Original Article

rs12904 polymorphism in the 3'-untranslated region of ephrin A1 ligand and the risk of sporadic colorectal cancer in the Iranian population

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ABSTRACT

Background: Colorectal cancer (CRC) is rated as the second cause of cancer death. Genetic determinants are considered as driving forces in the development of sporadic CRC. Single-nucleotide polymorphisms (SNPs), due to their abundance in the human genome with collectively huge effect on cellular signaling pathways, are attributed as the main genetic factor in disease susceptibility including cancers. MicroRNAs are contributing to posttranslational gene regulation. They exert their regulatory function by binding to their specific recognition sequences located at 3'-untranslated region (UTR) of mRNAs. In the present study, we have elucidated the role of rs12904, a naturally occurring SNP, in the recognition site of miR200c in the 3'UTR of ephrin A1 ligand gene, in the development of sporadic CRC in the Iranian population.

Materials and Methods: A case–control study using 152 CRC patients and 160 noncancerous counterparts was conducted to determine the rs12904 genotypes using polymerase chain reaction–restriction fragment length polymorphism method.

Results: The results revealed no significant association between the rs12904 and sporadic CRC (odds ratio = 0.97, 95% confidence interval = 0.70–1.34). The frequency of genotypes and also alleles of the mentioned polymorphism were not significantly different between case and control groups ($P = 0.765$ and $P = 0.847$, respectively).

Conclusion: The results suggest that this polymorphism probably has not a crucial role in the Iranian CRC risk and is not an important potential risk factor in molecular diagnostics of mentioned disease among the Iranian population.

KEY WORDS: Ephrin A1 ligand gene, Iran, single-nucleotide polymorphism, sporadic colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is the third most diagnosed cancer type in the world.[1] Colon cancer ranks as the fourth most common type of cancer in Iran, but its incidence rate is increasing over the last decades.[2] Environmental risk factors such as gender, age, body mass index (BMI), physical activity, smoking status, and nonsteroidal anti-inflammatory drugs (NSAIDs) consumption as well as genetic variants in related genes are huge contributors in the etiology of CRC and therefore could contribute to the CRC risk and development.[3-5] Here, we specifically focus on functional variants in microRNA (miRNA) binding sites that can modulate cancer risk. miRNAs are evolved as short-conserved noncoding RNAs (≈17–20 bp) with posttranscriptional gene regulation activity through binding to complementary sites at 3'-untranslated region (UTR) of target mRNAs. Given the fundamental role of miRNAs in multiple cellular processes such as cell proliferation, differentiation, and apoptosis, polymorphisms in miRNAs binding sites could result in altered susceptibility to cancer possibly by modifying the ability of miRNA binding and change the level of target mRNA consequently.[6] One of such functional polymorphisms is rs12904 in 3'UTR of ephrin A1 ligand (EFNA1) gene, which could effect on miR-200c binding capacity.[7] EFNA1 is a glycosylphosphatidylinositol-linkage ligand with 205 amino acids that binds to the receptor tyrosine

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kinase EphA2 at sites, where cell–cell contact occurs. Such sites leading to contact-dependent bilateral signaling and play an important role in tumor neovascularization and progression.[8] EFNA1 and its receptor EphA2 regulate integrin-mediated adhesion, migration, proliferation, and differentiation of cells and are considered as major mediators in the development and maintenance of many different types of tumors.[9] They are involved in multiple oncogenesis signaling pathways such as MAP/ERK and PI3K and could influence both tumor initiation and progression.[10,11] EFNA1 overexpression is significantly associated with TNM stages and lymph node metastasis in human gastric adenocarcinoma.[12] Furthermore, EFNA1 overexpression was seen in 57% of gas chromatography (GC) tissue samples and could play an important role in GC susceptibility.[8]

EFNA1 and its receptor have also known as angiogenesis factors[13] and seem to be expressed by human breast cancer cells and human Kaposi's sarcoma cells during tumor neovascularization.[14]

EFNA1 knockdown declines tumor-induced endothelial cell migration *in vitro* and its overexpression accelerates the endothelial cell migration toward tumor cells *in vivo*. Luciferase assays showed that rs12904 G > A could result in altered regulation of EFNA1 by miR-200c in gastric cancer cell lines and EFNA1 expression was significantly higher for rs12904 AA genotype than for AG or GG genotype. GA variation of seed site belongs to miR-200c in 3'UTR of EFNA1 disrupt the binding capacity of miRNA and resulted in constitute expression of EFNA1.[7] Its elevated level has been observed in many cancers such as gastric,[8] bladder,[15] hepatocellular carcinoma,[16] and more recently prostate cancers.[17] In an investigation of human colon cancer carried out by Kataoka *et al*., it has been revealed that CRC expresses EPHA2 and EFNA1 significantly, more abundantly than normal tissue, and it also associated with the tumor clinicopathological features.[18] A distinct study reported that mRNA expression of EFNA1 augmented from rectal adenoma to carcinoma.[19] Western blot analysis displayed that most CRC cell lines expressed abundant EFNA1 and its overexpression involved in invasion, migration, and proliferation *in vitro*.[20] Thus, EFNA1 overexpression could be regarded as a malignant factor and could induce the risk of tumorigenesis; however, the expression pattern of ephrin A1 in various tumors does not seem to be the same. In fact, EFNA1 exhibits low expression levels in both GBM and breast cancer cells.[21,22] These findings, therefore, suggest a potential feedback loop mechanism between EFNA1 and its receptor, EphA2.

Considering the important role of EFNA1 in carcinogenesis, it is not surprising that genetic variants, especially polymorphisms on 3'UTR of EFNA1, as mentioned could interfere with expression pattern, downstream pathways, and individual susceptibility to CRC consequently.

Based on these data, we intend to evaluate the possible association between rs12904 polymorphism in the 3'UTR of EFNA1 with CRC risk in the Iranian population for the first time and its interaction with established risk factors.

MATERIALS AND METHODS

Study population

CRC patients were recruited from the Colonoscopy Unit of Al-Zahra Hospital and CRC center of Seyed-Al-Shohada Hospital between mid-2013 and mid-2015. Eligible cases were incident and histologically confirmed CRC. Healthy controls, which had negative colonoscopy result, with no previous history of cancer were recruited in parallel. A total of 152 cases and 160 controls were included in the current study. This study was approved by the University Ethics Committee and all participants provided the written informed consent. The study participants were interviewed and data on gender, age, smoking status, NSAID usage, physical activity, and other factors including family history of cancer were obtained using a short questionnaire.

Genotyping of polymorphism

Approximately, 5 ml of blood sample was collected into EDTA anticoagulant tubes and stored at − 70°C for DNA isolation. DNA was extracted using DNA isolation kit (GeNet Bio). The DNA purity and concentration were assessed by agarose gel electrophoresis and spectroscopy. A genomic DNA fragment was amplified by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) methods to determine EFNA1 genotypes. The 25-mL PCR reaction system contained 2/5 mL ×10 PCR buffer, 1 mM MgCl, 200 μM dNTPs, 0.2 μM each primer, 100 ng of genomic DNA, and 1 U of Taq DNA polymerase. Thermal cycling included an initial denaturation of 94°C for 5 min, and then 34 cycles including 94°C for 30 s, 62.6°C for 30 s, and 72°C for 30 s after these cycles, the final extension of 72°C for 5 min. The PCR products were digested with SspI endonucleases overnight and were analyzed in 2% polyacrylamide gels. Primer sequence, characteristic of PCR product, and digestion reaction are shown in Table 1. Finally, randomly selected samples have been sent for direct sequencing for confirmed obtaining results.

Statistics

Hardy–Weinberg equilibrium was tested among cases and controls for genotype frequencies using Chi-square test. Demographic and lifestyle characteristic distribution such as gender, smoking status, and NSAIDs consumption, assessed by Pearson Chi-square test and continuous variables including age and BMI, was compared with Student's *t*-test between cases and controls. Associations between EFNA1 (rs12904) polymorphism with susceptibility to sporadic CRC were examined by logistic regression analysis. Significance of the association was determined using odds ratios (ORs) and 95% confidence intervals (CIs). The significance level was set at $P < 0.05$. The SPSS 22 (IBM, Armonk, NY: IBM Corp) was used for statistical analyses.

Table 1: Primer sequence and characteristic of polymerase chain reaction product and digestion reaction

SNP ID	Primer sequence	PCR product length (bp)	Restriction enzyme (Ta°C)	RFLP fragment size (bp)
rs12904	F: ACAGGCTGAAGAGAGGGACA R: AACTTCTCTGTGGCAGCTCC	494	*SspI* (37°C)	GG: 494 AA: 124+369 AG: 494+369+124

PCR=Polymerase chain reaction, RFLP=Restriction fragment length polymorphism, SNP=Single-nucleotide polymorphism

RESULTS

Demographic and lifestyle characteristics

In our study, we investigated a total of 152 patients (77 males and 75 females with mean age of 57.29 ± 12.54) and 160 control (61 males and 99 females with mean age of 47.37 ± 14.52) for EFNA1 (rs12904) polymorphisms. The distributions of selected characteristics of the cases and controls are presented in Table 2. There were no statistically significant differences between patients and controls in terms of BMI and smoking status ($P = 0.162$ and $P = 0.509$, respectively). However, adjusted data (by age and gender) for physical activity demonstrated that there is a significant difference between case and control groups ($P < 0.001$). Relatively, more aspirin or NSAID consumers (23.1% vs. 10.5%, $P = 0.003$) were found among controls compared to sporadic CRC cases.

Table 2: Baseline characteristics of colorectal cancer patients and controls in the study

	Controls (*n*=160)	Cases (*n*=152)	*P*
Age (mean±SD)	47.37±14.52	57.29±12.54	<0.001*
Gender, *n* (%)			
Male	61 (38.1)	77 (50.7)	0.026*
Female	99 (61.9)	75 (49.3)	
Smoking, *n* (%)			
Yes	24 (15)	27 (17.8)	0.509
No	136 (85)	125 (82.2)	
NSAIDs, *n* (%)			
Irregular	123 (76.9)	136 (89.5)	0.003*
Regular	37 (23.1)	16 (10.5)	
Physical activity, *n* (%)			
Very low	3 (1.9)	48 (31.6)	<0.001*
Low	91 (56.9)	63 (41.1)	
Moderate	39 (24.4)	35 (23.0)	
High	27 (16.9)	6 (3.9)	
BMI (mean±SD) kg/m^2	25.37±3.9	26±3.9	0.162

*P<0.05. SD=Standard deviation, NSAIDs=Nonsteroidal anti-inflammatory drugs, BMI=Body mass index

Genotype and allele distribution

The genotype frequencies in both groups were in agreement with those predicted by Hardy–Weinberg equilibrium. In this evaluation, when comparing genotype and allele frequencies of the EFNA1 polymorphism between the two groups, no significant differences were observed ($P = 0.765$ and $P = 0.847$, respectively). The frequency of the variant allele was slightly higher in the affected population (33.2%), as compared to controls (32.5%); however, this difference was not statistically significant [Table 3]. The frequency of the rs12904 genotypes, GG, AG, and AA were 45.4%, 42.8%, and 11.8% in the case group and 48.1%, 38.8%, and 13.1% in the control group, respectively. When we compared combined genotype AG + GG/AA as variant genotype, the AG + GG genotype had no risk or protective effect in relation to sporadic CRC ($P = 0.46$) [Table 4]. We further conducted stratification analysis of EFNA1 genotypes and risk of CRC. As shown in Table 4, the risk of CRC associated with the rs12904 AA variant and combined genotypes (rs12904 AG + GG) did not differ in patients stratified by age, drug usage, BMI, and smoking status ($P > 0.05$ for all subgroups). However, the relation of rs12904 genotypes with CRC risk was observed only in never smokers ($P < 0.047$); in this group, the combined AG + GG genotype acts as a risk genotype (OR = 2.09, 95% CI = 0.87–5.03, $P = 0.047$).

Table 3: Association between genotypes and allele frequency with colorectal cancer risk

	Case, *n* (%)	Control, *n* (%)	*P**	OR$^+$ (95% CI)$^\#$
Genotype frequency				
GG	69 (45.4)	77 (48.1)	<0.765	0.97 (0.70-0.34)
AG	65 (42.8)	62 (38.8)		
AA	18 (11.8)	21 (13.1)		
Allele frequency				
G	203 (66.8)	216 (67.5)	<0.847	1.03 (0.74-1.44)
A	101 (33.2)	104 (32.5)		

*P<0.05. $^+$OR=Odds ratio, $^\#$CI=Confidence interval

DISCUSSION

Examination of functional variants in some of the loci of genome, especially polymorphisms in 3'UTR of mRNAs, could provide a powerful tool to interpret the possible connections between genotype and environmental risk factors in complex diseases such as cancer.[23] EPHA2 and its major ligand EFNA1 implicated with carcinogenesis and angiogenesis in various cancers, including colon cancer,[20] gastric cancer,[8] breast cancer,[24] glioma,[25] urinary bladder carcinoma,[15] hepatocellular carcinoma,[16] and malignant mesothelioma.[26] Overexpression of EFNA1 has been reported in colon cancer compared to adjacent normal tissues and different colon cancer cell lines. rs12904 in 3'UTR of EFNA1 may have a potential impact on regulation of expression by miR-200c. G⟶A variation disrupts the binding ability of miR-200c and resulted in increased expression of EFNA1. As well in gastric cancer cell line, EFNA1 expression was significantly higher for rs12904 AA genotype than for AG or GG genotype. It could be realized that individuals carrying the A allele have a high risk of developing cancer.[7] Since the incidence of CRC in Iran is on the steady rise and based on the critical role of EFNA1 in colon cancer susceptibility,

Table 4: Association of age, nonsteroidal anti-inflammatory drugs consumption, cigarette smoking, and high body mass index with genotype

Group	Genotype		*P*	OR (95% CI)
	AA (%)	AG + GG (%)		
Overall				
Case	18 (46.2)	134 (49.1)	0.46	1.31 (0.64-2.7)
Control	21 (53.8)	139 (50.9)		
Age <55				
Case	6 (33.3)	59 (38.1)	0.69	1.23 (0.44-3.45)
Control	12 (66.7)	96 (61.9)		
Age ≥55				
Case	12 (57.1)	75 (63.6)	0.58	1.3 (0.51-3.36)
Control	9 (42.9)	43 (36.4)		
Irregular NSAIDs consuming				
Case	15 (53.6)	121 (52.4)	0.90	0.95 (0.43-2.09)
Control	13 (46.4)	111 (47.6)		
Regular NSAIDs consuming				
Case	3 (27.3)	13 (31)	0.81	1.19 (0.27-5.25)
Control	8 (72.7)	29 (69)		
Smoker				
Case	10 (71.4)	17 (45.9)	0.10	0.34 (0.09-1.28)
Control	4 (28.6)	20 (54.1)		
Nonsmoker				
Case	8 (32)	117 (49.6)	0.047*	2.09 (0.87-5.03)
Control	17 (68)	119 (50.4)		
BMI >25				
Case	12 (50)	80 (54.4)	0.69	1.19 (0.5-2.83)
Control	12 (50)	67 (45.6)		
BMI ≤25				
Case	6 (40)	54 (42.9)	0.83	1.12 (0.38-3.35)
Control	9 (60)	72 (57.1)		

*P<0.05. NSAIDs=Nonsteroidal anti-inflammatory drugs, BMI=Body mass index, OR=Odds ratio, CI=Confidence interval

we undertake this study to evaluate the relationship of this polymorphism with risk of sporadic CRC as well as the influence of other variables such as age, gender, smoking status, and aspirin or NSAID consumption in the Iranian population. No significant correlation is established between allele and genotype frequencies of rs12904 polymorphism and sporadic CRC risk in population under study. Neither combined G/G and G/A genotypes nor AA genotype affected the risk of CRC in our populations. This is apparently contradictory with two previous reports, indicating the elevated colorectal and gastric cancer risk for individuals with AA genotype in Chinese population. In the subgroup of nonsmokers, combined AG + GG genotype has proved to be a risk factor for developing CRC in the population under study. While in the Chinese population, AG + GG genotype decreased the risk of CRC in smoker subgroups. This suggested the involvement of ethnic diversity with specific genetic background and highlights the necessity of association replicative studies in different populations, to validate these results.

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Conflicts of interest

There are no conflicts of interest.

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