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ORIGINAL ARTICLE

Relevance of *GSTM1*, *GSTT1* and *GSTP1* Gene Polymorphism to Breast Cancer Susceptibility in Mizoram Population, Northeast India

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Abstract The enzymes encoded by glutathione S-transferase mu 1 (*GSTM1*) and theta 1 (*GSTT1*) genes are involved in the metabolism of wide range of carcinogens that are ubiquitous in the environment. Homozygous deletions of the *GSTM1* and *GSTT1* genes are commonly found and result in lack of enzyme activity. This study was undertaken to evaluate the association between *GSTM1*, *GSTT1* and *GSTP1* gene polymorphism and breast cancer risk in Mizoram population. Odd ratio (OR) and 95% confidence interval (CI) from conditional logistic regression model were used to estimate the association between genetic polymorphism and breast cancer risk. The *GSTM1* and *GSTT1* null genotypes were associated with an increased risk of breast cancer [OR = 10.80 (95% CI 1.16–100.43)]. The risk of breast cancer associated with the *GSTT1* null genotype was observed to be low among postmenopausal women. When considered together, *GSTM1* and *GSTT1* genotypes were found to be associated with an increased risk of breast cancer. The relationship between *GSTM1* and *GSTT1* gene deletions and breast cancer risk was substantially altered by consumption of Smoked Meat/Vegetable. In the present study, *GSTP1*Ile105Val (rs1695) polymorphism was related to breast cancer susceptibility or phenotype. Our data provides evidence for substantially increased risk of breast cancer associated with *GSTM1* and/or *GSTT1* homozygous gene deletions in Mizoram population.

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Introduction

Breast cancer (BC) is the third leading cause of cancer death, accounting for 9.7% of total cancer in the world and is the second most common cancer in India (Murthy et al. 2009). Mizoram belonging to North Eastern state of India has a high age-standardised breast cancer rate of 14.1 per 1,00,000 population (NCRP 2013). Studies have shown that consumption of alcohol, tobacco and peculiar food habits, especially high fat intake, are important risk factors for breast cancer (Ghatak et al. 2014; Sieri et al. 2008).

GSTs are polymorphic genes and are associated with various chemical detoxification mechanisms. GSTs have three major isoenzymes: GSTM (mu)1, GSTT (theta)1, and GSTP (pi)1 which are highly polymorphic in nature. The risk for cancer to a individual with polymorphism in one of the GST genes is observed to be low, but develops into a major risk factor when the frequency in that population becomes high. GSTM1 and GSTT1 genes reveal deletion polymorphism or null genotype, resulting in lack of enzyme activity. Tobacco smoke contains several types of carcinogens which require detoxification by different enzymatic pathways and in GSTP1, an amino acid substitution (lle105val) reported due to that an A-G polymorphism at nucleotide 313 position is linked with tobacco consumption. This substitution is present in the active site of the enzyme, and it affects the enzyme activity (Zimniak et al. 1994).

It is not so far understandable whether the GST polymorphism alters the risk for breast cancer. Most of the published data found no or less association, but these reports are based on few cases and on selected populations which could lead to false-negative conclusion. Hence, there is a need to study the correlation of GST polymorphism in different populations and their association with breast cancer. In the present study, a case–control study was performed to estimate the significance of GSTM1, GSTT1 and GSTP1 gene polymorphism to BC phenotype and to assess their association with demographic factors in this high-risk Mizo population, North east India.

Materials and Methods

Subjects

A total of 22 newly diagnosed breast cancer patients were recruited at Mizoram State Cancer Institute, Aizawl, Mizoram between May 2013 to July 2014. Patients who were histopathologically confirmed as breast adenocarcinoma were selected for this study. Demographic data and family history for BC were collected by a structured questionnaire administered by trained personnel as previously described (Ghatak et al. 2014). One ml of peripheral blood was collected from each patient including 10 control samples (HC) from healthy volunteers with no previous history of breast cancer and matched by gender, age (±5 years) and area of residence with the cancer patients. All participants gave written informed consent to the study

protocol which was approved by the Ethical Committee of the Civil Hospital, Mizoram and Mizoram University, India.

Extraction of Genomic DNA from Blood Sample

The lymphocytes from whole blood were separated by lysing the RBCs using a hypotonic buffer (ammonium bicarbonate and ammonium chloride, Hi-media) with minimal lysing effect on lymphocytes. Three volumes of RBC lysis buffer were added to the blood sample, mixed by vortexing and inverting thoroughly for 5 min and centrifuged (Eppendorf 5415R, Germany) at $2000 \times g$ for 10 min. The supernatant was discarded leaving behind about 100 μl to prevent loss of cells. To the pellet, three volumes of RBC lysis buffer were added and vortexed. The sample was mixed thoroughly by inverting and centrifuged repeatedly for 2–3 times until a clear supernatant and a clean white pellet was obtained. After final wash, the supernatant was discarded completely, and the pellet was resuspended in 500 μl PBS, followed by addition of 400 μl of cell lysis buffer (100 mM Tris–HCl, 50 mM EDTA, 50 mM Nacl 10% SDS, pH 7.5) and 10 μl of Proteinase K (10 mg/ml stock) (Hi-media). The sample was vortexed to dissolve the pellet completely and incubated for 2 h at 56°C in a water bath (Jeio-Tech, CW-30G) for lysis. An equal volume of phenol (equilibrated with Tris, pH 8) was subsequently added to the tube and mixed well by inverting for 1 min. The tube was centrifuged at $10,000 \times g$ (at 4°C) for 10 min, and the aqueous upper layer was transferred to a fresh tube containing equal volumes (1:1) of phenol and chloroform: Isoamyl alcohol (24:1). The mixture was mixed by inverting for 1 min and centrifuged for 10 min at $10,000 \times g$ (at 4°C). The supernatant was transferred to a fresh tube, and 10 μl of 10 mg/ml RNase A (Fisher Scientific, Fermentas, Germany) was added. The sample was incubated at 37°C for 30 min before an equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed by inverting the tube for 1 min and centrifuging at $10,000 \times g$ (at 4°C) for 10 min. The supernatant was transferred to a fresh tube and twice the volume of absolute alcohol (Merck) was added and inverted gently a few times and incubated at −20°C, followed by centrifugation at $10,000 \times g$ at (4°C) for 20 min. After decanting the supernatant, 250 μl of 70% ethanol was added, and the pellet was gently tapped, followed by centrifugation at $10,000 \times g$ for 10 min and gently decanting the supernatant. The pellet was air dried in a laminar air flow, and the dried pellet was resuspended in 50 μl of Nuclease-free water or 1X TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.6) buffer and stored in −20°C (Ghatak et al. 2013).

Genotyping of *GSTM1*, *GSTT1* and *GSTP1* Gene Polymorphism

Null genotype polymorphism of *GSTM1* and *GSTT1* were detected by multiplex polymerase chain reaction (PCR) according to previously described method (Arand et al. 1996). Primer sequences and conditions are summarised in Table 1. The reaction mixture (25 μl) contained 50–100 ng of genomic DNA in $1 \times$ Taq buffer, 0.2 mM of each dNTP, 0.2 μM of each primer, and 1 U of Taq DNA polymerase. The reaction mixture was heated to 94°C for 5 min, followed by 35 cycles each

Table 1 Primers and reaction conditions used for the detection of *GSTM1*, *GSTT1* and *GSTP1* gene polymorphisms

Gene	Primer (5′ to 3′)	Annealing temperature	Type of polymorphism	Amplified products/Alleles
GSTM1	F: GAACTCCCTGAAAAGCTAAAGC	60°C—1 min	Gene deletion	– Null genotype
	R: GTTGGGCTCAAATATACGGTGG			+ 219 bp
GSTT1	F: TTCCTTACTGGTCCTCACATCTC		Gene deletion	– Null genotype
	R: TCACCGGATCATGGCCAGCA			+ 459 bp
Albumin	F: GCCCTCTGCTAACAAGTCCTAC		–	Internal control
	R: CCCTAAAAAGAAAATCGCCAATC			349 bp
GSTP1 I105V (rs1695)	F: AATACCATCCTGCGTCACCT	59°C—40 s	*BsmAI* RFLP	A-digested fragment (308, 258 bp)
	R: TGAGGGCACAAGAAGCCCCTT			G-digested fragment (258, 219 and 89 bp)
GSTP1 V114A (rs1138272)	F: ACAGGGATTTGGTACTAGCCT	50°C—40 s	*AciI* RFLP	C-digested fragment (143, 27 bp)
	R: AGTGCCTTCACATAGTCATCCTTG			T-intact fragment (170 bp)

consisting of 30 s denaturation at 94°C, 30 s annealing at 60°C, 1 min of extension at 72°C and a final 5 min extension at 72°C. Amplified products were analysed by electrophoresis on 8% polyacrylamide gel which resulted in a 215-bp fragment for *GSTM1,* 480-bp fragment for *GSTT1* and 350-bp fragment for albumin gene as an internal control. The absence of the specific *GSTM1* and/or *GSTT1* fragments specify the parallel null genotype, whereas the presence of the 350-bp albumin gene fragment confirms that the accepted null genotype was not due to PCR failure.

The samples were genotyped by PCR–RFLP-based method for detecting the two amino acid substitutions at codons 105 (Ile → Val) (Zimniak et al. 1994) and 114 (Val → Ala) (Ali-Osman et al. 1997) due to the SNPs in the *GSTP1* gene. *GSTP1*Ile105Val (rs1695) polymorphic site was PCR amplified with specified primers, and the reaction mixture was heated to 94°C for 5 min, followed by 30 cycles each consisting of 30 s denaturation at 94°C, 30 s annealing at 60°C, 1 min of extension at 72°C and a final 5 min extension at 72°C. PCR products were digested with 1 unit of *BsmAI* (NEB, India) for 2 h at 55°C. Digests were electrophoresed on 8% polyacrylamide gel resulting in three fragments of 305, 135 and 128 bp (allele A) or in four fragments of 222, 135, 128 and 83 bp (allele G). PCR conditions for the *GSTP1*Ala114Val (rs1138272) polymorphism were denaturation for 5 min at 94°C followed by 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s with a final elongation step at 72°C for 5 min. PCR product was digested with 2 unit of *AciI* (NEB, India) for 90 min. at 37°C and electrophoresed on a 8% polyacrylamide gel. The T allele was concluded by the presence of a fragment of 170 bp and the C allele by the presence of two fragments of 143 and 27 bp (Table 1).

Statistical Analysis

The association between the different polymorphism and demographic factors was estimated for each group using odd ratio (OR) and 95% confidence interval (CI). *GSTT1* and *GSTM1* were combined as having no deletions or having homozygous deletions (*GSTT1*-null and *GSTM1*-null genotype, respectively), whereas *GSTP1* polymorphism association studies with demographic factors were also analysed using odd ratio (OR) and 95% confidence interval (CI). In addition, logistic regression analyses were carried out to calculate the influence of lifestyle factors for BC as the dependent variable. All statistical analyses were performed using SPSS and Sys-Stat statistical package.

Results and Discussion

Clinical and Demographic Characteristics

Correlation study showed that smoking habit and a positive family history for BC were found to be risk factors for the development of BC (Table 2). A strong association between post-menstrual BC risk and fat consumption (OR: 3.50; 95% CI 1.39–8.86; $P = 0.001$) was observed, and intake of smoked meat or vegetable was

Table 2 Demographic and clinical characteristics of healthy controls and breast cancer patients

Demographic factor	HC ($n = 10$)	BC ($n = 22$)	ORs (95% CI; RR)
Age years $\pm$ SD (range)	52.18 $\pm$ 12.35	58.7 $\pm$ 9.76	–
Cigarette smoking	65%	70%	1.26 (0.49–3.20; 1.07)
Fat intake	40%	70%	3.50 (1.39–8.86; 1.75)
Family history of breast cancer	12.5%	15%	1.24 (0.34–4.43; 1.20)
Smoked meat/vegetable	62.5%	90%	5.40 (1.60–18.20; 1.44)
Alcohol	42.5%	50%	1.16 (0.40–3.40; 1.13)

also significantly associated with BC risk (OR: 5.40; 95% CI 1.60–18.20; $P < 0.0004$).

Genotyping

Genotype frequencies of *GSTM1* and *GSTT1* polymorphism classified the breast cancer and control samples into two separate groups under the Hardy–Weinberg equilibrium (Table 3; Fig. 1). Highly significant differences in carriage rate and genotype distribution were observed between patient and controls when BC patients and controls were analysed as a single group. The frequencies of *GSTM1* and *GSTT1* null genotypes in BC patients differ significantly from controls (91% in BC vs. 60% in HC for *GSTM1*, and 63.6% in BC vs. 40% in HC for *GSTT1*). In addition, simultaneous presence of both the *GSTM1* and the *GSTT1* null genotypes differed in both the groups (30% in BC vs. 7.5% in HC; OR: 5.29, 95% CI 1.36–20.53). Strong significant dissimilarities in carriage, genotype or allele frequencies of the *GSTP1* Ile105Val polymorphism were observed between BC patients and controls rather than *GSTP1* Val114Ala polymorphism (Fig. 2).

Since the first publication by Lizard-Nacol et al. (1999) relating to the association between the *GST*s null genotype and the increased risk of BC, a large number of epidemiological studies have been conducted. In the present study, the *GSTM1* gene deletion was associated with a higher risk of BC than *GSTT1*. These results are contrary to expectations, since an individual with a *GSTM1*-null polymorphism was shown to have an increased vulnerability to cancer (García-Closas et al. 1999). Notably, the simultaneous presence of both the *GSTM1* and the *GSTT1* null genotypes were not identical in BC and control groups (54.54% in BC vs. 10% in HC; OR: 10.80, 95% CI 1.16–100.43). However, simultaneous deletions of *GSTM1* and *GSTT1* genes were found to be associated with BC risk in studies carried out in different geographical populations (Buchard et al. 2007), similar to the Mizoram population in the present study.

Some reports revealed that GST gene family was not associated with an increased susceptibility to breast cancer. Unlü et al. (2008) and Samson et al. (2007) demonstrated that the *GSTT1*, *GSTM1* null alleles and *GSTP1* (Ile105Val) were not associated with susceptibility to breast cancer in both Europe and Asian woman. A probable explanation for this inconsistency in GST profiling is due to the ethnic and geographic variations. Such variation is of particular interest in the case of *GSTT1*

**Table 3** Genotype distribution of *GST* gene polymorphisms in gastric cancer patients and healthy controls

Genotypes	Total HCs (n = 10)	Total BC patients (n = 22)	OR	95% CI	RR
GSTM1					
(+)	4 (40%)	2 (9%)	0.15	0.02–1.03	0.22
(−)	6 (60%)	20 (91%)	6.66	0.97–45.79	1.51
GSTT1					
(+)	6 (60%)	8 (36.36%)	0.38	0.08–1.76	0.60
(−)	4 (40%)	14 (63.63%)	2.62	0.56–12.17	1.59
GSTM1/T1					
(+/+)	2 (20%)	2 (9.09%)	0.40	0.04–3.34	0.45
(−/+)	4 (40%)	6 (27.27%)	0.56	0.11–2.71	0.68
(+/−)	3 (30%)	2 (9.09%)	0.23	0.03–1.69	0.30
(−/−)	1 (10%)	12 (54.54%)	10.80	1.16–100.43	5.45
GSTP1 105 (rs1695)					
A/A	10 (100%)	15 (68.18%)	0.001	0.51–0.90	0.68
A/G	0	5 (22.72%)	2.64	0.26–26.24	2.27
G/G	0	2 (9.09%)	0.90	0.07–11.25	0.90
GSTP1 114 (rs1138272)					
C/C	10 (100%)	21 (95.45%)	0.001	0.87–1.04	0.95
C/T	0	1 (4.54%)	0.42	0.02–7.63	0.45
T/T	0	0	Na	Na	Na
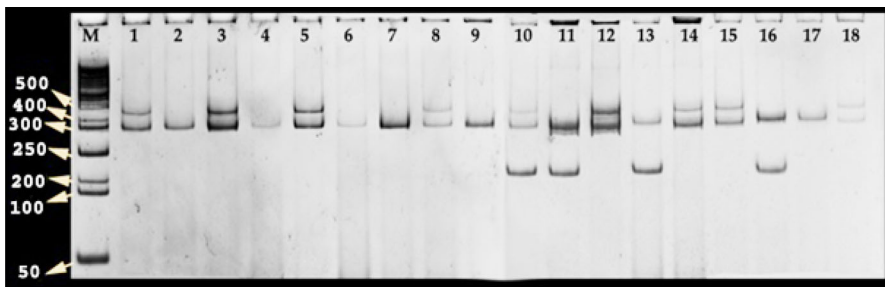
Fig. 1 Multiplex PCR-amplified products of *GSTT1* (459 bp), *GSTM1* (219 bp) and internal control gene albumin (349 bp). *M* low range ruler plus; *1–9* breast cancer samples; *10–18* healthy control samples

and *GSTM1* null frequencies which differ significantly between Asians and Caucasians. In this context, the European Prospective Investigation into Cancer and Nutrition study (EPIC study) reported geographic variations for the *GSTT1* deletion among European population with prevalence above 20% (Spain and the Netherlands) and below 10% (United Kingdom and Sweden) (Agudo et al. 2006).

Fig. 2 PCR-RFLP of GSTP1.1 (rs1695) and GSTP1.2 (rs1138272) regions in breast cancer samples. *S1—BsmAI* RFLP (A-digested fragment); *S2—BsmAI* RFLP (G-digested fragment). *S3—AciI* RFLP (C-digested fragment); *S4—AciI* RFLP (T-intact fragment)
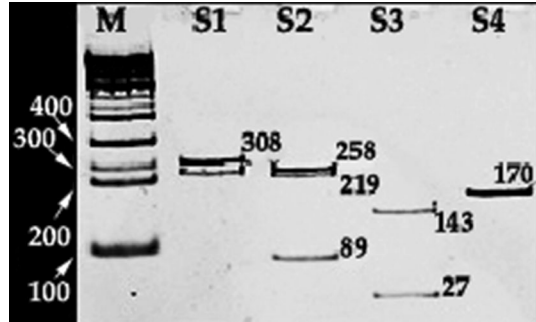
Conclusion

In summary, our data showed that *GSTM1* and *GSTT1* polymorphism along with consumption of either fat, smoked meat or vegetable are major risk factors for determining the individual susceptibility to BC risk and phenotype in Mizo population. However, *GSTP1* polymorphism was not significantly relevant in Mizo population. Nevertheless, BC is a multifactorial chronic inflammatory disease involving a complex interplay among the host, environmental factors and life style habits. Like many other complex disease, it is very difficult to investigate the influence of each factor involved in its pathogenesis, particularly the involvement of genetic factors. Future studies with larger sample size analysing gene–environment interactions in different geographic areas and ethnic groups are needed to conclusively assess the relevance of each factor in BC development.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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