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RESEARCH

# Association of GSTM1, GSTT1, GSTP1 and CYP2E1 Single Nucleotide Polymorphisms with Colorectal Cancer in Iran

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**Abstract** Colorectal cancer is a major cause of morbidity and mortality both globally and in Iran. The aim of this study was to determine the association between genetic polymorphisms of glutathione S-transferases P1, M1 and T1 (GSTP1, M1, T1) and susceptibility to colorectal cancer (CRC). Genotyping of GSTP1, GSTM1 and GSTT1 was performed by the use of pyrosequencing. One hundred cases and healthy controls were enrolled into this study. Mean GSTT1 polymorphism type was significantly ($P<0.01$) higher in cases as compared to controls ($P<0.0001$: OR, 2.43: 95% CI, 1.47-4). On the other hand there is no significant association between GSTM1, GSTP1 and colorectal cancer. GSTs measurement may be useful as a colorectal marker in colorectal cancer and biopsies obtained at colonoscopy can be used to measure tumor markers.

**Keywords** Glutathione S-transferases · Colorectal cancer · Cytochrome P4502E1 · Iran

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## Introduction

Most chemical carcinogens require metabolic activation for DNA-damaging capabilities, a step widely believed to be essential in carcinogenesis [1–3] .On the other hand carcinogens may also be detoxified before damaging DNA by in vivo metabolic detoxifying systems [4, 5] .In recent years, a relatively new field of cancer research has focused on the interaction between genes and environment to understand the etiology of cancer [6]. Primary candidates for gene-environment interaction studies are those which encode enzymes related to the metabolism of established cancer risk factors. It has been known that most carcinogens require metabolic activation in the human body for their carcinogenic effects. Two major enzyme systems can metabolize potential carcinogens in the body, classified as phase I (activation) and phase II (conjugation) enzymes [7–9].

The major enzymes involved in the metabolic activation of chemical carcinogens are CYPs2 [10], a multigene superfamily of enzymes (phase I). Of the 25 or more CYP enzymes known to be expressed in the liver and also at significant levels in human colon or other extrahepatic tissues, CYP2E1 is believed to be involved in the activation of most carcinogenic nitrosamines [11, 12]. Furthermore, this

enzyme has the ability to metabolically activate many low molecular weight carcinogens [11] and to produce reactive free radicals from ethanol [13], which also might be of importance in carcinogenesis [14].

Glutathione S-transferases (GSTs), a superfamily of dimeric phase II metabolic enzymes, play an important role in the cellular defense system [15]. GSTs are a large and diverse family of enzymes, and in humans, there are at least 13 GST enzymes belonging to five families, namely $\alpha$(GSTA), μ (GSTM), π (GSTP), δ (GSTS), and θ (GSTT) [16, 17]. GSTs detoxify diverse electrophiles, including carcinogens, chiefly by conjugating them with glutathione [18, 19]. The subclass GSTP1 is widely expressed in normal human epithelial tissues and has been shown to be highly overexpressed in colon cancer [20]. The GSTM1 and GSTT1 defects seem to be associated with increased risk of certain cancers [21, 22].

Colorectal cancer is a common cause of mortality unless diagnosed early in its course when it is the most amenable to cure. Efforts for early diagnosis of colorectal cancers have become widespread over the past two decades with limited success, and tumor markers are appealing tools for this purpose [23, 24].

Epidemiologic studies that have sought to investigate the relation between variants in CYP and GST genes and colorectal neoplasia have thus far yielded conflicting results and a consensus regarding their etiologic importance has yet to be reached [25–29]. One method for investigating the protective role of GSTs is studying polymorphisms in GSTM1, GSTT1, and GSTP1 and CYP2E1 genes on susceptibility to colorectal cancer [30–32].

## Material and Methods

### Study Sample

The samples were obtained from patients who underwent colonoscopy and had colon cancer in the Research Center for Gastroenterology and Liver Disease, Taleghani Hospital, Shaheed Beheshti Medical University, Tehran, Iran from 2008 to 2010. None of the patients received prior treatment. In addition, it is necessary to mention that the samples of patients obtained from right colon, left colon and rectum. As a matter of fact, our main aim was to clarify GSTs measurement may be useful as a colorectal marker for CRC. Controls were selected from patients who had undergone colonoscopy with a completely normal result and no polyps on their examination. Genomic DNA was extracted from EDTA-treated peripheral blood by a standard phenol-chloroform extraction technique.

### Polymerase Chain Reaction and Pyrosequencing

Polymerase chain reactions (PCR were performed using 40 ng of DNA, 2.5 mmol/L MgCl2, and 1 pmol of each primer. All PCR-primers were designed using Assay Design Software version 1.0.6, Biotage AB, primer design software. Pyrosequencing was then performed as described by the manufacturer in a standard way.

### Pyrosequencing Reaction

20 μl of biotinylated PCR products are immobilized on Streptavidin-coated Sepharose beads. In a 96-well PCR plate containing 20 μl of a well optimized PCR reaction, high purity water was added to 40 μl per well, then 37 μl of the Binding buffer(Biotage AB, Sweden) and 3 μl of Streptavidin Sepharose bead (Streptavidin Sepharose HP, GE healthcare, Bioscience AB, Sweden) mixed. The plate was shaken 200 rpm at room temperature for 10 min. Biotinylated strands of PCR products were separated by alkaline denaturation and using Vacuum Prep. Workstation (Pyrosequencing, Sweden). Then separated single stranded PCR products were transferred into 40 μl mixture of sequencing primer and annealing buffer (Biotage AB, Sweden) and incubated at 80°C for 2 min, and the samples cooled to room temperature. After that the samples were sequenced by using Pyro Gold SQA reagent (Biotage AB, Sweden) in PSQ 96 MA pyrosequencer (Pyrosequencing, Sweden) and analyzed by PSQ 96 MA software ver 2.1.1 (Biotage AB, Sweden)

### Statistical Analysis

Comparisons between case and control groups were conducted using Pearson's $\chi^2$ test for nominal data. Odds ratios (OR) and 95% confidence intervals (CI) were calculated from 2×2 tables with the Mantel-Hanzel technique. Unconditional multivariate logistic regression was applied to adjust for confounding factors such as (age, sex) and for the covariates found to be associated with CRC in the study. Statistical analyses were performed with the SPSS 13.0 for Windows (Standard version, release September 2004).

## Results

### GSTM1 Genetic Polymorphism and Allele Frequency

DNA samples were subjected to PCR and pyrosequencing subsequently and resulted in three genotypes of GSTM1. The frequency of the alleles among cases and controls for the different variation studied and their respective genes are listed in Table 1.

**Table 1** The genotype frequencies of GSTM1, GSTP1, GSTT1, CYP2E1 polymorphisms

| Gene | Polymorphism | Total control | Tatal cases | Wild/wild | | Wild/Mut | | Mut/Mut | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Control | Cases | Control | Cases | Control | Cases |
| GSTM1 | Rs17072 Exon8 289 | 87 | 71 | 68(78.2%) | 62(87.3%) | 18(20.7%) | 8(11.3%) | 1(1.1%) | 1(1.4%) |
| | Rs10654 Exon8 11 | 100 | 99 | 90(90%) | 91(91.9%) | 10(10%) | 8(8.1%) | 0(0.0%) | 0(0.0%) |
| | Rs10586 Exon8 06 | 100 | 99 | 87(87%) | 91(91.9%) | 8(10%) | 8(8.1%) | 0(0.0%) | 0(0.0%) |
| GSTP1 | Rs1695 Exon5 | 100 | 99 | 53(53%) | 54(54.5%) | 42(42%) | 39(39.4%) | 5(5%) | 6(6.1%) |
| | Rs49869 Exon5 48 | 98 | 80 | 62(63.3%) | 49(61.3%) | 30(30.6%) | 27(33.8%) | 6(6.1%) | 4(5%) |
| | Rs11382 Exon6 72 | 95 | 73 | 83(87.4%) | 53(72.6%) | 12(12.6%) | 19(26%) | 0(0.0%) | 1(1.4%) |
| GSTT1 | Rs2234953Exon4 | 99 | 98 | 90(90.9%) | 94(95.9%) | 9(9%) | 4(4.1%) | 0(0.0%) | 0(0.0%) |
| | Rs2266637 Exon4 | 70 | 91 | 37(52.9%) | 34(37.4%) | 26(37.1%) | 26(28.6%) | 7(10%) | 31(34%) |
| | Rs4630 3' UTR | 100 | 80 | 100(100%) | 79(98.8%) | 0(0.0%) | 1(1.3%) | 0(0.0%) | 0(0.0%) |
| | Rs2266633 Exon4 | 100 | 94 | 81(81%) | 71(75.5%) | 11(11%) | 19(20.2%) | 8(8.0%) | 4(4.3%) |
| CYP2E1 | rs6413419 Exon4 | 100 | 90 | 95(95%) | 80(88.9%) | 5(5%) | 10(11.1%) | 0(0.0%) | 0(0.0%) |
| | rs2854140 Promoter Upstream | 91 | 86 | 88(96.7%) | 83(96.5%) | 3(3.3%) | 3(3.5%) | 0(0.0%) | 0(0.0%) |

Rs17072289 was the first polymorphism analyzed. Its frequency of wild homozygous, heterozygous and mutated homozygous variant genotype in the controls and CRC patients was 78.2% (68/87), 20.7% (18/87) and 1.1% (1/87) vs. 87.3% (62/71), 11.3% (8/71) and 1.4% (1/71) respectively (Table 1). The difference was not significant. Rs1065411 was the second polymorphism analyzed. Its frequency of wild homozygous, heterozygous and mutated homozygous variant genotype detected in the controls and CRC was 90% (90/100), 10% (10/100) and 0.0% (0/100): 91.9% (91/99), 8.1% (8/99) and 0.0% (0/99) respectively (Table 1). The difference was not significant. The frequency of wild homozygous, heterozygous and mutated homozygous variant genotype detected in the controls and CRC in Rs1056806, the third polymorphism analyzed, was 87% (87/100), 13% (13/100) and 0.0% (0/100) : 91.9% (91/99), 8.1% (8/99) and 0.0% (0/100) respectively . The difference was not significant.

## CYP2E1 Genetic Polymorphism and Allele Frequency

The distribution of CYP2E1 allele frequencies was found to be in Hardy Weinberg equilibrium in controls and cases. The frequency of *wild* homozygous genotype 95%(95/100) and 88.9%(80/90), heterozygous genotype 5%(5/100) and 11.1%(10/90) in rs6413419 was detected in controls and cases respectively (Table 1). The frequency of wild homozygous genotype and heterozygous genotype in rs6413419 for controls and cases were 96.7%(88/91) and 96.5%(83/86): 3.3%(3/86) and 3.5%(3/86) respectively. The results were marginally different in patients in comparison to controls but the difference was not statistically significant in both polymorphisms (Table 1). (We found no mutant homozygote in cases and controls.)

## GSTP1 Genetic Polymorphism and Allele Frequency

In the control subjects the rs1695 frequencies of the wild homozygous allele, heterozygote allele and mutant homozygote allele were detected by pyrosequencing and found to be 53% (53/100), 42% (42/100) and 5% (5/100) respectively, in the CRC subjects, and those for the mentioned genotype were found to be 54.5% (54/99), 39.4% (39/99) and 6.1% (6/99), in controls (Table 1). The difference was not significant. The rs4986948 frequencies of the wild homozygous allele, heterozygote allele and mutant homozygote allele were detected by pyrosequencing and found to be 63.3% (62/98), 30.6% (30/98) and6.1% (6/98) respectively, in the CRC subjects, and those for the mentioned genotype were found to be 61.3% (49/80), 33.8% (27/80) and 5% (4/80), for controls (Table 1). The difference was not significant. The rs4986948 frequencies of the wild homozygous allele, heterozygote allele and mutant homozygote allele detected by pyrosequencing were found to be 87.4% (83/95) and 12.6% (12/95) respectively, in the CRC subjects, and those for the mentioned genotype were found to be 72.6% (53/73), 26% (19/73) and1.4% (1/73), in controls (Table 1) ($P<0.024$: OR,2.48: 95% CI, 1.04–5.96).

GSTT1 Genetic Polymorphism and Allele Frequency

The distribution of GSTT1 allele frequencies was found to be in Hardy Weinberg equilibrium in controls and cases. For rs2234953 the frequencies of wild homozygous, heterozygous and mutated homozygous variant genotype detected in the controls and CRC cases was 90.9% (90/99) and 9% (9/99): 95.9% (94/98) and 4.1% (4/98) respectively (Table 1). The difference was not significant. For rs2266637 the frequencies of wild homozygous, heterozygous and mutated homozygous (polymorph) variant genotype detected in the controls and CRC cases was 52.9% (37/70), 37.1% (26/70) and 10% (7/70) : 37.4% (34/91), 28.6% (26/91) and 34.1% (31/91) respectively (Table 1). ($P$<0.0001: OR, 2.43: 95% CI, 1.47–4)

In rs4630 the frequencies of wild homozygous, heterozygous and mutated homozygous variant genotype detected in the controls and CRC was 100% (100/100): 98.8% (79/80) and 1.3% (1/80) respectively (Table 1). The difference was not significant. In rs2266633 the frequencies of wild homozygous, heterozygous and mutated homozygous variant genotype detected in the controls and CRC cases was 81% (81/100), 11% (11/100) and 8% (8/100) : 75.5% (71/94), 20.2% (19/94) and 4.3% (4/94) respectively (Table 1). The difference was not significant.

## Discussion

Under similar environmental carcinogens exposure, different individuals respond differently to environmental exposures. The different liability to cancer is called genetic susceptibility to cancer. Genetic susceptibility can affect in every step of carcinogenesis, including modifying the effect of environmental carcinogens [33–35]. Cancer susceptibility genes include types I and II metabolism enzyme genes, DNA repair genes and those affecting cell proliferation rates [36].

In recent years, the evidence has been accumulated to support the hypothesis that cancer susceptibility genes may be of importance in determining individual susceptibility to cancer[37, 38]. CRC is a disease determined by multifactors, including environmental risk factors and genetic factors. However, little is known about the impact of GSTM1, P1, T1 and CYP2E1 genetic polymorphisms on the susceptibility to CRC in the Iranian population. This is the study that simultaneously evaluated the GSTM1, P1, T1 and CYP2E1 polymorphisms in Iranian patients with CRC.

The gene of GSTs, one of the most important phase 2 enzymes, has attracted much attention with reference to CRC. GSTs can detoxify a number of reactive electrophilic compound substances, including the carcinogens polycyclic aromatic hydrocarbons (PAHs). In individuals with GSTs polymorphic genotype, probably the ability of detoxifying the carcinogens is decreased. The polymorphic genotype leads to loss of enzyme activity and individuals with GSTs: polymorphisms could have increased risk of cancers [39, 40].

Because the etiology of colorectal cancer has been hypothesized to be associated with environmental nitrosamine exposure, we investigated whether genetic factors that might modulate the activation and/or detoxification of these carcinogens could have an impact on the risk of developing this malignant disease. We analyzed genetic polymorphisms at the CYP2E1, GSTM1, GSTT1, and GSTP1 loci. GSTP1 gene has an A:G transition that causes replacing Isoleucine with Valin and a G:T transition in exon6 that result in substitution of valin with alanin. These SNPs leads to substitution in the enzyme active site and thus decrease the enzyme activity(). On the other hand the polymorphisms of GSTT1 gene (Table 1) are nonsynonymous. The rs2266637 SNP in exon4 results in substitution of Val with Ileu. We found that the GSTT1 and GSTP1 polymorphisms were associated with susceptibility to colorectal cancer. The polymorphism (rs6413419) 0f CYP2E1 gene exists in exon4 and results in substitution Val with Ileu. This variation doesn't have any effect on physic-chemical property of protein. The rs28514 polymorphism of CYP2E1 exists in promoter upstream. This SNP can change the expressin of gene. GSTM1 SNPs that presented in Table 1 are potentially functional SNPs but in our study there was not any significant association between CYP2E1 and GSTM1 polymorphisms and CRC. In our study one polymorphism (rs1138272) of GSTP1 and one polymorphism (rs2266637) of GSTT1 had a noticeable difference between controls and cases but the difference in GSTP1's polymorphism was not statistically significant. On the other hand we observed no association between either both CYP2E1 or GSTM1 polymorphisms and CRC. According to the difference in genotypes between cases and controls, we assume that if this study was performed on a greater sample of controls and patients, the results may have been statistically meaningful.

Several studies have examined the association between GSTM1, GSTT1, CYP2E1, GSTP1 and colon cancer risk: however, these results have been conflicting. They found a significant excess of the GSTM1 deletion among 196 cases of colorectal cancer (56% among cases versus 42% among 225 controls) and noted a stronger association for proximal colon cancers [41]. There was no association between the GSTM1 genotype and colorectal cancer in several case–control studies [42], although in some studies observed an association limited to distal colorectal cancers [43]. Deakin et al. observed a positive association between the GSTT1 null genotype and colorectal cancer (OR 1 .9: 95% CI, 1.3–2.8), but no association with the combined GSTT1/GSTMJ null genotype [44]. A positive association of the GSTT1 phenotype was observed in another small study for

combined gastric and colon adenocarcinoma and in a larger study of 132 cases in which the association for the GSTT1 null genotype was present only among people older than 70 years [42].

Based on our knowledge it is the first time that pyrosequencing technique was applied to detect any association between GSTM1, GSTT1, CYP2E1, GSTP1 polymorphisms and CRC. We recognized that there is a strong association between GSTT1 and CRC and there is no association between GSTM1, CYP2E1, GSTP1 and CRC. In our study we could not detect any association between GSTM1 and CYP2E1 polymorphisms and CRC. We are reevaluating these associations with greater number of patients and controls.

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