RESEARCH ARTICLE

Intronic Polymorphisms of the SMAD7 Gene in Association with Colorectal Cancer

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Abstract

Based on genome-wide association studies (GWAS) a linkage between several variants such as single nucleotide polymorphisms (SNPs) in intron 3 of *SMAD7* (mothers against decapentaplegic homolog7) were, rs12953717, rs4464148 and rs4939827 has been noted for susceptibility to colorectal cancer (CRC). In this study we investigated the relationship of rs12953717 and rs4464148 with risk of CRC among 487 Iranian individuals based on a case-control study. Genotyping of SNPs was performed by PCR-RFLP and for confirming the outcomes, 10% of genotyping cases were sequenced with RFLP. Comparing the case and control group, we have found significant association between the rs4464148 SNP and lower risk of CRC. The AG genotype showed decreased risk with and odds ratio of 0.635 (adjusted OR=0.635, 95% CI: 0.417-0.967, p=0.034). There was no significant difference in the distribution of *SMAD7* gene rs12953717 TT genotype between two groups of the population evaluated (adjusted OR=1.604, 95% CI: 0.978-2.633, p=0.061). On the other hand, rs12953717 T allele showed a statistically significant association with CRC risk (adjusted OR=1.339, 95% CI: 1.017-1.764, p=0.037). In conclusion, we found a significant association between CRC risk and the rs4464148 AG genotype. Furthermore, the rs12953717 T allele may act as a risk factor. This association may be caused by alternative splicing of pre mRNA. Although we observed a strong association with rs4464148 GG genotype in affected women, we did not detect the same association in CRC male patients.

Keywords: *SMAD7* - single nucleotide polymorphism - cancer - *TGF-β* - gender variation

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Introduction

Cancer is one of the most complex diseases caused by complicated interactions such as signaling pathways that regulate tumor initiation , invasion, and metastasis (Zhang et al., 2013). It is quite renowned that transforming growth factor-β (*TGF-β*) is one of the most important signaling pathways that could play a role as a tumor suppressor in the early stages of tumorigenesis and its influence has been shown in some variants Such as polymorphisms in *SMAD2,4,7* in colorectal cancer (Massague, 2008; Thompson et al., 2009)

Based on genome-wide association studies (GWAS) a linkage between several loci and the susceptibility to colorectal cancer such as various variants within *SMAD7* has been identified (Massague, 2008). *SMAD7* is an inhibitory *SMAD*, and due to its role as a negative regulator of the *TGF-β* signaling pathway; it elevates the anti-inflammatory effects of *TGF-β* pathway (Monteleone et al., 2008). Thus the act of *SMAD7* could remarkably decrease the *TGF-β* signaling and results in increasing the risk of cancer (Li et al., 2011; Akbari et al., 2014). Molecular biology and studying the signaling pathways are now going to make an evolutionary challenge through presenting the new generation of medicine (Ginsburg and McCarthy, 2001).

The molecular variations associated with specific diseases may lead to give the medicine a new look as a preventive medicine, that not only is limited to different populations but also is specialized for individuals. Moreover, the development of technology and specially the individualized genome characterization is leading to have the personalized medicine in the near future. Single nucleotide polymorphisms (SNPs) have revealed as significant markers for predicting susceptibility to serious diseases like cancer and a road to get to personalized medicine (Mias and Snyder, 2013). Since colorectal cancer is one of the leading causes of death in the world and there are so many findings regarding the influence of single polymorphisms on alternation the risk of CRC, we have carried out our research on it (Azimzadeh et al., 2011; Azimzadeh et al., 2012; Milanizadeh et al., 2013; Khorshidi et al., 2014).

Among the most remarkable related SNPs in intron 3

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of *SMAD7* which revealed an association with colorectal cancer and have identified by GWAS were, rs12953717, rs4464148 and rs4939827 (Tomlinson et al., 2008). Considering the importance of mentioned SNPs, the aim of our study was to recognize the relationships between *SMAD7* SNPs (rs12953717, rs4464148) and colorectal cancer in an Iranian population based on a control-case project.

Materials and Methods

A total of 487 Iranian individuals included in this case-control study. The case group consisted of 234 patients with a sporadic CRC diagnosis confirmed with positive colonoscopy and pathology result, who were referred to Taleghani Hospital, Tehran, Iran. The control group included 253 healthy control were required to be free of inflammatory of bowel disease, adenomatous polyps and without family history of colorectal cancer. All controls had eligible colonoscopy results with no malignant tumors. The study was admissible by ethics committee of the RCGLD, Shahid Beheshti University of Medical Sciences and all subjects gave informed consent to the study.

Genomic DNA was extracted from 5 mL of peripheral blood drawn from study participants using a standard salting out method (Miller et al., 1988). Genotyping of mentioned two *SMAD7* SNPs (rs12953717, rs4464148) were performed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method. The sequences of PCR primers and characteristics of restriction enzymes used for RFLP are summarized in Table 1. To confirm the results, 10% of genotyping result with RFLP were sequenced by genetic analyzer 3130xl (Applied Biosystems, USA).

The chi-square test was used for comparison of the distribution of the allele and genotype frequencies test. The Hardy-Weinberg equilibrium was determined by the chi-square test to compare the observed genotype frequencies among studied cases and controls with the expected genotype frequencies. The Student's t test was accomplished to testing the statistical comparison of age and body mass index (BMI) between case and control groups. Logistic regression method was applied to compute 95% confidence intervals (CIs) and odds ratios (ORs) and to adjust the data for confounding factors, such as age and gender. P-values less than 0.05 were considered significant. Logistic regression on the general mode was used to construct receiver-operating characteristic (ROC) curves and calculate the areas under the curve (AUCs). We assessed classification performance of clinical SNPs risk, age+gender and SNPs×age+gender using the ROC curve. For all the statistical analysis we used the SPSS

Results

Originally, we genotyped 487 individual CRC cases and controls. 108 (46.2%) males and 126 (53.8%) females of total 234 individual CRC cases and 152 (60.1%) male and 101 (39.9%) females of 253 control group non-cancer fellow included in this study. In case group, 189 (74.7%) individual demonstrated tumors in the colon and the rest of 64 (25.3%) cases in the rectum. The mean age and gender among CRC cases and controls group had significant differences ($p<0.05$), while the Smoking and BMI showed no significant differences between case and control groups when we investigated them with Statistical analysis ($p>0.05$). To adjust the data according to probable confounding variables such as age and gender, we exploited the logistic regression method. There was no significant deviation in control group genotype distribution from the Hardy-Weinberg equilibrium.

When we compared the group of CRC patients and the healthy control group in this investigation (Table 2), we have found significant association between the rs4464148 SNP and lower risks for CRC. The AG genotype showed decreased risk for colorectal cancer with odds ratio 0.635 (adjusted OR=0.635, 95% CI: 0.417-0.967, p=0.034). There was no significant difference in the distribution of *SMAD7* gene rs12953717 TT genotype between two groups of the population evaluated (adjusted OR=1.604, 95% CI: 0.978-2.633, p=0.061). On the other hand, rs12953717 T allele shows statistically significant association with CRC risk (adjusted OR=1.339, 95% CI: 1.017-1.764, p=0.037).

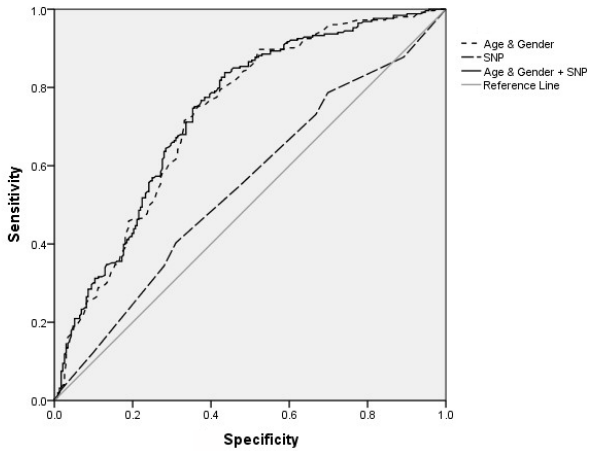

Figure 1. ROC Curves for Age+Gender×SNPs Risk, Age+Gender and SNPs Risk

Table 1. Information for SMAD7 SNPs that Included in this Study

SNPs	Location	Primer Sequence	PCR Product Size	Restriction Enzyme	RFLP Fragment Size
rs12953717	(C/T)	Forward Primer : 5'-GGGTGCCACAGGGTCTCC-3'	297bp	BbvI	C: 297
		Reverse Primer : 5'-GTGCAGCACTCTCCACAAGC-3'			T: 35 + 146 + 114 + 2
rs4464148	(A/G)	Forward Primer : 5'-GGAGGAGCGAGAAGAGTAAT-3'	246bp	MaeII (TaiI)	A: 246
		Reverse Primer : 5'CTGGGGGATGAAGGAAAT-3'			G: 154 + 92

Table 2. Comparison of CRC Patients and Healthy Control Samples of Allele and Genotype Distribution and Assortments Analysis of Gender Group for Two Studied Polymorphisms

SNP	Total				Male				Female			
Variable	Control	Case	*OR (95% CI)	p-value	Control	Case	OR (95% CI)	p-value	Control	Case	OR (95% CI)	p-value
rs12953717												
Genotypes n (%)												
CC	68 (26.9)	78 (33.3)	1.00 (Reference)		38 (25.0)	38 (35.2)	1.00 (Reference)		30 (29.7)	40 (31.7)	1.00 (Reference)	
CT	97 (38.3)	90 (38.5)	1.377 (0.857-2.212)	0.186	63 (41.4)	40 (37.0)	1.610 (0.840-3087)	0.151	34 (33.7)	50 (39.7)	1.139 (0.569-2.283)	0.713
TT	88 (34.8)	66 (28.2)	1.604 (0.978-2.633)	0.061	51 (33.6)	30 (27.8)	1.701 (0.854-3.388)	0.131	37 (36.6)	36 (28.6)	1.496 (0.736-3.040)	0.266
Alleles n (%)												
C	233 (46.0)	246 (52.6)	1.00 (Reference)		139 (45.7)	116 (53.7)	1.00 (Reference)		94 (46.5)	130 (51.6)	1.00 (Reference)	
T	273 (54.0)	222 (47.4)	1.339 (1.017-1.764)	0.037	165 (54.3)	100 (46.3)	1.380 (0.944-2.017)	0.097	108 (53.5)	122 (48.4)	1.293 (0.867-1.928)	0.207
rs4464148												
Genotypes n (%)												
AA	138 (54.5)	113 (48.3)	1.00 (Reference)		86 (56.6)	48 (44.4)	1.00 (Reference)		52 (51.5)	65 (51.6)	1.00 (Reference)	
AG	78 (30.8)	101 (43.2)	0.635 (0.417-0.967)	0.034	49 (32.2)	49 (54.4)	0.563 (0.317-1.001)	0.050	29 (28.7)	52 (41.3)	0.725 (0.388-1.356)	0.314
GG	37 (14.6)	20 (8.5)	1.719 (0.896-3.299)	0.103	17 (11.2)	11 (10.2)	0.785 (0.315-1.956)	0.604	20 (19.8)	9 (7.1)	3.499 (1.382-8.859)	0.008
Alleles n (%)												
A	354 (70.0)	327 (69.9)	1.00 (Reference)		221 (72.7)	145 (67.1)	1.00 (Reference)		133 (65.8)	182 (72.2)	1.00 (Reference)	
G	152 (30.0)	141 (30.1)	1.046 (0.776-1.409)	0.768	83 (27.3)	71 (32.9)	0.742 (0.490-1.122)	0.157	69 (34.2)	70 (27.8)	1.506 (0.981-2.314)	0.061

*Adjusted for age as a confounder variable

We analyzed genotyping and allele distribution data according to gender status and observed a substantial result, all data were summarized in Table 2. In women, the rs4464148 GG genotype was statistically associated with an increased risk of colon cancer (adjusted OR=3.499, 95% CI: 1.382-8.859, p=0.008). For men p-value of the rs4464148 AG genotype was 0.050. This stratified analysis has revealed no association between gender difference and rs12953717.

To assess the ability of age+gender×SNPs risk score to better classify colorectal cancer susceptibility, ROC curve analysis was used (Figure 1). We observed an AUC of 0.548 (95% CI=0.496-0.599) for the SNPs risk alone, compared with AUC of 0.729 (95% CI=0.684-0.774) for age+gender and AUC of 0.737 (95% CI=0.693-0.782) for the combination of age+gender×SNPs risk.

Discussion

According to the surveys conducted on the role of *TGF-β* in cancer, reduction in *TGF-β* signaling, increases the risk for cancer. It has also proven that *SMAD7* has an important regulatory role in the *TGF-β* signaling pathway. *SMAD7* is an intracellular *TGF-β* receptor hostile, by inhibiting the *TGF-β* signaling pathway (Massague, 2008; Monteleone et al., 2008). Moreover, *SMAD7* have significant increase in colorectal cancer (Pittman et al., 2009). Patients with CRC have high level expression of SMD7 mRNA in their CRC cell lines (Broderick et al., 2007). In recent years, numerous studies have investigated several genetic variations within *SMAD7*. Two of the most important intronic SNPs in *SMAD7* (on 18q21) that has been revealed to have significant effects on regulation of *TGF-β* in CRC patients are rs12953717 and rs4464148 (Broderick et al., 2007; Tomlinson et al., 2008; Pittman et al., 2009). In 2007, a Genome-wide association study by Broderick et al. (2007) demonstrated that the *SMAD7* intron 3 SNPs had high level association with polymorphic sites defined by rs12953717, rs4464148 and rs4939827. In addition, it has depicted that they map to the block of linkage disequilibrium (LD) of intron 3.

Here, we conducted a case-control study for *SMAD7* SNPs rs12953717 and rs4464148 to describe feasible association between those two SNPs and increment in the risk of CRC among Iranian population. We have found statistical differences between the distributions of rs4464148 AG genotype in the CRC patients and healthy controls. It is consistent with several surveys around the world emphasized on the effect of rs4464148 in the CRC risk (Broderick et al., 2007; Tomlinson et al., 2008; Pittman et al., 2009; Loh et al., 2011; Dai et al., 2012; Hoskins et al., 2012). On the other hand, Slattery M.L., et al. couldn't find any significant association between rs4464148 and CRC susceptibility even when laminated them by age, tumor site or family history (Slattery et al., 2010). Furthermore, in two distinguished researches that have been investigated in Asia, same results have been accomplished (Ho et al., 2011; Li et al., 2011).

In 2009 Thompson, et al. reported strong association between rs4464148 GG genotype and colon cancer that was limited to women as well as Our investigation revealed the same results when we performed gender-specific analysis, rs4464148 GG genotype showed strong association with CRC in women, however the results in men group was in contrast with the mentioned findings.

To the best of our knowledge, several studies corroborated the role of rs12953717 polymorphism in cancer susceptibility (Broderick et al., 2007; Tomlinson et al., 2008; Curtin et al., 2009; Middeldorp et al., 2009; Pittman et al., 2009; Slattery et al., 2010; Ho et al., 2011; Li et al., 2011). According to previous cancer-susceptibility-studies which mainly revealed the remarkable association between the *SMAD7* SNP and the risk of CRC, our study demonstrated that allele T at rs12953717 can increase the risk of CRC. Our results was in consistence with a meta-analysis study of Zhang H, et al. in 2013 performed a study to review 14 case-control survey that previously presented. They have found a considerable association between *SMAD7* rs12953717 and cancer risk. Among all caner types that have been studied, colorectal cancer was the strongest associated type, while the molecular epidemiological investigation has conflicting results about rs12953717 in cancer risk (Zhang et al., 2013).

Although, only Wijnen, et al. in 2009 performed a study regarding colorectal cancer risk in patients with Lynch Syndrome (LS), which have not found any

significant relation between rs12953717 and CRC risk. It has suggested that perhaps rs12953717 is involved in the TGFbetaRII signaling and the *TGFbeta RII* pathway is usually disrupted in LS cancers.

In conclusion, we found a significant association between CRC risk and rs4464148 AG genotype. Furthermore, rs12953717 T allele may act as a risk factor. Although we observed a strong association with rs4464148 GG genotype in affected women, we didn't detect the same association in CRC male patients. These associations may cause by alternative splicing of pre mRNA mainly in two specific modes including retained intron and exon extended. So that targeted SNPs in intronic regions could be a reason for susceptibility to diseases such as colorectal cancer (Modrek and Lee, 2002; Najjar Sadeghi et al., 2013; Watson, 2013). In order to get a precise evaluation of *SMAD7* polymorphisms and risk of CRC there is a need of enlarging the sample size. At the end we suggest that future study in functional activity of these polymorphisms and their effects on tumorigenesis would help us to understand the underlying mechanisms in CRC development.

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