Original Article

Relationship between genetic polymorphism of *glutathione S-transferase-p1* and p53 protein accumulation in Iranian esophageal squamous cell carcinoma patients

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Abstract

BACKGROUND: It has been reported that the activity of glutathione S-transferase (GST) is over-expressed in plasma and esophagus biopsies in Iranian patients suffering from esophageal squamous cell carcinoma (SCC). The aim of this study was to find out the frequency of *GST-P* genotypes in these patients. Moreover, the association of *GST-P* genotypes with p53 protein accumulation in esophageal epithelium was investigated. **MATERIALS AND METHODS:** DNA isolated from paraffin-embedded tissue biopsies from patients suffering from esophageal SCC (n = 56) were collected. polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using *Alw261* enzyme was applied to determine *GST-P* genotypes (Ile 105 Val). All the samples were also subjected to immunohistochemistry (IHC) for p53. **RESULTS:** The frequency of *GST-P* genotypes in Iranian esophagus SCC patients for Ile/Ile, Ile/Val and Val/Val was 73.2, 21.5 and 5.3%. There was no association between *GST-P* polymorphism and p53 accumulation in esophageal epithelial cells. **CONCLUSIONS:** The frequency of *GST-P* polymorphism was not associated with p53 protein accumulation in esophagus epithelium. The frequency of polymorphic variants of GST-P, Ile/Ile, Ile/Val and Val/Val in SCC patients may suggest that Ile to Val substitution in *GST-P* gene dose not represent susceptibility to SCC in high-risk Iranian population.

Key words: Esophageal squamous cell carcinoma, glutathione S-transferase, Iran, p53, polymorphism

Introduction

Esophageal squamous cell carcinoma (SCC) is a predominant histological subtype of esophageal cancer which is characterized by high mortality rate and geographic difference in incidence.[1] It has been reported that the incidence of esophagus SCC is very high in Northern Iran. The incidence of this disease in this high-risk region is believed to be 200 times greater as compared to that in populations living in low-risk regions.[2] Higher incidence rates in certain geographic areas may suggest that environmental factors contribute to the carcinogenesis of esophageal cancer.[3] The contribution of genetic polymorphism to risk of esophageal cancers including adenocarcinoma has been reviewed.[4]

Most environmental chemical carcinogens undergo activation by phase I enzymes, often in an oxidation reaction, and detoxification by phase II enzymes such as glutathione S-transferase (GSTs).[4] GSTs are a family of enzymes involved in detoxification of a wide range of chemicals, including carcinogens. *GST-P1* is a major *GST* isoform expressed in human epithelial esophagus[5,7] and has been shown to be genetically polymorphic.

Many investigators have shown the genetic polymorphism of carcinogen-metabolizing enzymes is associated with susceptibility to some kind of cancers.[8,9] *GST-P* is a polymorphic gene and there are two genetic variants of *GST-P1* gene, one at codon 105 with isoleucine (Ile) to valine (Val) polymorphism and the other at 114 (Alanine to Val transition) which result in significant differences in catalytic activity.[10] The *GST-P1* polymorphism of a single base substitution in exon 5 leading to replacement of the amino acid Val with Ile has been reported in patients with esophageal adenocarcinoma (ADC). Such changes are believed to be implicated in reduced activity and impaired detoxification activity of the enzyme.[11] According to Jain and co-worker,[12] there is no association between genetic polymorphism of *GST-P1* with risk of esophageal factors in an Indian population suffering from ADC or SCC. Although the association between the *GST-P* polymorphism and risk of esophageal SCC has been examined by epidemiologic studies,[13] results have been conflicting.[14,15]

Earlier we reported that *GST-P* is overexpressed in plasma as well as esophagus tissue biopsies obtained from patients suffering from esophagus SCC, when compared to that the normal tissues.[16] In the present study, the frequency of *GST-P* polymorphism was determined to find out if a particular type of *GST-P* genotype represents susceptibility to SCC in a population believed to be at high risk of the disease. Moreover, tissue biopsies were divided based on p53 protein status ($p53^+/p53^-$) and *GST-P* polymorphism was determined in terms of accumulation of p53 protein in esophageal epithelium.

Materials and Methods

A total of 56 esophageal tissue biopsies (formalin-fixed, paraffin-embedded esophageal tissue) from individuals with SCC pathological confirmed (including 29 male, 27 female with the mean age of 61), were collected from Imam Khomeini and Shariati hospitals in Tehran. In this study, hospital records were used to verify patient's data and the study protocol confirms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in the guidelines of the Medical Ethics Committee, Ministry of Health, I.R. Iran.

Serial sections of 10-µm thickness were prepared from tissue blocks. Genomic DNA was extracted from esophageal epithelial tissues that were fixed on slides. After dewaxing, DNA was isolated using phenol/chloroform extraction procedure as described.[17]

Analysis of *GST-P1* polymorphism: The two variant alleles in the *GST-P* gene were differentiated by Restriction Fragment Length Polymorphism (RFLP) as described by Harris *et al.* [17] PCR for *GST-P* genotyping was carried out using exon 5 specific primers.[18] The sequence of primers used is as follows:

Forward; 5'-ACC CCA GGG CTC TAT GGG AA-3' and Reverse; 5'-TGA GGG CAC AAG AAG CCC CT-3'. Using these primer sequences, the samples underwent an initial denaturation at 95°C for 3 min, followed by denaturation at 95°C for 45 sec, anneling at 55°C for 1 min, polymerization at 72°C for 30 sec and finally the elongation step at 72°C for 5 min. The PCR product of 176p was then digested with 5U *Alw261* (Fermentase Inc., USA) at 37°C in a total volume of 20 µl and the products were electrophoresed and visualized on 12% polyacrylamide gel after silver staining. The genotypes were classified as Ile/Ile (A/A) homozygote (176 bp), Ile/Val (A/G) heterozygote, (176 bp, 91 bp, 85 bp), Val/Val (G/G) homozygote (91 bp and 85 bp).

In addition, direct sequencing of exon 5 of *GST-P* with different genotypes was carried out to confirm the genotypes detected with PCR-RFLP.

Immunohistochemistry (IHC): Paraffin-embedded tissue blocks were cut into 4 mm sections and mounted on to polylysine-coted slides. Sections were de-waxed in xylene, rehydrated in descending alcohol concentrations. The sections were then placed in 95°C solution of 0.01 M sodium citrate buffer (pH = 6) for antigen retrieval. Subsequently endogenous peroxidase activity blocked with hydrogen peroxide 0.1% for 15 min. After rinsing, slides were incubated overnight at 4°C with primary antibodies. Incubation with appropriate biotinylated secondary antibody was carried out for 30 min, followed by incubation in avidin-biotin-peroxidase reagent and visualization with 3, 3-diaminobenzidine. Finally, the sections were counterstained with hematoxylin, dehydrated, cleared, and mounted. The sections were incubated with monoclonal anti-human p53 protein produced in mouse (Cat. #BO-7; Dako, Denmark). IHC analysis was first evaluated by showing that in absence of the primary antibody there was no staining.

Validation of staining scoring of all the preparations was carried out independently by two pathologists (SMT and MJZ) without prior knowledge to samples. Tumors classified positive if >5% of stained cells were positive for p53. Negative controls by the respective primary antibody omitting were also performed.

Distribution of *GST-P* polymorphism in samples was stratified according to p53 status and assessed by Logistic regression analysis. Odds ratio (ORs) with 95% confidence intervals (95% CIs) were also calculated. The

GSTP1 genotypes were analyzed by the Chi-square test or Fisher's exact probability test.

Results

In this study, DNA samples were collected from tissue biopsies and subjected to PCR followed by enzymatic *(Alw26I)* digestion. The *GST-P* polymorphisms were detected for three genotypes.

Table 1 shows the *GST-P* genotype frequency in esophageal SCC patients. The Ile/Ile genotype was most common in patients with frequency of about 73.2%. IHC of p53 showed that out of 56 SCC samples 36 tissues were $p53^+$. The association of *GST-P* genotyping with accumulation of p53 protein showed that the frequency of *GST-P* Ile/Ile homozygote in $p53^+$ and $p53^-$ samples is 75% and 70%, respectively.

GST-P1 heterozygous and homozygous for Val allele (Ile/Val and Val/Val) in $p53^+$ and $p53^-$ tissues was 25% and 30% respectively [Table 1]. This data showed that there is no association between *GST-P* genotyping and accumulation of p53 in cancerous esophagus (P = 0.79).

Direct sequencing of exon 5 of *GST-P* with different genotypes confirmed that in cases with Ile/Ile genotype (*GST-P*, AA) homozygote, there was an adenine (A) base in position 1578 whereas, in case of Ile/Val (AG) heterozygote, both A and G bases were detected (results not shown).

Discussion

The etiology of esophagus SCC particularly in people living in high-risk regions such as Caspian Sea coastal region of Iran is unclear. The disease can be influenced by polymorphic forms of genes that are involved in detoxification of environmental carcinogens. In addition, since GSTs are involved in modulation of expression of other drug metabolizing proteins, polymorphism in *GST* may influence other defense mechanisms, thereby indirectly conferring a differential risk for development of cancer.[19]

Table 1: The frequency of *GST-P* genotypes and relationship between genotypes and p53 accumulation in esophagus squamous cell carcinoma patients

Semple	*GST-P* genotype		
	Ile/Ile	Ile/Val	Val/Val
SCC (n = 56)	41 (73.2%)	12 (21.5%)	3 (5.3%)
$p53^+$ (n = 36)	27 (75%)	7 (19%)	2 (6%)
$p53^-$ (n = 20)	14 (70%)	6 (30%)	(0%)
P-value	0.73 (n.s)	0.99 (n.s)	0.99 (n.s)

n.s = not significant

GSTs, particularly its class-Pi are predominantly expressed in human epithelial cells in the normal squamous esophagus epithelium[16,20-22] and it has been used as the marker of gastrointestinal (G.I) carcinoma. Earlier we reported that the level of class P GST is elevated in tissue biopsies obtained from SCC patients who underwent surgery or upper gastro-digestive endoscopic operation when compared to normal samples. This observation was further substantiated by showing a relatively higher levels of *GST-P* in plasma samples from SCC patients as compared to the control group.[16] More recently, differences in *GST-P* levels in normal and malignant esophagus was approved by measuring *GST-P* specific mRNA levels using RT-PCR-ELISA method.[20]

The difference in the *GST-P* levels in normal and malignant epithelium, which is reflected in plasma, is probably not related to the genetic polymorphism of *GST-P*. The similarity of the frequency of *GST-P* polymorphism in control and SCC cases with those reported in normal subjects attests to this observation. The frequencies of the variant genotypes of *GST-P* could be dependent on the ethnic groups being considered. The frequencies of *GST-P* Ile/Ile, Ile/Val and Val/Val genotypes in a normal Iranian population (n = 25) was found to be 68%, 28% and 4% respectively (Unpublished results). The pattern of *GST-P* frequencies was found to be similar to those reported in a Japanese population (68.8% of Ile/Ile; 29.3% of Ile/Val and 1.4% of Val/Val).[14] Cai *et al.*,[23] also reported similar frequency for *GST-P* genotypes in a Chinese population living in Taixing city of Jiangsu province (67.4% of Ile/Ile; 29.5% of Ile/Val and 3.1% of Val/Val). The data presented in this study revealed that the frequency of valine allele (Ile/Val and Val/Val) is much higher as compared to that reported in normal individuals living in France. The frequency of Ile/Ile, Ile/Val and Val/Val was observed to be 48%, 45% and 7% in this normal population.[24] Almost similar frequencies were reported from Canada by Casson and co-workers.[25]

Regardless of the difference in the frequencies of *GST-P* variants among the population in different ethnic groups, there are no differences in *GST-P* genotypes between normal (data not shown) and esophagus SCC patients, suggesting that the genetic variants of *GST-P* gene is not associated with the development of esophageal SCC. As shown in Table 1 in Iranian esophageal SCC patients, the frequency of the polymorphism of *GST-P* Ile/Ile, Ile/Val, and Val/Val is 73.2%, 21.5% and 5.3% respectively suggesting that an Ile to Val substitution in

Table 2. Relationship of p53 accumulation and GST-P genotypes in esophagus squamous cell carcinoma patients

p53 accumulation	*GST-P genotype*		
	Ile/Ile	**Ile/Val**	**Val/Val**
p53 (n = 36)	27 (75%)	7 (19%)	2 (6%)
p53 (n = 20)	14 (70%)	6 (30%)	0 (0%)
OR (95% CI)	1	0.00 (0.00^{-})	0.00 (0.00^{-})
LRT (d.f)	0.60 (2)	0.00 (1)	0.00 (1)
P-value	0.73	0.99	0.99

the *GST-P* gene may not represent susceptibility to this type of cancer.

Another observation in the present study was that the frequency of *GST-P* between control and cases was not related to accumulation of p53 protein in squamous esophagus epithelium. Taking into consideration, the presence of unusual profile of p53 gene mutations in Iranian esophageal SCC patients,[17] we scrutinized all the samples into two groups of $p53^{+}$ and $p53^{-}$ samples. For this purpose, initially all samples were subjected to imunohistochemistry (IHC) to detect p53 protein accumulation in the esophageal biopsies. Table 2 shows that *GST-P* genotypes are not significantly different among $p53^{+}$ or $p53^{-}$ samples.

Many papers have shown inconsistent association between the *GST-P* polymorphism and p53 protein mutations in different cancers, but association with p53 protein expression has not been reported. The evidences presented in this study showed that irrespective of the p53 status, the frequency of *GST-P* Ile/Ile homozygote in SCC patients was between 68-75%. Likewise, the frequency of *GST-P1* heterozygous for Val allele (Ile/Val and Val/Val) was between 25-30% in patients irrespective of the p53 status in the esophagus tissue as judged by IHC. It was also demonstrated that there is no association between p53 accumulation with other factors such as age, gender and histology grade of the tissues (Results not presented). The results presented in this paper, clearly show that there is no risk of esophageal SCC associated with Ile or Val variants of codon 105 of *GST-P* in Iranians possessing any genotypic combination. Moreover, there was no significant association between *GST-P* genotypes and p53 protein expression in esophagus tissue. Further studies in larger populations in this high-risk region would be desirable to confirm these findings.

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