plasmid construct with GSH-SF treatment). ii) Western blotting analysis of GSTM1 in the siRNA-treated HeLa cells. iii) Western blot analysis of GSTM2 in siRNA-treated HeLa cells. iv) GFP profile of transfected HeLa cells after siRNA treatment. All the blots are representative cropped images and every set have been processed under similar conditions as detailed in the methods section.

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[29,30]. 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 $mUmg^{-1}min^{-1}$.

Screening of *GSTM1*-null samples. For identifying and confirming the *GSTM1*-null variant, two exons (exon 2 and exon 7) were amplified separately[31]. 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′-CTGTCTGCAGAATCCACAGC-3′, and RP, 5′-CTGCAGCTGCTCCACACTT-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 fluoro-spectrometer (LS 55, Perkin Elmer) at Ex/Em of 380/460 nm[32]. 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 MMuLV-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 min, 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 $2^{-\Delta\Delta CT}$ following the general guidelines discussed by Schmittgen and Livak[33]. 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-human beta-actin antibody (Santa Cruz Biotechnologies, CA, USA) were used in 1 : 1000 dilutions, followed by goat anti-rabbit IgG (GE Healthcare) as the secondary antibody (1 : 2000 dilutions).

Table 1 | Primers used in gene expression profiling

Primer	Sequence (5′ → 3′)
M1FP	AGCGGCCATGGTTTGCAGGAA
M1RP	TTCTCCAAGCCCTCAAAGCGG
M2FP	CCAGAGCAACGCCATCCT
M2RP	GATTCCCCGCACAGGTTGT
M3FP	TCACCATGTCGTGCGAGTCGT
M3RP	TCATAGTCAGGAGCTTCCCCGCA
M4FP	TGGAGAACCAGGCTATGGACGT
M4RP	CCAGGAACTGTGAGAAGTGCTG
M5FP	AAGCACAACCTGTGTGGGGAGA
M5RP	AGCACAGTCTGACCAGCTCCAT
P1FP	TATTTCCCAGTTCGAGGCCGCT
P1RP	AACTTGGGGAGCTGCCCGTATA
T1FP	CCTGCCGCGCTGTTTACATCT
T1RP	GCCACACTCTCCGTCAAGGTGA
β-actin F	CGAGCACAGAGCCTCGCCTT
β-actin R	TCATCATCCATGGTGAGCTGGCG

Cell culture and transfection. HeLa cells were obtained from the national cell repository of the National Center for Cell Science (Pune, India). GFP-tagged plasmid constructs for *GSTM1* and *GSTM2* were purchased from OriGene Technologies Inc. (Rockville, MD, USA). The plasmids were propagated in *E. coli* DH5alpha 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 prepared in molecular-grade water (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*i* *GSTM2* transfection (over the background 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[34]. At 48 hours post-*GSTM2* transfection, the expression efficiency was estimated to be ~ 80%, as based on GFP reporter expression. The cells were serum starved overnight and were treated with 15 μM GSH-SF or vehicle for additional 48 hours[22]. 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.

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Acknowledgements

We sincerely thank Council of Scientific and Industrial Research (CSIR), India, for providing the Senior Research Associateship (to P.B.) and Senior Research fellowship (to P. Banerjee). This work was supported by the CSIR Network Project NWP-0052 and CSIR-Emeritus Scientist Grants No 1(53)/2012/AKG to A.K.G. and CSIR Network Project BSC 0206 to A.B. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Bhattacharjee, P. *et al.* Functional compensation of glutathione S-transferase M1 (*GSTM1*) null by another GST superfamily member, *GSTM2. Sci. Rep.* **3**, 2704; DOI:10.1038/srep02704 (2013).