P21 (Waf1/Cip1) Gene Polymorphisms and Possible Interaction with Cigarette Smoking in Esophageal Squamous Cell Carcinoma in Northeastern Iran: A Preliminary Study

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Abstract:

Background: The incidence of esophageal squamous cell carcinoma (ESCC) is very high in northeastern Iran. However, the genetic predisposing factors to ESCC in this region have not been clearly defined. The P21 (waf1/cip1) gene is involved in the arrest of cellular growth, as induced by the p53 tumor suppressor gene. Two polymorphisms of p21 gene in codon 31 (p21 C98A, dbSNP rs1801270) and the 3'UTR (p21 C70T, dbSNP rs1059234) may affect protein expression and play a role in cancer susceptibility. The present study aimed to investigate the association of p21 polymorphisms in codon 31 and the 3'UTR, and cigarette smoking on the risk of ESCC in northeastern Iran.

Methods: A case-control study was carried out to detect the p21 polymorphism in the 3'UTR and codon 31 of samples from 126 ESCC cases and 100 controls from 2006 to 2007. There were no significant differences of age and sex between cases and controls. Genotyping of p21 polymorphisms were determined with the PCR-RFLP method. Conditional logistic regression was used to adjust for potential confounders.

Results: None of the p21 genotypes were significantly associated with risk of ESCC, even after adjusting for age and gender (*P*=0.52, OR=1.24; 95%CI: 0.63 – 2.42). However, the presence of these polymorphisms in combination with cigarette smoking had a synergistic interaction in ESCC carcinogenesis in northeastern Iran (*P*=0.02, OR=8.38; 95%CI: 1.03 – 67.93).

Conclusions: Our data suggests that these two p21 polymorphisms, both alone and in combination, are not genetic susceptibility biomarkers for ESCC. However, their interaction with cigarette smoking may influence the susceptibility to ESCC development in northeastern Iran.

Keywords: cigarette smoking - esophageal squamous cell carcinoma - p21 polymorphism

Introduction

Esophageal cancer ranks as the sixth most common cause of cancer deaths worldwide.[1,2] The geographic distribution of incidence varies greatly due to environmental and genetic factors.[3–5] Golestan Province is a part of the Turkmen plain in northeastern Iran, located at the western end of the "Esophageal Cancer Belt," a region with very high rates of esophageal squamous cell carcinoma (ESCC). This region stretches from China westward through central Asia to northern Iran.[6,7] The incidence of esophageal cancer is very high in northern Iran (over 100 cases per 10^5 person years, for both men and women), and ESCC is considered the predominant type.[8] However, the specific genetic factors that predispose people in this region to this disease have not been clearly defined.[3,8]

Various genetic and environmental factors play an important role in esophageal cancer development.[5,9]

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Environmental factors such as cigarette smoking and heavy alcohol consumption, which account for many esophageal cancer cases in European and North American countries, are not considered to be major risk factors in this region.[8,10,11] However, a recent study in Golestan Province has shown a correlation between tobacco use and ESCC.[12]

Some studies have suggested that genetic polymorphisms might explain individual differences in susceptibility to specific malignancies, including esophageal cancer.[13] Several genes involved in DNA repair and cell cycle control are associated with esophageal carcinogenesis.[4] In response to DNA damage, wild-type *p53*, a tumor suppressor gene, assembles several downstream target genes including *p21*. *P21* protein is a cell cycle regulator that induces G1 arrest, leading to DNA repair or apoptosis.[14–18]

Several studies have shown that *p21* polymorphisms may affect protein expression and activity and play a role in susceptibility to cancer.[19,20] Two major *p21* polymorphisms in codon 31 (*p21* C98A, dbSNP rs1801270) and in the 3'UTR (*p21* C70T, dbSNP rs1059234), both alone and/or in combination, may have an effect on carcinogenesis.[19] The *p21*C98A polymorphism results in a non-synonymous serine to arginine substitution in the protein, which affects the DNA-binding zinc finger motif. The other polymorphism, *p21* C70T, occurs 20 nucleotides downstream of the stop codon in the 3'-UTR region. This region is considered to be an important site for cell differentiation, proliferation and tumor suppression.[21–23] Hence, it affects mRNA stability by inducing rapid message degradation, [24–27] leading to an alteration in protein expression level.[19,28–30]

To our knowledge, no one has studied the effect of *p21* gene polymorphisms on esophageal cancer risk in the high-risk area of northeastern Iran. To investigate the genetic susceptibility of ESCC in this region, we conducted a preliminary study with a case-control design to pursue the association of cigarette smoking, opium use, *p21* polymorphisms in codon 31 and the 3'UTR, and ESCC.

Materials and Methods

Subjects

Recruited subjects in this case-control study were selected from individuals referred to the Atrak Clinic between May 2006 and December 2007. The Atrak Clinic is considered as a referral center for upper GI disorders in Gonbad, the principle city in eastern Golestan. We estimate that approximately 95% of upper GI cancer patients in this region are referred to this clinic.[31] A total of 126 ESCC patients (108 female and 118 male; ages ranging from 32 – 89 years) who were diagnosed by upper GI endoscopy and confirmed histologically were considered as the case group. A total of 100 eligible controls that included healthy volunteers randomly selected from individuals who visited the Atrak Clinic for upper GI health examinations. They were diagnosed as healthy by physical examination and esophagoscopy, followed by histological confirmation. The controls had no previous history of any cancer, were not being treated for any diseases, and were genetically unrelated to the cases. There was no significant difference of age and sex between cases and controls. Both case and control groups included different ethnicities (Turkmen, Turk, Kurd, and Fars) and were restricted to people who had lived in Golestan Province for at least ten years, meaning that they shared the same geographic origin and culture. Using a standardized questionnaire, trained interviewers collected demographic characteristics and information about cigarette smoking and opium use from both cases and control groups. After discharge, all subjects were regularly followed at the Atrak Clinic once monthly for cases and every three months for controls. All eligible patients and control individuals signed an informed consent form according to institutional guidelines, and the study was approved by the Research Ethics Committee of Tehran University of Medical Sciences.

Specimens

Tumor tissue was obtained from 126 histologically confirmed ESCC patients. Tumors were histologically verified as ESCC and sub-typed as well differentiated, moderately differentiated or poorly differentiated. We also performed the same assays using blood samples and normal adjacent tissue to optimize our results. Blood samples and normal esophageal tissue were also taken from 100 healthy controls. All tissue specimens were obtained by endoscope. The biopsies were fixed in 70% alcohol and processed in paraffin blocks for histological examination.

Genotyping

Genomic DNA was extracted from esophageal tumor and normal tissues with the QIAamp DNA mini kit (QIAGEN, Canada) and from whole blood cells with the Flexi Gene DNA kit (QIAGEN, Canada), according to the manufacturers' instructions.

PCR-RFLP for p21 codon 31

The status of the *p21* C98A polymorphism in codon 31 was determined by PCR-RFLP. We performed the PCR analysis according to the TaqPCR Core kit (TaqPCR Core Kit, QIAGEN, Canada) manufacturer's instructions. The PCR mixture contained 10X concentrated Coralload PCR buffer, which contains Tris-HCl, KCl, $(NH4)_2SO_4$, 15 mM $MgCl_2$ gel loading reagent, orange dye and red dye (pH 8.7), 5X concentrated Q-Solution buffer, 10mM dNTP mix, RNase-free water, 1 pmol of each primer, 0.5 U of Taq DNA polymerase and template DNA. The amplification primers for the 272 bp region in exon 2 of the *P21* gene were: 5'-GTC AGA ACC GGC TGG GGA TG-3' (forward) and 5'-CTC CTC CCA ACT CAT CCC GG-3' (reverse).[32] The amplification conditions for codon 31 were: initial denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 30 seconds, 63.7°C for 30 seconds, 72°C for 40 seconds, and a final 5 minute extension step at 72°C. The 272 bp PCR-amplified fragment of *p21* exon 2 was subsequently digested with the Bpu1102I restriction enzyme (Fermentas Co., Canada). Digestion of the wild-type allele (Ser) created DNA fragments of 89 and 183 bp whereas the Arg allele, which lacks a Bpu1102I site, yielded the original 272 bp fragment. The restriction-digested DNA was subjected to electrophoresis on a 2% agarose gel and stained with ethidium bromide. All genotypes were confirmed by direct sequencing at the start of genotyping (Figure 1).

PCR- RFLP for the 3'UTR of p21

The PCR primers used for the amplification of a 298 bp region of the *p21* genomic 3'UTR were: 5'-CCCAGGGAAGGGTGTCCTG-3' (forward) and 5'-GGGCGGCCAGGGTATGTAC-3' (reverse). [30] Thermocycler parameters for this PCR were an initial cycle at 95°C for 5 minutes followed by 30 cycles at 95°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds, and a final 5 minute extension step at 72°C.The 298 bp fragment of *p21* exon 3 was digested with the PstI restriction

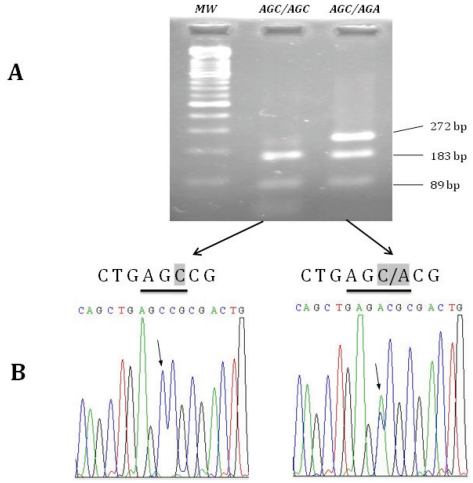

Figure 1. PCR assay and direct-sequencing to detect genetic polymorphism at p21 codon 31 (C98A). A) p21 codon 31 polymorphism, AGC-to-AGA change which resulted in an amino acid substitution from serine (AGC) to arginine (AGA). The Ser alleles with BPU1102I site generated two 89-bp and 183-bp fragments. A heterozygous form of AGC/AGA yielded three fragments (272, 183, and 89 bp). B) p21 codon 31 polymorphism was confirmed by direct sequencing.

enzyme (Fermentas Co., Canada). PstI digestion of the wild-type allele, with one intact PstI site, leads to two DNA fragments of 126 and 173 bp. The C to T polymorphism causes the loss of the PstI site, resulting in one uncut DNA fragment of 298 bp. The restriction-digested DNA was subjected to electrophoresis on a 2% agarose gel and stained with ethidium bromide. Direct sequencing was performed to confirm the genotypes (Figure 2).

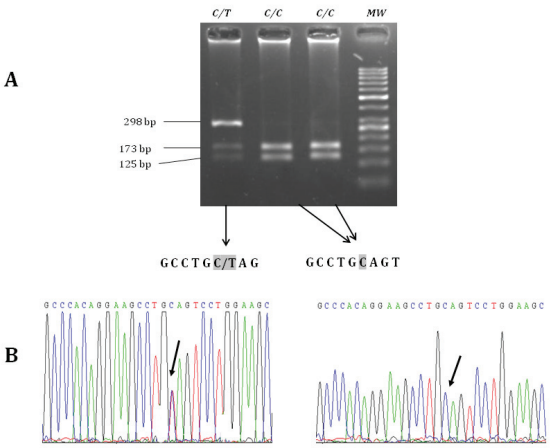

Figure 2. The PCR-RFLP analyses and direct sequence result of p21 C70T in 3'UTR. A) The genotype of p21 C70T was identified by a C allele with fragment length of 173 and 125 bp and a heterozygous form of C/T yielded three fragments (298, 173, and 125 bp). B) p21 C70T polymorphism was confirmed by direct sequencing.

Statistical analysis

The distribution of demographic characteristics and substance use was examined using Chi-square statistics and Fisher's exact test for dichotomous variables and Student's *t*-test for continuous variables. Unconditional logistic regression model has been used for confounder effect adjustment. Genotypes were analyzed for Hardy-Weinberg equilibrium. Associations between ESCC and polymorphisms were evaluated by calculating the OR and 95%CI. To evaluate the presence of gene-environment interaction we used ORs in a multiplicative interaction model and calculated the Synergy Index Multiplicative (SIM) as representative of the presence of synergistic multiplicative interaction when SIM is more than one. The Statistical Package for the Social Sciences software version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. A two-sided *P* value<0.05 was considered statistically significant.

Results

A total of 126 ESCC cases and 100 healthy controls were examined in this study. The mean age overall was 62.55±11 years, ranging from 32 to 89 years with a median of 62 years. The mean age of cases was 63.72±12 years with a median of 64 years and the mean age of controls was 61.08±10 years with a median of 61 years. There were no significant differences of age and gender between cases and controls. One hundred and eighteen subjects (52.21%) were men and 108 (47.79%) were women. The distribution of demographic variables, smoking status, and opium use for the cases and controls are summarized in Table 1.

Codon 31 and 3'UTR polymorphisms of the p21 gene and ESCC risk

No deviation from Hardy-Weinberg equilibrium was found for any of the observed loci for the *p21* C98A polymorphism in codon 31 and the *p21* C70T polymorphism. The frequencies of the wild type and variant genotypes for each of these SNPs were the same and equal to 78.57% for the wild type genotype and 21.43% for the variant genotype, respectively, among cases and 82% and 18%, respectively, among controls; after adjusting for some potential confounder variables that included smoking, opium use, and ethnicity, this difference was not statistically significant (*P*= 0.52) (Table 2). No A/A or T/T genotypes were observed in either polymorphism.

In addition, we analyzed the two polymorphisms of the *p21* gene, in combination. Four genotypic combinations emerged from the *p21* C98A and *p21* C70T polymorphisms. We then investigated the risk of ESCC for each group versus all other genotypes. The frequency of "double-wild-type" genotype (C/C

Table 1. Comparability of age, gender, cigarette smoking, opium use, and ethnicity in cases and controls*

Factor		Case *n*=126	Control *n*=100	χ^2	*P* [a]
Age (year)					
	<60	42 (33.3%)	39 (39.0%)	0.77	0.37
	≥60	84 (66.7%)	61 (61.0%)		
Gender					
	Male	70 (55.6%)	48 (48%)	1.27	0.25
	Female	56 (44.4%)	52 (52.0%)		
Smoking					
	Yes	33 (26.6%)	15 (15.2%)	4.28	0.03
	No	91 (73.4%)	84 (84.8%)		
Opium use					
	Yes	45 (36.3%)	24 (24.2%)	3.74	0.05
	No	79 (63.7%)	75 (75.8%)		
Ethnicity					
	Turkmen	64 (51.6%)	48 (52.7%)	0.02	0.86
	Non-Turkmen	60 (48.4%)	43 (47.3%)		

[a]The differences between cases and controls were evaluated by two-sided chi square for discontinuous variables; *There were no data available for some study subjects for some variables

Table 2. P21 genotype and allele frequencies of cases and controls and their association with ESCC risk.

	P21 C98A polymorphism			Adjusted [a] OR (95% CI)	P21 C70Tpolymorphism			Adjusted [a] OR (95%CI)
	C/C	C/A	A allele frequency		C/C	C/T	T allele frequency	
Cases [*n* (%)]	99 (78.6%)	27 (21.4%)	0.107	1.24 (0.63–2.41)	99 (78.6%)	27 (21.4%)	0.107	1.24 (0.63–2.41)
Controls [*n* (%)]	82 (82%)	18 (18%)	0.09		82 (82%)	18 (18%)	0.09	
Total	181	45			181	45		

[a] The OR was adjusted for covariates (ethnicity, smoking, and opium use)

for both) was 98 (77.78%) in cases and 82 (82%) in controls. The frequency of "C98A variant, C70T variant" genotype (C/A-C/T) in cases and controls was 28 (22.23%) and 18 (18%), respectively. There was neither the "C98A wild-type, C70T variant" genotype (C/C-C/T) nor the "C70T wild-type, C98A variant" genotype (C/C-C/A) in either cases or controls. There were no statistically significant differences between combination groups.

In addition, the data were subsequently stratified into subgroups based on smoking status, opium use, and ethnicity. We evaluated the ESCC risk in each subgroup by estimating the ORs associated with the p21 C98A or C70T polymorphisms. There were no significant differences in any subgroups between individual genotypes or their combination.

Interaction between cigarette smoking and C98A (C/A) or C70T (C/T) genotypes

A synergistic interaction between cigarette smoking and the *p21* C98A polymorphism was observed in the recruited subjects (Table 3). Also, the p21 C70T polymorphism showed the same interaction with cigarette smoking. We also observed a complete linkage between two polymorphisms of *p21*, with a perfect LD of 1.0 (D-prime=1; *P*=0.000).

Discussion

This is the first study to investigate the effect of both polymorphisms of p21 gene in the codon 31 and in the 3'UTR on increased risk of ESCC in northeastern Iran. However, no association was detected between these two polymorphisms and ESCC development. Furthermore, we have found cigarette smoking not only is considered as a risk factor for ESCC (*P*=0.03, OR=2.03; 95%CI: 1.03 – 4.00), but it also interacts with *p21* polymorphisms in susceptibility to ESCC (*P*=0.02, OR=8.38; 95%CI: 1.03 – 67.93).

A previous study in Golestan Province also showed that tobacco use was associated with a higher risk of developing ESCC (OR, 95%CI: 1.70, 1.05 – 2.73).[12]

Given that cigarette smoking increases the risk of ESCC, it is plausible that some mutagenic com-

Table 3. Interaction of smoking and p21 C98A genotype.

Smoking	P21C98A	Cases	Controls	OR	OR95%CI	χ^2	*P*
No	No	73	68	1.00	—	—	—
Yes	No	24	14	1.6	3.34–0.76	1.56	0.21
No	Yes	18	16	1.05	2.22–0.49	0.01	0.90
Yes	Yes	9	1	8.38	67.93–1.03	5.50	0.02

SIM=8.38/(1.6×1.05)=4.99

pounds such as polycyclic aromatic hydrocarbons (PAH), which are important carcinogenic components of tobacco smoke, would influence the ESCC carcinogenesis in individuals with genetic alterations.[33]

On the other hand, different genetic backgrounds not only may affect individuals' susceptibility to cancer, but may also modify the effects of environmental carcinogens.[34–41] It is possible that the variant forms of these two *p21* polymorphisms are associated with determinants of ESCC in response to certain environmental factors, including tobacco smoking. This polymorphism may consequently be a candidate genetic marker for screening ESCC risk in association with exposure to particular environmental carcinogens.

Regarding the *p21* C98A polymorphism, we observed that the frequency of the A allele was higher in ESCC cancer patients than the control group; however, it was not statistically significant. This observation is consistent with the recent studies by Shih et al. and Lai et al. in the Taiwanese and Su et al. in the Caucasian population, in which no associations were detected between the polymorphism at codon 31 in the *p21* gene and the risk of developing several types of cancer.[42–44] However, other studies have actually reported an association of the Ser (C) allele with the risk of some specific types of cancer, including esophageal cancer.[28,32,45–48] This conflict in results might be attributed to one of several possible reasons, including: non-random sampling, limited sample size, and different molecular mechanisms in carcinogenesis or ethnic disparity. The frequency of the Arg allele at codon 31 is significantly different between Caucasian (0.063 – 0.074)[19,47] and Asian (0.408 – 0.571) populations.[28,32,44,45] Our results showed no allelic differences in the Arg allele of codon 31 between Turkmen and non-Turkmen ethnic groups, which is consistent with a recent study in this region that detected no significant differences between ethnic subgroups in the frequencies of several genetic polymorphisms associated with esophageal cancer.[49] Furthermore, another study suggested that ESCC risk was not much higher in Turkmen compared to non-Turkmen in Golestan Province.[8] All of the findings from this region thus argue against the effect of ethnicity as a predominant ethnological factor.

Regarding the *p21* C70T polymorphism, our results are in agreement with a report by Facher et al. that showed no significant association between this polymorphism and squamous cell carcinoma of the head and neck,[30] though this association was indeed detected in a different study.[48] Our findings demonstrated, despite a higher rate of polymorphic allele frequency (T allele) in ESCC cases, that there was not a significant association between the *p21* C70T polymorphism and ESCC risk.

Conclusion

This preliminary data suggested that (a) the *p21* polymorphisms C98A and C70T were not associated with development of ESCC in northeastern Iran, and that (b) gene-environment interaction analysis showed that cigarette smoking may have synergistic interactions with the *P21 C/A* and *p21* C/T genotype in ESCC carcinogenesis in this region. Although we could not detect any association between *p21* gene polymorphisms in the codon 31 or 3'UTR regions, we could not reject the modest effect of these polymorphisms on ESCC development in the studied population due to limited sample size. Since this preliminary study is the first report on ESCC risk in northeastern Iran relative to *p21* polymorphisms, further studies with larger sample sizes and modified designs in diverse Iranian ethnic populations are needed to confirm our findings.

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