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GENETIC POLYMORPHISM OF GLUTATHIONE S-TRANSFERASES GENES GSTM1, GSTT1 AND GSTP1 AND SUSCEPTIBILITY TO BREAST CANCER

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Abstract
The family of Glutathione S-transferases (GSTs) is comprised of phase II metabolic enzymes capable of catalyzing the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification. Decreased GST enzyme activity is linked to the risk of developing cancer due to reduced detoxification efficiency .In this context; we aimed to investigate the association of GST gene polymorphisms with breast cancer risk. The polymorphisms of GSTT1, GSTM1 and GSTP1 genes among breast cancer patients and healthy controls were studied by allele specific PCR and PCR-RFLP methods. Our results showed no significant association of GSTT1 null, GSTM1 null and GSTP1 heterozygous (AG) and GSTP1homozygous (GG) genotypes with the breast cancer risk. However the breast cancer risk was seen to be increased by 2.23 fold among the women who carried a combination of GSTT1 and GSTM1 null genotypes (p=0.05).Women suffering from hypertension and having increased BMI were also seen to be at a greater risk of developing breast cancer.

Key Words: Glutathione S- Transferases (GSTS).

Introduction

Breast cancer is the most common cancer in India and second most common cancer in the world. The incidence of breast cancer in India in 2012 was 27% of all the cancers. This incidence has increased from 7.2 to 33.4/100,000 within 10 years. The ratio of female to male was > 100:1 [27]. Thus, breast cancer remains the leading cause for cancer deaths in Indian women similar to the rest of the world. The risk factors for breast cancer include age, menopausal status, family history of the disease and various reproductive factors [18].

The family of Glutathione S-transferases (GSTs) is comprised of phase II metabolic enzymes capable of catalyzing the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification. Deficiency of these enzymes may increase sensitivity to certain environmentally derived carcinogens as well as endogenously generated reactive oxygen species (ROS) which may pose a risk of creating various malignancies. In humans, 4 major subfamilies of GSTs can be distinguished and are designated as GST α, GST θ, GSTμ and GST π. Each of these subfamilies is composed of several members, some of which display genetic polymorphism [17]. Reduced GST enzyme activity is linked to the risk of developing cancer due to reduced detoxification efficiency [25].

The GSTM1 gene belongs to μ class and is located on chromosome no. 1 p13.3 and consists of 8 exons. The GSTT1 gene belongs to ɵ class and is located on chromosome no.22p 11.2 and consists of 6 exons. The homozygous deletion of GSTT1 and GSTM1 genes are common and results in complete loss of enzyme activity. The GSTP1 gene belongs to π class and is located on chromosome no.11q13 and consists of seven exons [6, 13]. GSTP1 gene possess two variations in coding region , an A→ G transition at codon 105 and C→T transition at codon 114[3,33]. The variants of GSTP1 gene have shown over expression in wide variety of tumours and are also associated with differences in chemotherapeutic response and cancer susceptibility [29]. The genetic variants of GSTT1, GSTM1 and GSTP1 have been associated with an increased risk of developing various types of cancers [20, 32]. In the present study, an attempt has been made to determine the association, if any, of these variants with susceptibility to breast cancer.

Materials and Methods

Subjects

A total of 200 patients diagnosed with breast cancer were selected from Mohan Dai Oswal Cancer Hospital, Ludhiana, Punjab. The control group was comprised of 100 healthy individuals matched with patients in terms of age and sex. The demographic and clinical information of patients were collected from medical records of Mohan Dai Oswal Cancer Hospital, Ludhiana. All the patients were followed up from January, 2012 to Oct, 2014 .A brief questionnaire that included age, weight, menopausal status and marital status etc was also filled each of the normal healthy control women. Written consent was obtained from both the cases and controls. Our study was approved by ethical committee of the Punjabi University, Patiala, Punjab. Blood samples were collected in EDTA coated vials from each individual by a trained technician. Samples were stored at 4◦ C till use.

Genotyping

Genomic DNA was extracted from all the blood samples using salting out method of Miller et al. 1988 with some modifications. Polymorphisms of GSTT1 and GSTM1 were performed by multiplex PCR with albumin as control. Primers used are given in Table1. Multiplex allele specific PCR was performed and reaction was initiated by denaturation for 5 minutes at 95◦C .The PCR reaction was set at 35 cycles at 95◦ C for 1 minute, 57.8◦ C for 1 minute and 72◦ C for 1 minute with a final elongation of 7 minutes at 72◦ C. The presence of GSTT1 and GSTM1 allele (non-null genotype) or its complete deletion (null genotype) was evaluated by electrophoresis, using 2% agarose gel. The gel was stained with ethidium bromide and viewed under UV transillumintor.

The GSTP1 polymorphism was determined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method. After initial denaturation at 95◦C for 5 min, amplification was carried out for 30 cycles at 94◦Cfor 30 sec and 72◦ C for 30 sec followed by final extension at 72◦C for 5 min. The amplified product (176 bp) was then submitted to digestion with BsmA1 (NEB) in a total volume of 15µl and products were separated by electrophoresis in 3.5% agarose gel containing ethidium bromide. Digestion of PCR product enzyme yielded 176, 91 and 85 bp fragments for the GSTTP1 AG heterozygous genotype, 176 bp fragment for wild type GSTP1 AA genotype and 91 and 85 bp fragments for mutant GSTP1 GG genotype.

Statistical Analysis

The association between GSTT1, GSTM1 and GSTP1 polymorphisms and breast cancer risk was evaluated by using odds ratio (OR) at 95% confidence interval and Chi Square test and Fisher's exact test. Statistical analysis was carried out by SPSS software version 22.0 (SPSS Chicago. IL, USA). Level of significance for comparison was set as $p< 0.05$.

Results

Distribution of different variables including age ,BMI ,marital status ,menopausal status ,exercise ,dietary habits ,obstetric history ,history of hysterectomy, hormone replacement therapy ,history of infertility and other disease history studied in cases and controls of present study are given in Table 2. The mean age of patients was 49.40 ±8.742 year (age range 25 to 70 years) and that of control group was 47.66 ±6.836 years (age range 30 to 68 years). All the women included in the study were grouped into three age groups: <=35 years, 36-45 years and >45 years in order to find out the modal group of the patients. The highest frequency (59%) was observed in the cases in the age group of > 45 years. Out of the eleven variables studied, the frequency distribution of five variables viz BMI, marital status, bad obstetric history, hyperthyroidism and hypertension, was significantly different in cases vs controls. The frequency of breast cancer women having more than 25 BMI was significantly higher as compared to the frequency of control women having more than 25 BMI (51% vs 23%)(p=0.0001*). Regarding marital status, one woman (30 years of age) among cases and 09 women (average age of 26.44 years) among controls were unmarried and the difference was statistically significant (p=0.0002). Obstetric history of women showed that 49 (24.5%) women among cases and 14 (14%) women among controls had bad obstetric history The difference was statistically significant in the incidence of bad obstetric history amongst cases and controls was (p=0.03). Regarding other disease history, only cases had the history of hypertension, (14.5%) and hyperthyroidism (5%) and thus the differences in the incidence of these two diseases were statistically significant (p=0.0001, and 0.034 respectively).

Distribution of the genotypes of GSTT1, GSTM1 and GSTP1 were in Hardy Weinberg Equilibrium. The frequencies of GSTT1 null, GSTM1 null, GSTP1 heterozygous (AG) and homozygous mutant (GG) genotypes among cases vs controls were 36.5% vs 34%, 51% vs 41% ,47.5% vs 49% and 14.5%vs12% respectively and are given in Table 3 (p=0.670, 0.102 and0.866). The frequencies of GSTT1null, GSTM1 null and GSTP1 homozygous variant were higher among cases as compared to controls but the differences were not statistically significant. The number of individuals carrying a combination of GSTT1 and GSTM1 null genotypes was significantly higher among cases vs controls (p=0.05) and showed a 2.23 fold risk of developing breast cancer. The frequency of individuals carrying a combination of GSTT1 null, GSTM1 null and GSTP1 variant genotypes was found to be higher among cases as compared to controls but the difference was not statistically significant.

Distribution of different variables in relation to GSTT1 null, GSTM1 null and GSTP1 variants were also studied, in order to find out the confounding effect of these variables and are given in Table 4. The frequency of women having GSTT1 null, GSTM1 null and GSTP1 variant genotypes and having BMI > 25 was significantly higher among cases vs controls. The frequency of women having GSTM1 null genotype and having vegetarian diet was also significantly increased among cases vs controls. The frequency of women having GSTM1 null genotype, GSTP1 variants and also having hypertension was significantly higher among cases vs controls. Though the frequencies of other reproductive variables like menopausal status,

use of HRT, hysterectomy and history of infertility and other confounding factors i.e exercise etc were higher in cases vs controls but the differences were not statistically significant.

Table 1 PCR Primers for Amplification of GST Genes

Gene	SNP	Forward and reverse primers		PCR Product
GSTT1	Gene deletion	(F) 5'-TTCCTTACTGGTCCTCATCTC -3' (R) 5'-TCACCGGATCATGGCCAGCC -3'	Multiplex PCR	459 bp
GSTM1	Gene deletion	(F)5'-GAACTCCCTGAAAAGCTAAAGC -3' (R) 5'-GTTGGGCTCAAATATACGGTGG- 3'		219 bp
Albumin	Positive control	(F)-5'-GCCCTCTGCTAACAAGTCCTAC-3' (R)5'-GCCCTAAAAAGAAAATCGCCAATC-3'		350 bp
GSTP1	313 A>G (rs1695) Exon 5	(F)5'- ACC CCA GGG CTC TATGGGAA- 3' (R)5'-TGAGGGCACAAGAAGCCCC - 3'	PCR-RFLP using *BsmA1* restriction enzyme	176 bp

Table 2 Frequencies of Different Variables among Cases and Controls

Variables	Cases (N=200) n (%)	Controls (n=100) n (%)	p-value
Age (in years)			
<=35 35-45 >45	10 (5) 72 (36) 118 (59)	02 (2) 45 (45) 53 (53)	0.192
BMI			
<25	89 (44.5)	77 (77)	0.0001*
>25	111 (55.5)	23 (23)	
Marital status			
Married women Unmarried women	199 (99.5) 01 (0.5)	91 (91) 09 (9)	0.0002*
Menopausal status			
Premenopausal Post menopausal	85 (42.5) 115 (57.5)	53 (53) 47 (47)	0.085
Exercise			
Yes No	44(22) 156 (78)	24 (24) 76 (76)	0.806
Dietary habits			
Vegetarian Non vegetarian	182 (91) 18 (9)	87 (87) 13 (13)	0.283
Bad obstetric history of women			
Yes No	49 (24.5) 151 (75.5)	14 (14) 86 (86)	0.03*
History of hysterectomy			
Yes No	19 (9.5) 181 (90.5)	16 (16) 84 (84)	0.298
Hormone replacement therapy			
Yes No	18 (9) 182(91)	13 (13) 87 (87)	0.383
History of other diseases			
Diabetes	23 (11.5)	10 (10)	0.695
Hypertension	29 (14.5)	0	0.0001*
Hyperthyroidism	10 (5)	0	0.034*
History of infertility			
Fertile women** Infertile women	193 (96.5) 07 (3.5)	91 (100) 00	0.163

(*p<0.05) (** Average married life span and average age of infertile women with breast cancer was 21.5 and 44.42 years respectively)

Table 3 Frequency Distribution of GSTT1, GSTM1 and GSTP1 Gene Polymorphisms among Cases and Controls

Genotype/Allele		Genotype frequencies		Allele frequencies			p value
		Cases (N=200) n(%)	Controls (N=100)n(%)	P value	Cases	Controls	
GSTM1	Null	102 (51)	41 (41)	0.1029	NA**	NA	
	Non Null	98 (49)	59 (59)				
GSTT1	Null	73 (36.5)	34 (34)	0.6701	NA	NA	
	Non Null	127 (63.5)	66 (66)				
GSTP1	AA	76 (38)	39 (39)	0.8666 (AA vs AG& GG	A- 247 (61.7) G-153 (38.3)	A- 127 (63.5) G-73 (36.5)	0.67 (0.17)*
	AG	95 (47.5)	49 (49)	0.9845 (AA vs AG)			
	GG	29 (14.5)	12 (12)	0.5866(AA vs GG)			
GSTT1& GSTM1	Null	36 (18)	9 (9)	0.0521*	NA	NA	
	Non Null	61 (30.5)	34 (34)				
GSTT1 GSTM1 GSTP1	Null Null AG or GG	23 (11.5)	6 (6)	0.2236	NA	NA	
GSTT1 GSTM1 GSTP1	Non Null Non Null AA	27 (13.5)	14 (14)				

*Chi square value, **NA- not applicable

Table 4 Frequencies of Different Variables among Cases and Controls having GSTT1 Null, GSTM1 Null and GSTP1 Variants

Variables	GSTT1 null			GSTM1 null			GSTP1AG+GG		
	Cases n=73	Controls n=34	p value	Cases n=102	Controls n=41	p value	Cases n=124	Controls n=61	p value
Age (in years)									
<=35	04(5.4%)	01 (2.9%)	0.835	05(4.9%)	01(2.4%)	0.634	04(3.2%)	01(1.6%)	0.458
35-45	26(35.6%)	13 (38.2%)		40(39%)	14(34.1%)		44(35.4%	27(44.2%)	
>45	43(58.9%)	20 (58.8%)		57(55.8%)	26(63.4%)		76(61.2%)	33(54%)	
BMI									
<25	31(42.4%)	28(82.3%)	0.0003*	44(43.1%)	31(75.6%)	0.0009*	57(45.9%)	46(75.4%)	0.0003*
>25	42(57.5%)	06(17.6%)		58 (56.8%)	10(24.3%)		67(54%)	15(24.5%)	
Menopausal status									
Premenopausal	35(47.9%)	16(47%)	0.92	39(38.2%)	21(51.2%)	0.216	49(39.5%)	32(52.4%)	0.131
Postmenopausal	38(52%)	18(52.9%)		63(61.7%)	20(48.7%)		75(60.4%)	29(47.5%)	
Dietary habits									
Vegetarian	64(87.6%)	29(85.2%)	1	92(90.1%)	31(75.6%)	0.044*	110(88.7%)	53(86.8%)	0.92
Non vegetarian	09(12.3%)	05(14.7%)		10(9.8%)	10(24.3%)		14(11.2%)	08(13.1%)	
Exercise									
Yes	15(20.5%)	10(29.4%)	0.446	23(22.5%)	12(29.2%)	0.527	27(21.7%)	15(24.5%)	0.806
No	58(79.4%)	24(70.5%)		79(77.7%)	29(70.7%)		97(78.2%)	46(75.4%)	
History of hysterectomy									
Yes	06(8.2%)	03(8.8%)	0.791	09(8.82%)	07(17%)	0.261	12(9.6%)	10(16.3%)	0.277
No	67(91.7%)	31(91.1%)		93(91.1%)	34(82.9%)		112(90.3%	51(83.6%)	

Variables	GSTT1 null			GSTM1 null			GSTP1AG+GG		
	Cases	Controls	p value	Cases	Controls	p value	Cases	Controls	p value
	n=73	n=34		n=102	n=41		n=124	n=61	
Hormone replacement therapy									
Yes	06 (8.2%)	02(5.8%)	1	09(8.82%)	07(17%)	0.261	11(8.87%)	10(16.3%)	0.129
No	67 (91.7%)	32(94.1%)		93(91.1%)	34(82.9%)		113(91.1%)	51(83.6%)	
Bad obstetric history									
Yes	05 (6.8%)	01(2.9%)	0.718	06(5.8%)	02(4.8%)	0.862	08(6.4%)	04(6.5%)	0.777
No	68(93.1%)	33(97%)		96(94.1%)	39(95.1%)		116(93.5%)	57(93.4%)	
History of infertility									
Fertile women	70(95.8%)	34(100%)	0.841	99(97%)	41(100%)	0.639	122(98.3%)	61(100%)	0.555
Infertile women	03 (4.1%))	0		03(2.9%)	0		02(1.61%)	0	
History of other diseases									
Diabetes									
Yes	06 (8.2%)	05(14.7%)	0.493	12(11.7%)	05(12.1%)	0.823	18(14.5%)	06(9.8%)	0.512
No	67 (91.7%)	29(85.2%)		90(88.2%)	36(87.8%)		106(85.4%)	55(90.1%)	
Hypertension									
Yes	08 (10.9%)	0	0.106	15(14.7%)	0	0.02*	23(18.5%)	0	0.0008*
No	65 (89%)	34 (34%)		87(85.2%)	41 (100%)		101(81.4%)	61 (100%)	
Hyperthyroidism									
Yes	04 (5.4%)	0	0.399	03(2.9%)	0	0.639	04(3.2%)	0	0.377
No	69(94.5%)	34 (100%)		99(97%)	41(100%)		120(96.7%)	61 (100%)	

Discussion

The presence of polymorphisms in phase II detoxifying genes of GST family has been associated with an increased risk of developing the cancer of bladder, head, larynx, breast, skin, colon, stomach, lungs and testicles.[9,10,16,35]. Several environmental risk factors have also been associated with increased susceptibility to breast cancer. These include several aspects of reproductive history characterized by elevated and prolonged estrogens levels, lack of or reduced breast feeding, older age at first time full pregnancy, early menarche and late menopause (25). In the present study, besides genetic polymorphism eleven variables were also studied and out of these eleven variables, five variables viz. BMI, marital status, bad obstetric history of women, hypertension and hyperthyroidism showed significant risk for breast cancer. Higher value of BMI was found to be associated with the breast cancer risk in our study. Similar findings have also been reported by Zheng et.al (2002) on Caucasian women, by Li et.al (2005) on Hispanic women, Native American women and Anglo women, Gilani et.al (2006) on Pakistani women, Singh et.al (2013) on North Indian women and Ahmed et.al (2015) on Bangladeshi women.

Regarding the marital status, several studies have reported a strong association of breast cancer risk with ″unmarried status″ of women (Osborne et.al, 2005, Aggarwal et.al 2009, Pakseresht et.al 2009) but somehow in the present study rather a ″married status″ of women was seen as a risk factor. This could be due to the fact that the average age of the nine unmarried women of control group was 26.44 years which was much lower than the age of a single unmarried women found in the patient group (30 years) and also much lower than the mean age of cases (49.4) and controls (47.6) and there was plenty of scope of these women to get married in a span of 1 to 2 years.

We also found a significant higher breast cancer risk among women with the bad obstetric history (Women with two or more than 2 abortions were considered as women with bad obstetric history). A similar positive association of bad obstetric history

with breast cancer risk was also observed in the studies of Yanhuna et.al (2012) Kamath, et.al (2011) and Ahmed et.al (2015). We have found a positive association of hypertension with the breast cancer risk and our study supported the study of Pereira et. al (2012) who showed four fold increased risk of breast cancer among the patients with a history of hypertension . A positive association of hyperthyroidism among cases vs controls (p=0.034) was also found in our study but no such association has been reported in any of the previously reported studies.

The frequencies of GSTT1 null, GSTM1 null, GSTP1 Ile105Val andVal105Val variant genotypes among cases vs controls were 36.5% vs 34%, 51% vs 41%, 47.5% vs 49 %and 14.5%vs12% respectively. The frequencies of GSTT1null, GSTM1 null and GSTP1 variants were higher among cases as compared to controls but the differences were not statistically significant (p=0.671 ,0.102 and0.956) respectively. Similarly no significant association of GSTT1null ,GSTM1 null and GSTP1 variants with breast cancer risk was found by Ambrosone et al (1996), Vogl et al (2004), ,Hashemi et al (2012) ,Duggan et al.(2013) and Rodriguez et al(2014). Saadat at al(2003) studied the association of polymorphism of only two genes i.e. GSTT1 and GSTM1 and Khabaz et al (2014) studied the association of polymorphism of only one gene i.e GSTP1 with the breast cancer risk and authors of both the studies did not find any association of GST variants with breast cancer risk. However the study of Helzlsouer et al (1998) and Saxena et.al (2009) reported a positive association of GSTM1 and GSTP1 genotypes with the breast cancer risk.

In a meta analysis conducted by Theodoros et.al (2009), GSTT1 null genotype was found to be associated with elevated breast cancer risk in non Chinese population. Regarding GSTP1 Ile105Val, no statistically significant association of breast cancer risk was found in non Chinese population. However, authors did find an association between Val105Val genotype and an increased breast cancer risk in Chinese population. The overall findings of meta analysis revealed the involvement of GSTT1 and GSTP1 gene polymorphism in increasing the breast cancer risk in a race specific manner. An another meta analysis on GSTP1 Ile105Val polymorphism and breast cancer risk was conducted by Zhanwai et at (2011). Authors selected 30 published case –control studies (most of which were also included in the previous meta analysis reported by Theodoros et.al, 2009) and found no significant association between GSTP1 Ile105Val and breast cancer risk in overall population. However they did find a significant association between GSTP1Ile105Val and breast cancer risk in Asian women. The authors also observed a lot of inconsistency in the findings between hospital based studies and population based studies which the authors believed was due to the biases brought by hospital based studies. Authors were of the opinion that the controls in hospital based studies may be less representative of general population than the controls from population –based studies.

The number of individuals carrying a combination of GSTT1 and GSTM1 null genotypes was significantly higher among cases vs controls (p=0.05) and showed a 2.23 fold risk of developing breast cancer. Similar findings were also reported by Saadat et al (2003), Steck et al (2007), and Anton et al (2010). In the present study ,the frequency of individuals carrying a combination of all the three GST variants i.e. GSTT1 null, GSTM1 null and GSTP1 variants was found to be higher among cases as compared to controls but the difference was not statistically significant. However Saxena et al (2009) reported a positive association of a combination of GSTT1 null, GSTM1 null and GSTP1 variants with breast cancer risk in the Indian population.

When the different variables of both the groups were compared in relation to GSTT1 null, GSTM1 null and GSTP1 variants, significant differences were found in BMI, dietary habits and hypertension. A significant increased risk of breast cancer was observed among women having increased BMI and a GSTT1 null or GSTM1 null or GSTP1 variants (0.0003, 0.0009and 0.0003 respectively). Similar findings have also been observed by Zheng et. al (2002). Regarding dietary habits, a significant increased risk of breast cancer was observed among vegetarian women having GSTM1 null genotype (p= 0.044). Contrary to our findings, Zheng et al (2002) found a significant increased risk of breast cancer among non vegetarian women with GSTT1 or GSTM1 null genotypes. We also found a significant increased risk of breast cancer among hypertensive women having GSTM1 null genotype or GSTP1 variants. (p=0.02 and 0.0008 respectively). However, no such association has been reported in the previous studies.

Conclusion

No significant association of GSTT1 null, GSTM1 null and GSTP1 (AG) and GSTP1 (GG) genotypes were found to be significantly associated with the breast cancer risk. However the presence of more than one GST variant was found to be significantly associated with elevated breast cancer risk. Women suffering from hypertension and having increased BMI were also seen to be at a greater risk of developing breast cancer.

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