

## Study of cyp2E1 Gene RsaI/PstI Polymorphisms in Patients with Gastric Cancer in North of Iran

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### Abstract

**Background:** North of Iran is amongst high incidence rate areas of gastric carcinoma where environmental carcinogenic compounds especially agricultural pesticides are massively used. Cytochrome P450 2E1 (CYP2E1) enzyme metabolically activates a large number of low molecular mass xenobiotics. The polymorphic nature of cyp2E1 gene control elements is associated with interindividual differences for toxicity of its substrates and may be responsible for increased gastric cancer susceptibility. The current study investigated the allelic frequencies of cyp2E1 gene RsaI/PstI polymorphisms and its association with gastric cancer risk in north of Iran.

**Materials and Methods:** This case-control study comprised of 120 gastric cancer patients and a group of 135 healthy individuals as control. Genotyping of cyp2E1 gene PstI/RsaI polymorphisms were carried out by PCR-RFLP method. Statistical analyzes were performed by Logistic regression model and  $P < 0.05$  was considered as significance level.

**Results:** TNM classification showed that most patients (88%) were in advanced stages when the disease was diagnosed. Frequencies of C1C1 and C1C2 genotypes of PstI/RsaI polymorphisms were 96 and 4% in case, and 99 and 1% in control group, respectively whereas homozygote C2C2 genotype was not observed in any of the subjects. In Logistic regression model no significant association was found between RsaI/PstI allelic variants and gastric cancer risk ( $p = 0.443$ ,  $OR = 0.386$ ,  $CI = 0.034-4.395$ ). Furthermore, no significant correlation was seen between genotypic frequencies and clinicopathological characteristics.

**Conclusion:** No significant association was found between cyp2E1 gene PstI/RsaI allelic variants and gastric cancer risk or clinicopathological characteristics of gastric cancer patients in north of Iran.

**Keywords:** CYP2E1 gene; Gastric Cancer; PstI/RsaI polymorphism; North of Iran

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### Introduction

Gastric cancer is the fourth most frequent cancer, accounting for 8% of the total cancer cases, and the second leading cause of cancer related death resulting in 10.4% annual deaths worldwide (1, 2). Although the incidence rate of gastric cancer has decreased in the western world, its incidence and mortality have increased or remained stable in developing countries. Accordingly, in Iranian males and females it is the

leading and the third cause of cancer related death, respectively (3, 4). Northern parts of Iran along with Northwestern regions are the highest risk areas for gastric cancer with an age-standardized incidence rate (ASR) of 49.1 for males and 25.4 for females (4). Gastric cancer mostly occurs as a result of complex interaction between genetic factors such as single nucleotide polymorphism (SNP) within key genes,

and environmental risk factors such as *Helicobacter pylori* (*H. pylori*) colonization and exposure to diverse carcinogens (5, 6). Pesticides are among the most carcinogenic compounds that are massively used in north of Iran to protect agricultural products (7). Most exogenous (xenobiotics) and endogenous chemical carcinogens undergo biotransformation by phases I and II enzyme systems to activate and subsequently detoxify in the human body (8, 9, 10, 11). Genetic variation of the metabolizing enzymes may affect its activity or inducibility leading to individual differences in susceptibility to chemical carcinogen (12-14). Cytochrome P450 (CYP) is a superfamily of hemoproteins and a major kind of phase I enzymes detoxification that play a central role in the metabolism of many xenobiotics and endogenous compounds. It is also involved in activation of many carcinogens (15-16). CYP2E1, a member of the cytochrome P450 superfamily is an alcohol-inducible enzyme that plays a key role in the metabolic activation of many low molecular weight compounds such as benzene, vinyl chloride and N-nitrosamines (17-19). It is located on chromosome 10q26.3 as an 11.7 kb gene consisting of 9 exons and 8 introns that encodes for a 493 amino acid protein (20). Some functional cyp2E1 gene SNPs have been identified that alter the transcriptional activity of the gene and as a result influences the susceptibility to develop cancer (21). Since the coding region of the human cyp2E1 gene is highly conserved, it is likely that the variability is related to polymorphic sites at the control elements present in the upstream region (22). RsaI/PstI polymorphisms in the 5'-flanking region are composed of two point mutations including RsaI polymorphism at -1053C>T (ra2031920) and PstI at -1293 G>C (rs3813867) (22, 23). They are in complete linkage disequilibrium resulting in three genotypes; the individuals with predominant homozygous allele (C1/C1), the heterozygous allele (C1/C2), and the rare homozygous allele (C2/C2) of PstI/RsaI polymorphisms are named the wild-type homozygote, the heterozygote, and the rare homozygote, respectively. These polymorphisms are reported to alter transcriptional activity of the cyp2E1 gene and their association with greater risk for oral, pharyngeal, liver, lung and gastric cancers have been documented in numerous studies. Nevertheless, several studies conducted on the possible association of cyp2E1 RsaI/PstI genetic variants and gastric cancer risk produced controversial results. High incidence rate of gastric cancer in north of Iran where pesticides are being massively used and different toxicity of chemical carcinogens in relation to metabolizing enzymes variants has prompted us to analyze interindividual variation of cyp2E1 RsaI/PstI polymorphisms as a key enzyme for

xenobiotics metabolism. So, the aim of this research was to study the genotypic frequency of cyp2E1 gene RsaI/PstI polymorphisms and its association with the risk of gastric cancer in north of Iran.

## Materials and methods

### Study population

The present population based case-control study comprised of 120 gastric cancer patients who were diagnosed and underwent surgery in Ayatollah Rowhani and Shahid Beheshti hospitals in Mazandaran and Sayyad Shirazi Hospital in Golestan province during 2011 to 2013. A group of 135 healthy individuals randomly selected from blood donor volunteers without any familial history of the disease were also enrolled as control for statistical analysis. The healthy controls were age- and sex-matched and were from the same ethnic group. Five ml samples of peripheral blood were collected from each case and control subjects in tubes containing EDTA and stored at -20 °C. Demographic (e.g. age, sex) and clinicopathological (e.g. TNM stage) characteristics of the subjects were extracted from their medical records and histopathological reports.

Clinicopathological Variables	Number of patients (%)
Age	
≤40	10(9)
>40	104(91)
Type of cancer	
Cardia	10(14)
Non-cardia	60(86)
TNM staging	
I- II	12(12)
III – IV	86(88)
Family history	
Positive	23(21)
Negative	87(79)
Smoking	
Positive	35(33)
Negative	72(67)

### DNA extraction

Genomic DNA was extracted from whole blood cells by routine phenol-chloroform method. DNA concentration of each sample was measured by UV spectrophotometry and its purity was examined through agarose gel electrophoresis. DNA samples were stored at -20 °C for future use.

### cyp2E1 RsaI/PstI polymorphisms analysis

Genotyping of both PstI and RsaI polymorphic sites were determined by polymerase chain reaction – restriction fragment length polymorphism (PCR-

RFLP) method. A set of forward 5'-CATTGTCAGT TCTCACCTCG-3' and reverse 5'-GCTCCACATTG ACTAGCTTC-3' primers were designed using a cyp2E1 gene sequence (accession number: NG00838 3.1) available in GenBank which amplify a 537 bp fragment from nucleotide position -1392 to -855 encompassing both variable alleles. Amplification was carried out in a 25µl mixture containing 1X PCR buffer, 120 ng of genomic DNA, 1.5 mM of MgCl<sub>2</sub>, 200 µM of each dNTP, 0.3 PM of each forward and reverse primers and 2U of taq DNA polymerase (10U/µl). PCR cycling conditions consisted of one cycle initial denaturation at 95 °C for 4 min, 35 cycles including denaturation at 94 °C for 40s, annealing at 46 °C for 30s, and extension at 72 °C for 30s and one cycle final extension at 72 °C for 7min. Amplified products were electrophoresed in 1.5% agarose gel and visualized following staining with ethidium bromide. Concentration of amplified fragments was measured using spectrophotometry technique to apply equal amounts of each sample for restriction digestion. Allelic variants of both polymorphic sites were studied through restriction digestion with RsaI and PstI restriction enzymes to evaluate if they were at linkage disequilibrium as other genotypes. Digestion reaction was performed in a 25µl mixture containing 1X Tango buffer, 200ng amplified fragment, and 2U RsaI (10U/µl) restriction enzyme for RsaI polymorphism and 1X O buffer, 200ng amplified fragment, and 2U PstI restriction enzyme (10U/µl) for PstI polymorphism. Reaction mixture was incubated at 37 °C for 4 hours and the products were analyzed through electrophoresis at 2.5% agarose gel. Following digestion with RsaI restriction enzyme, homozygote CC genotype produces two 339 and 198 bp fragments, homozygote mutant TT genotype produces a single 537bp fragment and heterozygote CT genotype produces three 537, 339 and 198 bp fragments. In case of PstI polymorphism GG genotype produces a single 537bp fragment, CC genotype produces two 432 and 105bp fragments and heterozygote GC genotype produces three 537, 432 and 105bp fragments. At least one sample of each unique restricted pattern was sequenced in both directions at Bioneer company (Korea) to verify the results of RFLP genotyping.

### Ethics Statement

All patients and healthy controls provided their verbal informed consent to participate in this study.

### Statistical analysis

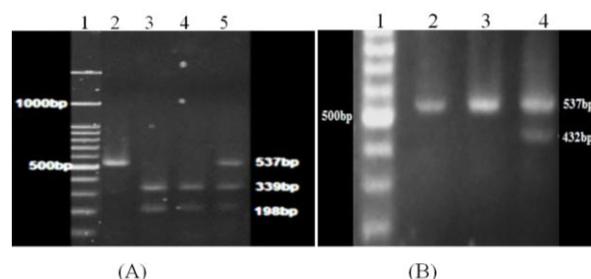
All Statistical analyzes were performed in SAS 9.1 statistics software and P<0.05 was considered as statistical significance level. The genotype and allele frequencies of cyp2E1 RsaI/PstI polymorphisms were

tested for Hardy-Weinberg equilibrium (HWE) using  $\chi^2$  test. The distribution of cyp2E1 RsaI/PstI polymorphisms between case and control groups were calculated using unconditional Logistic regression methods and Odds ratio (OR), confidence intervals (CI), and p-values adjusted for age were measured to study the association between genotypes and the risk of gastric cancer or some clinicopathological data (20).

## Results

### Demographic and clinicopathological data

This study was performed in 120 gastric cancer patients including 96 (80%) males and 24 (20%) females with an average age of 65.7±8.16 years (Table 1). The control group composed of 97 (72%) men and 38(28%) women with the mean age of 62.5±6.472 years. The Student's t test showed no significant differences in matching characteristics between cases and controls (p>0.1311). Thirty five out of 107 patients (33%) were smokers while this rate was 23% for the controls. So, significant association was found between smoking and disease incidence (P=0.02). Results of Chi-square test showed that the genotype frequency of case and control groups did not significantly diverge from HWE (both p>0.05). Pathological assays showed that most patients (88%) were in stages III and IV when the disease was diagnosed for the first time.



**Figure 1.** PCR-RFLP profile of CYP2E1 gene RsaI/PstI allelic variants in 2.5% agarose gel. A) RsaI RFLP: 1: 100bp ladder, 2: Amplified fragment (537bp), 3, 4: Homozygous wild type (339 and 198bp), 5: Heterozygous genotype (537, 339 and 198bp). B) PstI – RFLP: 1: 100 bp ladder, 2, 3: Homozygous wild type (537bp), 4: Heterozygous genotype (537, 432 and 105bp).

### The cyp2E1 gene RsaI/PstI genotype distribution and its association to clinicopathological characteristics

The allelic variants of cyp2E1 gene RsaI/PstI polymorphisms were determined according to the patterns created following digestion of amplified fragments by respective restriction enzyme (Figure 1) and resulting genotypic distributions in cases and controls are shown in table 2. Results of RsaI polymorphism analysis showed that the genotypic frequencies of C1C1 and C1C2 genotypes were 96 and 4% in case, and 98.5 and 1.5% in control group,

respectively whereas homozygote C2C2 genotype was not observed in any subjects. Similar genotypic distribution was found on PstI digestion of amplified

fragments in cases and controls verifying complete linkage disequilibrium of these two polymorphisms.

**Table 2.** Distribution of cyp2E1 gene RsaI/PstI polymorphisms and gastric cancer risk.

Polymorphisms	Number		Non-adjusted <sup>a</sup>			Adjusted <sup>b</sup>	
	Cases(n=120)	Controls(n=135)	P value	OR	P value	OR	CI (95%)
RsaI/PstI	C1C2	5(4%)	2(1.5%)	...	1.00	...	1.00
	C1C1	115(96%)	133(98.5%)	0.346	0.312	0.443	0.386

OR= Odd Ratio, CI= Confidence Intervals, <sup>a</sup> Logistic regression model, non-adjusted, <sup>b</sup> Logistic regression model, adjusted for diagnostic age.

In the logistic regression model no significant association was seen between RsaI/PstI allelic variants and gastric cancer risk (p value=0.443,

OR=0.386, CI=0.034-4.395). The association between RsaI/PstI genotypes and clinicopathological characteristics was analyzed and presented in table 3.

**Table 3.** Relationship between cyp2E1 RsaI/PstI polymorphisms and known clinicopathological variables.

Clinicopathological Variables	Number (%)	Genotype		Adjusted <sup>a</sup>	
		C1C1	C1C2	p value	OR
Age					
<40	10(9)	10(100)	0(0)	...	1.00
>40	104(91)	99(95)	5(5)	1.00	1.00
Type of cancer					
Cardia	10(14)	10(100)	0(0)	...	1.00
Non-cardia	60(86)	55(92)	5(8)	0.971	<0.001
TNM staging					
I- II	12(12)	12(100)	0(0)	...	1.00
III - IV	86(88)	81(94)	5(6)	0.982	<0.001
Family history					
Positive	23(21)	20(89)	3(11)	...	1.00
Negative	87(79)	84(97)	3(3)	0.311	4.962
Smoking habit					
Positive	35(33)	33(94)	2(6)	...	1.00
Negative	72(67)	69(96)	3(4)	0.595	2.167

<sup>a</sup> Logistic regression model adjusted for diagnostic age. All statistical tests were two-sided with a significance level of p<0.05

The results showed that none C1C1 and C1C2 genotypes were significantly correlated with demographic and clinicopathological characteristics including age at diagnosis (p=1.00), type of cancer (p=0.971), stage of disease (p=0.982), smoking habit (p=0.595) and family history (p=0.311) (Table 3).

## Discussion

In spite of decrease in the incidence rate of gastric cancer in western world, the North and northwest of Iran are still amongst high incidence rate areas of gastric carcinoma (24, 25). Cytochrome P450 2E1 (CYP2E1) enzyme metabolically activates a large number of low molecular mass xenobiotics and the polymorphic nature of the controlling elements of its gene is associated with interindividual differences for toxicity of its substrates and cancer risk (26, 27, 28). We investigated allelic frequency of cyp2E1 RsaI/PstI polymorphisms at the 5' flanking region and its association with gastric cancer risk in north of Iran.

The average age of patients was 65.7±8.16 years and most patients (80%) were males that is consistent with the results of other epidemiological researches in Iran (Table 1) (29, 30). The positive association between tobacco smoking and gastric cancer risk is also confirmed in this study (p=0.02). Pathological analysis showed that only 14% of patients had tumors in cardia region. This is in contrary with some other epidemiological studies documenting cardia as the major part for gastric cancer in north and northwest of Iran (4). TNM classification showed that 88% of patients were diagnosed in advanced stages of the disease (III and IV). Clinically, symptoms of gastric cancer tend to emerge late in the development of the disease (4, 31). So, regular periodic tests and public education about the early warning signs and diagnosis of the disease are necessary (31). The cyp2E1 gene C1/C1 and C1/C2 genotypes distribution of RsaI/PstI polymorphisms were 97.5% and 2.5% in case and control groups, respectively.

Interestingly, the mutant C2/C2 genotype was not detected in any samples of the cases and controls. Striking inter-ethnic differences were found between different races with respect to cyp2E1 RsaI/PstI polymorphisms. Studies of RsaI/PstI polymorphisms in Caucasian and Asian population showed that the frequency of mutant C2/C2 genotype was 6% and 0.1% while the heterozygote genotype was found in 37% and 5%, respectively (32,33). In this research, logistic regression model showed no significant association between RsaI/PstI polymorphism and gastric cancer risk ( $p = 0.443$ ,  $OR = 0.386$ ,  $CI = 0.034-4.395$ ). Furthermore, no significant correlation was detected between RsaI/PstI genotypes and demographic or clinicopathological characteristics. Previous researches on the association between cyp2E1 polymorphisms and gastric cancer risk has yielded conflicting results. Study of genotypic frequencies of CYP2E1 PstI/RsaI polymorphism in Chinese patients and a small Costa Rican population paradoxically demonstrated significant correlation; despite positive association of C2 allele with gastric cancer incidence in Chinese population, it was found that C2C2 genotype was associated with reduced risk of gastric cancer in Costa Rican population (32, 34). However, meta-analyses based on 24 case-control studies and a study on Japanese population revealed no significant associations between CYP2E1 RsaI/PstI polymorphism and gastric cancer risk (35, 36). In addition to inter-ethnic differences of cyp2E1 gene and their discrete effects on cancer susceptibility, the interaction of genetic and environmental risk factors may be involved in cancer development (37). Organophosphorus pesticides are amongst the most important environmental carcinogenic compounds which are massively used in north of Iran (7). We recently detected Diazinone pesticide in the blood serum of some gastric cancer patients (0.155 ppm) using GC-mass analysis (unpublished data). So, it is suggested that the influences of both genetic and environmental risk factors should be considered simultaneously on gastric cancer incidence. Particularly, the possible epigenetic effects of prevalent pesticides should be studied on cyp2E1 gene expression. Furthermore, due to low frequencies of heterozygote C1C2 and mutant C2C2 genotypes of RsaI/PstI polymorphisms researches with larger groups are needed to verify these points.

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#### **Authors' contributions**

AB and SK participated in the whole processes of the study including design of research work, data analysis and drafting manuscript. NN performed surgery and provided samples. MSh helped in providing some materials and equipment.

#### **Conflict of interest**

The authors declare that they have no conflict of interest in this work.

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