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Full Length Research Paper

Analysis of glutathione S-transferase (M1, T1 and P1) gene polymorphisms in Iranian prostate cancer subjects

Sahar B. Ansari[1], R. Vasudevan[1]*, Ali Bakhshi[1], M. Mirinargesi[1,2], I. Patimah[1], A. R. Sabariah[3] and P. Pasalar[4]

[1]Genetic Research Group, Molecular and Bioinformatics Unit, Department of Biomedical Science, Faculty of Medicine and Health Sciences, University Putra Malaysia, 43400 UPM Serdang, Selangor DE, Malaysia.
[2]Department of Genetics, Faculty of Medicine, Tonekabon Islamic Azad University, Tonekabon, Mazandaran, Iran.
[3]Department of Pathology, Faculty of Medicine and Health Sciences, University Putra Malaysia, 43400 UPM Serdang, Selangor DE, Malaysia.
[4]Department of Biochemistry, School of Medicine, Tehran University of Medical Science, Tehran, Iran.

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Glutathione S-transferase enzymes are active in detoxifying a wide number of endogenous and exogenous chemical carcinogens and subsequently, are crucial in protecting the DNA. Several studies show some differences in association of glutathione S-transferase M1, T1 and P1 genetic polymorphisms with the risk of prostate cancer in various populations. The current study was done with Iranian subjects to evaluate the association of the polymorphism of glutathione S-transferase subtypes (T, M and P) and the susceptibility of prostate cancer in Iranian patients as compared to controls. Blood samples were collected from 65 prostate cancer patients and 65 unrelated health individuals as controls from Milad hospital, Tehran, Iran. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to determine the polymorphism of glutathione S-transferase pi (GSTP1) -313 A/G gene, while multiplex PCR method was utilized to detect the glutathione S-transferase teta (GSTT) 1 and glutathione S-transferase mμ (GSTM) 1 null allele. There was no significant association in the -313 G allele (Val) of GSTP1 gene polymorphism and prostate cancer risk (odds ratio 0.61, 95% confidence intervals (CI) 0.08 - 4.60, p = 0.627). Moreover, no relationship was found between the polymorphism of GSTT1 (odds ratio 0.66, 95% CI 0.27 - 1.62) and GSTM1 (odds ratio 0.54, 95% CI 0.27 - 1.08) genes and higher risk of prostate cancer among Iranian subjects (p > 0.05). This study showed that either GSTP1-313 G polymorphism or GSTT1 and GSTM1 genes cannot be predisposing risk factors for prostate cancer among Iranian subjects.

Key words: Glutathione S-transferase, prostate cancer, polymorphism.

INTRODUCTION

The phase II metabolizing enzymes such as glutathione S - transferase (GST), N-acetyltransferase, epoxide hydroxylase and sulphotransferase are involved in detoxifying chemical carcinogens in the cells and subsequently protect DNA from damage (D'Errico et al., 1996). The GST genes encode enzymes which catalyze the conjugation of electrophonic compound to glutathione (Hayes and Pulford, 1995). The vital role of these enzymes is to protect DNA from oxidative damage since activating and inactivating oxidative metabolite of carcinogenic com-pound is associated with causing prostate cancer (Ryberg et al., 1997). Among the subtypes of glutathione S-transferase, the null allele of glutathione S-transferase teta (GSTT) 1, glutathione S-transferase mμ (GSTM) 1

*Corresponding author. E-mail: vasuphd@gmail.com.

Abbreviations: GSTT, Glutathione S-transferase teta; **GSTM,** glutathione S-transferase mμ; **GSTP,** glutathione S-transferase pi; **PCR,** polymerase chain reaction; **RFLP:** restriction fragment length polymerase; **OR:** odds ratio; **CI,** confidence intervals; **PSA,** prostate-specific antigen; **EDTA,** ethylenediamine-tetraacetic acid.

Table 1. Primer sequences used in PCR for GSTT1, GSTM1 and GSTP1 genes.

Gene	Primer	Sequences
GSTT1	Forward	5´-TTCCTTACTGGTCCTCACATCTC-3
	Reverse	5´-TCACCGGATCATGGCAGCA-3´´
GSTM1	Forward	5´-GAACACCCTGAAAAGCTAAAGC-3´
	Reverse	5´-CTTGGGCTCAAATATACGGTGG-3´
GSTP1	Forward	5´-GAACTGCCACTTCAGCTGTCT-3´
	Reverse	5´-CAGCTGCATTTGGAAGTGCTC-3´
CYP1A1	Forward	5´-ACCCAGGGCTCTATGGGAA -3´
	Reverse	5´-TGAGGGCACAGAAGCCCCT -3´

and the glutathione S-transferase pi (GSTP) 1-313 A/G substitution have been most investigated in many populations with conflicting results (Ali-Osman et al., 1997). The GSTP1-313 A/G single nucleotide polymer-phism leads to an amino-acid variation of isoleu-cine/valine at codon 105 in the protein. The substitution of valine amino acid decreases the enzyme activity. Also, the null allele of GSTT1 and GSTM1 genes is caused by lack of enzyme activity (Srivastava et al., 2004).

The increased evidences show that polymorphism of glutathione S-transferase genes can be associated with the risk of developing some types of cancer as these polymorphisms have been investigated in association with lung, bladder, colon (Ketterer et al., 1992), oral (Nair et al., 1992; Buch et al., 2002) and prostate cancers (Pemble et al., 1994; Steinhoff et al., 2000; Nakazato et al., 2003; Dekant and Vamvakas, 1993). Regarding prostate cancer, the conflicting results among different studies have shown a significant association in some populations (Steinhoff et al., 2000; Nakazato et al., 2003; Dekant and Vamvakas, 1993), whereas there was no association in others (Pemble et al., 1994; Medeiros et al., 2004; Kote-Jarai et al., 2001).

Nowadays, prostate cancer has become the second leading cause of cancer related mortality diagnosed in men worldwide (Mittal et al., 2004). The latest global estimation shows that the incidence of prostate cancer in Asian countries is 2.3–9.8 cases in 100,000 persons/year (Hsing et al., 2000). The incidence of prostate cancer in Iran is 5.1 in 100,000 persons/year (Safarinejad, 2006). The most significant factor of developing prostate cancer is age. The older someone is, the greater the risk (Ewis et al., 2002). It is quite rare that men less than 40 years old are at risk of developing this disease. As the affect of both genetic and environmental factors cannot be ignored in higher risk of prostate cancer, these factors seem to influence the metabolism of carcinogens (Rebbeck et al., 1999). Evidences show that genetic alterations are stronger than any other factor and play a major role in prostate cancer etiology (Mayer et al., 1999); however, some environmental factors such as diet and life style might also be a key to switch the disease. Previous reviews suggest that different racial and ethnic groups show different frequencies of gene polymorphism (Ryberg et al., 1997; Gsur et al., 2001; Srivastava et al., 2004). In this study, we attempted to find out whether the glutathione S-transferase genes polymorphism is associated with the higher risk of prostate cancer in Iranian patients. The A313G polymorphism of GSTP1 gene among cases and controls was resolved using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, while the absence/presence of the GSTT1 and GSTM1 gene frequencies for prostate cancer patients and controls were determined by multiplex-PCR.

MATERIALS AND METHODS

The study group included a total of 130 subjects comprising 65 men with prostate cancer and 65 healthy men. Samples were recruited from Milad hospital, Tehran, Iran. Informed consent was obtained from all participants. All patients who participated in the study were in advance level of cancer that had been referred to the hospital for treatment in 2008. The inclusion criteria for patients were between 40 - 50 years old (43 ± 10.24) suffering from prostate cancer based on diagnosis by physician. For the controls, over 40 years old men who were healthy with a prostate-specific antigen (PSA) level below <2 ng/ml were recruited. The present study was approved by the Ethical Committee of the Faculty of Medicine, Tonekabon Islamic Azad University, Tonekabon, Mazandaran, Iran.

Five millimeter of blood was collected in sterile ethylene-diaminetetraacetic acid (EDTA) vials from both cases and controls in Iran. DNA was extracted using genomic DNA extraction kit (Bioneer, South Korea). The purity and concentration of the extracted DNAs were quantified by spectrophotometry, using biophotometer UVette (Eppendorf, Hamburg, Germany). Study on the A313G polymorphism of GSTP1 was done using PCR-RFLP method. The forward and reverse primers sequences used for GSTP1 gene were those described by Srivastava et al. (2004). The PCR was performed in a volume of 25 µl including 60 - 100 ng of genomic DNA, 20 pmol of each of primers (Table 1), 5 µl of i-PCR 5X Master Mix (iDNA, Singapore) (which contain 0.1 unit/µl of *Taq* DNA polymerase, 1 mM of dNTP's, 10% of glycerol and 7.5 mM of $MgCl_2$) and 14.5 µl distilled water in each single reaction tube. For negative control, all materials except template DNA were added to a PCR tube. The PCR cycling conditions were carried out on iCycler (BioRad Laboratories, Hercules, California, USA). The PCR cycling conditions were set up as follows: 94°C for 3 min as initial denaturation, 94°C for 1 min, 59°C for 1 min and 72°C for 2 min for 30 cycles, and 72°C for 10 min as final extension. The sizes of the multiplex PCR products were clearly resolved in 4% agarose gel (Bioline, London, UK). 3 - 5 µl of 100 bp DNA marker (iDNA, Singapore) was run in the gel each time as a reference to estimate the PCR products' sizes. After the gel staining by ethidium bromide for 20 min, the gel was viewed under the UV light using Alpha Imager (Alpha Innotech, San Leandro, CA). The amplified PCR product for GSTP1 gene was digested by Alw26I restriction enzyme and electrophoresed in 4% agarose gel. The presence of restriction site resulted in two fragments (91 and 85 bp) which determined the G allele, and for the heterozygous situation (A/G polymorphism) there were three fragments which were 176, 91 and 85 bp.

Multiplex PCR technique was used to detect the presence or absence of GSTT1 and GSTM1 genes in the genomic DNA samples. The PCR forward and reverse primers sequences for both genes were obtained from Srivastava et al. (2004). Exon 7 of the CYP1A1 gene was co-amplified as positive internal control. The multiplex PCR was carried out in a volume of 25 µl including 60 - 100 ng of genomic DNA, 20 pmol of each of primers (Table 1), 5 µl of i-PCR 5X Master Mix (iDNA, Singapore) which contain 0.1 unit/µl

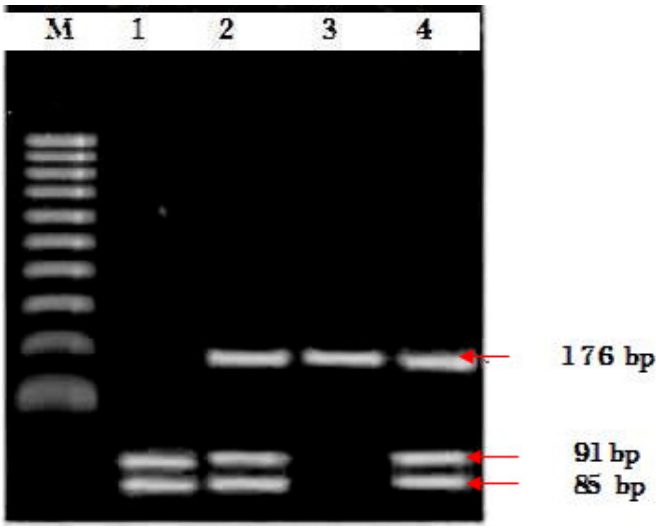

Figure 1. A313G polymorphism of GSTP1 gene in 4 case subjects. PCR-RFLP product was analyzed at 4% agarose gel electrophoresis. Lane N: Negative control; lane 1: shows that enzyme has two restriction site on the gene (91, 85 bp); lanes 2 and 4: show that the enzyme has only one restriction site (176, 91 and 85 bp); lane 3: shows that enzyme has no restriction site (176 bp).

of *Taq* DNA Polymerase, 1 mM of dNTP's, 10% of glycerol and 7.5 mM of $MgCl_2$. Negative control tubes included all materials except template DNA. The amplifications were executed on iCycler (Bio-Rad Laboratories, Hercules, California, USA) PCR instrument. The PCR cycling conditions for multiplex PCR were set up as follows: 94°C for 3 min as initial denaturation, 94°C for 1 min, 57°C for 1 min and 72°C for 2 min for 30 cycles, and 72°C for 10 min as final extension. The sizes of the multiplex PCR products were clearly resolved at 2% agarose gel (Bioline, London, UK). 3 - 5 µl of DNA marker (iDNA, Singapore) was added at each time in the gel as a reference for the amplified PCR products. The positive samples for GSTM1 and GSTT1 genotypes yielded bands of 215 and 480 bp, respectively. The internal positive control (CYP1A1) PCR product corresponded to 312 bp.

Statistical analysis

Allelic frequencies were calculated by gene-counting method and the genotype distribution with Hardy-Weinberg expectations by a chi-squared test. Odds ratios (OR) with 95% confidence intervals (CI) were estimated for the effects of high risk alleles. A level of $p < 0.05$ was considered statistically significant. All the statistical analysis was carried out by using the statistical package for the social sciences (SPSS) (Chicago, IL) software version 14.0 for Microsoft windows.

RESULTS

The present study showed the relation in A313G polymorphism of GSTP1 gene and the presence/absence of GSTT1 and GSTM1 allele in Iranian prostate cancer subjects. A total of one hundred and thirty Iranian subjects were collected to analyze the association of the homozygous and heterozygous genotype of GSTP1 gene and the null (homozygous deletion of the gene) versus the non-deleted allele (heterozygous or homozygous presence of the gene) of GSTT1 and GSTM1 genes between the cases and controls. In A313G polymorphism of GSTP1 gene, the presence of the restriction site results in only two fragments (91 and 85 bp) which indicate the G allele and three fragments of 176, 91 and 85 bp that confirm the A/G polymorphisms. Therefore, absence of restriction site in only one band (176 bp) illustrate the A allele of GSTP1 gene. Whereas, the DNA band for positive genotypes of GSTM1, GSTT1 and internal positive control corresponded to 215, 480 and 312 bp, respectively. Figure 1 show that the genotypes of Ile/Val polymerphism were allotted on the basis of the pattern of bands on the gel electrophoresis using standard methods. Figure 2 demonstrate the detection of GSTT1 and GSTM1 null allele homozygotes by sequence-specific amplification using CYP1A1 gene as an internal control. Table 2 explains the frequencies of GSTM1, GSTT1 and GSTP1 alleles and genotypes by case-control status and the association of GST genotypes with prostate cancer risk among Iranian subjects. In the control samples, the GSTP1 Ile allele was present in 58.5% in the homozygous state (Ile/Ile) while the Val allele was homozygous (Val/Val) in 3.1%; the remaining 38.5% were heterozygous (Ile/Val) and the frequency of GSTM1 null and GSTT1 null was 58.5 and 84.6%, respectively. Genotype distributions in controls were in agreement with the Hardy-Weinberg equilibrium. In addition, among cases, the homozygous state (Ile/Ile) present 35.5% where the homozygous state for (Val/Val) was 3.1%; the other 61.5% were heterozygous (Ile/Val).

DISCUSSION

To our knowledge, the current study is the first investigation done on Iranian subjects regarding the determination of genotypic distribution of GST genes in relation to prostate cancer. Several studies with inconsistent outcomes have been conducted in different populations to discover any association between GSTP1, GSTT1 and GSTM1 gene polymorphisms with the risk of prostate cancer. The observations of the present study concur with the results obtained for some populations, whereas, it also oppose some others. Our findings are consistent with that from Austrian and German populations (Gsur et al., 2001; Wiencke et al., 1995) which showed no association for GSTP1-313 A/G or G/G polymorphism in both Austria (OR = 0.65; 95% CI 0.38 – 1.09) and Germany (OR = 1.08; 95% CI 0.66 – 1.77). Results of our study also concur with that reported by Gsur et al. (2001) which found no association between null alleles of GSTT1 and GSTM1 with the risk of prostate cancer in Autriche. Another study carried out by Rebeck et al. (1997) in the USA suggested that the GSTM1 genotype distribution did not significantly differ between prostate cancer patients and

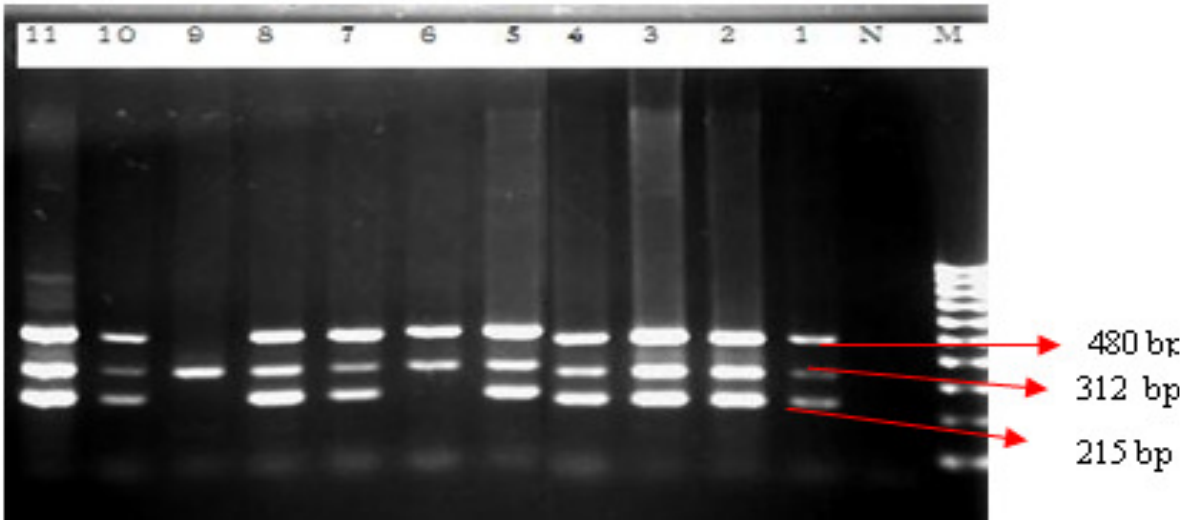

Figure 2. 11 case subjects which present polymorphism of GSTT1 and GSTM1 gene. Lane M: 100 bp stands for DNA marker; lane N: negative control; lane 1, 2, 3, 4, 5, 7, 8, 10 and 11: show GSTM1 (215 bp) and GSTT1 (480 bp) for both positive genotype; lane 6: indicates GSTT1 positive genotype (480 bp) with null allele for GSTM1 (215 bp); lane 9: demonstrates the null allele for both GSTT1 and GSTM1 genes and a band at 312 bp corresponding to internal control (CYP1A1 gene fragment). Multiplex PCR product was analyzed at 2% agarose.

Table 2. The distribution as n (%) of GST genotypes among prostate cancer subjects and controls.

Genotype	Control N (%)	Case N (%)	P value	Odds Ratio (95.0% C.I)
GSTT1	10 (15.40)	14 (21.50)	1.0	
Null	55 (84.60)	51 (78.50)	0.368	0.66 (0.27-1.62)
GSTM1	27 (41.50)	37 (56.90)	1.0	
Null	38 (58.50)	28 (43.10)	0.081	0.54 (0.27-1.08)
GSTP1 I/I	25 (38.50)	40 (61.50)	0.009*	0.39 (0.19-0.79)
I/V	38 (58.50)	23 (35.50)	0.649	1.60 (0.21-12.09)
V/V	2 (3.10)	2 (3.10)	0.627	0.61 (0.08- 4.60)

* $p < 0.05$.

controls. However, GSTT1-1 genotypes were significantly more common among cases than controls (adjusted OR = 1.83; 95% CI 1.19 – 2.80). On the contrary, in India, there was a greater risk of prostate cancer with GSTP1-313 A/G allele. Also, an association between GSTT1 and GSTM1 with higher risk of prostate cancer has been reported in this population (Srivastava et al., 2004). Moreover, an association between GSTP1-313 A/G or G/G polymorphism and the null genotype of GSTM1 with a greater risk of prostate cancer has been shown in Japanese population (Nakazato et al., 2003). Therefore, it is clear that the association of prostate cancer and G allele of GSTP1 and null genotype of GSTM1 and GSTT1 greatly vary in different populations. Our results did not provide the information that the A/G genotype of GSTP1, null genotype of GSTT1 and GSTM1 increase the risk of prostate cancer in Iranian subjects, however, they showed an association between a combination of these genes and higher risk of prostate cancer (Table 3). The GSTs genes are involved in detoxification of carcinogenic electrophiles and toxic compounds such as industrial chemicals, tobacco products and drugs by conjugating with glutathione (Vander-Gulden et al., 1992). As in the null genotype of GSTT1 and GSTM1, there are inactive forms of the GSTs enzymes, thus deto-xification of activated carcinogen is reduced, and it might be followed by progression to develop any type of cancer (Guengerich et al., 1995). The GSTP1-313 A/G polymorphism at the nucleotide level leads to an amino-acid variation of isoleucine/valine at codon 105 in the protein. Valine amino-acid substitution results in decreased enzyme activity (Srivastava et al., 2004). Many studies have shown that GSTs are important for maintaining cellular genome (DNA) from damage, and their absence may result in cancer susceptibility (Ketterer et al., 1992; Wiencke et al., 1995). Prostate cancer is a multi-factorial

Table 3. The distribution of GST genotypes in the patients with prostate cancer and controls.

Genotype (Triple)	Controls N (%)	Cases N (%)	*P value*	OR (95%CI)
M1 &T1(+/+)&P1(I/I)	13 (20.0)	2 (18.5)	0.824	0.91 (0.38-2.17)
M1(-/-),T1(-/-)&P1(I/VorV/V)	2 (3.1)	4 (6.2)	0.567	2.03 (0.18-22.98)
M1(-/-), T1(-/-)&P1(I/I)	2 (3.1)	3 (4.6)	0.650	1.52 (0.25-9.44)
M1(+/+), T1(-/-)&P1(I/I)	2 (3.1)	4 (6.2)	0.412	2.07 (0.36-11.69)
M1(+/+),T1(-/-),P1(I/VorV/V)	4 (6.2)	3 (4.6)	0.699	0.74 (0.16-3.44)
M1(-/-), T1(+/+)&P1(I/I)	8 (12.3)	1 (32.3)	0.008*	3.40 (1.38-8.40)
M1(-/),T1(+/+),P1(I/VorV/V)	15 (23.1)	9 (13.8)	0.254	0.59 (0.23-1.47)
M1&T1(+/+)&P1(I/Vor V/V)	19 (29.2)	9 (13.8)	0.036	0.39 (0.16-0.94)

*p < 0.05, M (+/-) Genotypes of GSTM1 gene, T (+/-) genotypes of GSTT1 gene, P1 (I-Isolecuine, V –Valine) genotypes of GSTTi gene.

disease influenced by complex genetic as well as environmental factors as noted by Kolonel et al. (2004). The differences in results obtained by studies on different populations can be attributed to their different ancestries which can influence their whole genome, as well as environmental conditions and life styles that can affect their level of gene expressions. Some studies have shown that improved nutrition, stress management techniques, exercise and psycho-social support actually changed the expression of over 500 genes in men with early-stage prostate cancer (Ornish et al., 2008).

It is worth noting that there are however, some limitations to this study. The sample size is relatively small as compare to other genetic epidemiological studies. Also, this sample size cannot represent the whole Iranian population as there are many descents dispersed over the country, and it is suggested to investigate on more samples collected from different provinces of the country in a more extensive study. Moreover, the genotypic frequencies of GSTP1, GSTT1 and GSTM1 gene polymerphisms were not demonstrated in level of expression in this study and we did not enter any function and mechanism of these polymorphisms on the clinical behavior of prostate cancer. Besides, we considered no environmental factors interfering in comparing between cases and controls.

Conclusion

Based on this study, there is no association between GSTP1, GSTT1 and GSTM1 gene polymorphisms with the higher risk of prostate cancer among Iranian subjects.

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