RESEARCH ARTICLE

Associations of ICOS and PD.1 Gene Variants with Colon Cancer Risk in The Iranian Population

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Abstract

Background: Positive and negative co-stimulatory molecules are important factors determining the outcome of immune responses to the presence of tumors. Since co-stimulatory molecule expression may be affected by gene polymorphisms, we aimed to investigate associations between variants of PD.1 and ICOS and susceptibility to colon cancer. **Material and methods:** ICOS (-693A/G), ICOS (+1720C/T) and PD.1 (-538G/A) gene polymorphisms were evaluated by the PCR-RFLP method in 76 colon cancer patients and 73 healthy controls. **Results:** The frequencies of the GG genotype and the G allele at position -693 of the ICOS gene were significantly higher in the patient group (P=0.014 and p=0.0002), while the AA genotype was significantly more common in controls (P=0.0016). At position -538 of PD.1, GG genotype and G allele frequencies were higher in the patient group (P<0.0001and P<0.0001). Again, AA and also AG genotypes significantly predominated in controls (P<0.0001 and P=0.012). Regarding genotypes and alleles of ICOS at position +1720. Frequencies of GCG and GTG haplotypes were higher in patients compared to those of controls (P=0.016 and P<0.0001), while, frequencies of GTA, ATA and ATG haplotypes were higher in controls (P=0.0017, P<0.0001 and P=0.015). GTG/GTG and GTG/GCG double haplotypes were more frequent in patients compared to controls (P=0.0147 and P=0.0071). **Conclusion:** Our study clarified that PD.1 (-538G/A) and ICOS (-693A/G) gene polymorphisms can be considered as genetic risk factors for the development of colon cancer among Iranian patients.

Keywords: Colon cancer- PD.1- ICOS- polymorphism

Introduction

Colon cancer is one of the most frequent malignant tumors worldwide (Parkin, 2001). Studies showed that in patients with colon cancer, immune system is generally compromised (Gasser et al., 2006; Jass, 2006). The reduced antitumor immunity in patients suffering from colon cancer is the result of immune suppression and tumor evasion from immune system (Mannie, 1999). Since T lymphocytes have central role in adaptive immune response to cancers (Teramoto et al., 2013), expression and functionality of molecules regulating T cell activity can affect cancer susceptibility.

PD.1 or programmed death protein-1 (also known as CD279), is a negative regulator of the immune system expressed on CD4+ T cells, CD8+ T cells, NKT cells, B cells and monocytes. This molecule is highly expressed on exhausted T cells. Studies showed that blockade of PD.1 can enable T cells to proliferate and produce effecter cytokines (Gonzalo et al., 2001). Correlation of PD-1 expression and numerous types of cancer such as colorectal cancer have been well illustrated (Dilmec et

al., 2008). Furthermore, it is shown that level of PD.1 expression is associated to tumor prognosis. The elevated level of PD.1 expression arises the poorer prognosis (Thompson et al., 2007). PD.1 has two ligands, PD-L1 and PD-L2, which are members of the B7 family. PD-L1 is expressed on macrophages and dendritic cells. Studies showed PD-L1 is applied by tumors to escape from immune system. Increase the expression of PD-L1 in renal carcinomas (Thompson et al., 2006), gastric carcinomas, breast carcinomas (Keir et al., 2007), and esophageal cancers with poor clinical prognosis is clearly observed (Ohigashi et al., 2005).

Inducible co-stimulator (ICOS) is a costimulatory molecule belongs to the CD28 and CTLA-4 cell-surface receptor family (Hutloff et al., 1999). Although CD28 is expressed on T cells constitutively to emerge signal for resting T cells to full activated, ICOS is up regulated only after T cell activation. This molecule provides positive signal to enhance T cell proliferation. Studies showed that blocking of ICOS results in the inhibition of Th1 and Th2 immune responses. Moreover, recent researches revealed that impaired function of CD4+ and CD8+ T cells are

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observed in ICOS-deficient patients (Dong et al., 2001; Gonzalo et al., 2001; Rottman et al., 2001; Takahashi et al., 2009). It is indicated that susceptibility to different diseases such as cancers may be the outcome of abnormal expression of ICOS (Cheng et al., 2006; Bouwhuis et al., 2010; Xu et al., 2011).

Studies have shown that the expression and function of proteins varies among individuals and correlates with single nucleotide polymorphisms (SNPs) in their genes. Furthermore, it is shown that gene polymorphisms could play important roles in susceptibility of different people to the same cancers. Considering the importance of PD.1 and ICOS in the defense against cancers, this study aimed to study the probable association between PD.1 (-538G/A), ICOS (-693A/G) and ICOS (+1720C/T) gene polymorphisms and susceptibility to colon cancer in Iranian patients.

Materials and Methods

Subjects

Patient group included 76 Iranian patients suffered from colon cancer; ranging age from 18 to 84 years old (mean 46.72 years). All of them were recruited from the department of Surgery Colon Cancer Center from 2009 to 2011 and were diagnosed from surgical and pathological symptoms. All cases were histopathologically confirmed (Table 1). Based on the American Joint Committee on Cancer (AJCC) TNM staging system, tumors were staged. The control group included 73 healthy individuals referring to Shiraz blood transfusion organization for blood donation, without the history of personal and familial malignancy and autoimmune disorders, mean age 35.61 years. Ethical board approval was obtained from Research Ethics Committee of Shiraz University of Medical Sciences. Each of the cases and controls signed the written informed consent, conforming to the ethical guidelines of the 1975 Helsinki declaration and provided 5 ml venous blood.

Determination of PD.1 (-538G/A), ICOS (-693A/G) and ICOS (+1720C/T) genotypes: Buffy coat were obtained from whole blood of patients and controls. Genomic DNA was extracted from Buffy coat, using a QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. The PD.1 (-538G/A), ICOS (-693A/G) and ICOS (+1720C/T) gene polymorphisms were assessed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). For each sample, three PCR reactions were set up using three specific sets of primers. Each PCR mixture contained 500 ng DNA, 0.5 units Taq DNA polymerase, 10X PCR buffer, 0.2 mM dNTPs mix, specific concentration of MgCl2 (all from CinnaGen, Iran) and 0.5 µM specific primers set (Primm, Italy). Amplification were performed by a thermo cycler set (PC-700, Fukuoka, Japan) under the following thermal conditions: a denaturation step for 5 min at 94 °C, then 30 cycles of 1 min. at 94 °C, 1 min. at 56 °C, 1 min. at 72 °C and finally an extension step for 5 min. at 72 °C. The PCR products were then digested by specific restriction enzyme (RE) and visualized on agarose gel containing ethidium bromide. The sequences of the primers and restriction enzymes are shown in Table 2.

Statistical analysis: Allele and genotype frequencies were calculated in patient and control subjects by direct gene counting. Using the Statistical Package for the Social Sciences (SPSS), version 16, Statistical evaluation was done. The frequencies of the alleles and genotypes were compared in cases and controls by Chi-square test and Fisher's exact test. Odds ratios and 95% confidence intervals (CIs) for relative risks were assessed. Hardy-Weinberg and haplotype frequencies were measured using Arlequin V311 software. All reported p-values were two-tailed. P<0.05 was considered to indicate a statistically significant difference. LD2SNPing program V 2.0 (http://www.bio.kuas.edu.tw/LD2SNPing) was applied to evaluate the linkage disequilibrium (LD).

Results

Genotype and allele frequencies

The frequencies of genotypes in colon cancer patients and control group were in agreement with the Hardy–Weinberg equilibrium. The genotype and allele frequencies of ICOS (-693A/G) and PD.1 (-538G/A) were significantly different between healthy controls and colon cancer patients (Table 3).

As shown in Table 3, the frequency of GG genotype and G allele at position -693 of ICOS gene were found to be significantly higher in patient group compared to those in normal group (p=0.014, OR=2.27, 95% CI=1.11-4.66, Study Power=70% and p=0.0002, OR=0.41, 95% CI=0.25-0.68, Study Power=97%, respectively). On the contrary AA genotype at this position was found to be significantly higher in control group compared to those in patient group (p=0.0016, OR=0.29, 95% CI=0.12-0.68, Study Power=90%).

The frequency of CT genotype at position +1720 of ICOS gene was higher in patient group compared to those of normal group, but it did not tolerate Bonferroni correction (p=0.02, OR=2.32, 95% CI=1.07-5.1, Study Power=4%). The difference of frequency of other genotype and alleles at this position was not significant

Table 1. Demographic Data and Clinical and Pathologic Features of Colon Cancer Patients

Feature Number	
Age	
<30	12
30-60	51
>60	13
Sex	
Male	18
Female	58
2002 primary tumor classification	
Tis-T	6
T2	5
Lymph node involvement	
N0	7
N1/N2	5

Table 2. The Primers and Types of PCR for the ICOS and PD.1

Locus	PCR primers	Restriction enzyme
ICOS (-693A/G)	F: 5´ATTTCTATCTTATGCTAGGTGCTCCA-3´	BseGI
	R: 5´ATCTTGGAAGCGCGTTCAGACTT-3´	
ICOS (+1720C/T)	F: 5´TTACCAAGACTTTAGATGCTTTCTT-3´	NcoI
	R: 5´GAATCTTTCTAGCCAAATCATATTC-3 ’	
PD1.1 (-538G/A)	F: 5´CTCAACCCCACTCCCATTCT-3´	MspI
	R: 5´TTCTAGCCTCGCTTCGGTTA-3´	

(Table 3).

At position -538 of PD.1, GG genotype and G allele frequencies were higher in patient group compare to normal group (p<0.0001, OR=11.46, 95% CI=5-26.73, Study Power=100% and p<0.0001, OR=0.1, 95% CI= 0.05-0.19, Study Power=100%, respectively). On the contrary, AA and AG genotypes at this position were significantly higher in control group compared to those in patient group (p<0.0001, OR=0.02, 95% CI=0.0-0.17, Study Power=100% and p=0.012, OR=0.4, 95% CI=0.18-0.88, Study Power=73%, respectively) (Table 3).

Single haplotype and double haplotype frequencies

To assess the combined influence of three SNPs, haplotypes were constructed. Pooled results show that the most frequent haplotypes (ICOS position -693A/G, ICOS position +1720C/T and PD.1 position -538G/A) in the patients were GTG (32.2%), GCG (29.6) and ACG (17.8), while the most frequent haplotypes in the controls were ATG (19.9%), ATA (17.8%) and GCG (17.8%). Further analysis revealed that the frequencies of GCG and GTG haplotypes were higher in patients compared to those of controls (P=0.016 and P<0.0001, respectively). On the contrary, frequencies of GTA, ATA and ATG haplotypes were higher in controls compared to those of patients (P=0.0017, P<0.0001 and P=0.015, respectively). ACG haplotypes was higher in patients compared to those of controls, but it did not tolerate Bonferroni correction (P=0.040) (Table 4).

Double haplotype analysis showed that GTG/GTG and GTG/GCG double haplotypes were more frequent

Table 3. The Frequencies of PD.1, ICOS -693A/G, ICOS +1720C/T Genotypes and Alleles in Patients with Colon Cancer and Controls

Locus	Patient group N (%)	Control group N (%)	X^2	P value	OR (95%CI)	Study power (%)
ICOS(-693A/G)						
Genotypes						
GG	40 (52.6)	24 (32.9)	5.93	0.014*	2.27 (1.11- 4.66)	70%
AA	11 (14.5)	27 (37)	9.93	0.0016**	0.29 (0.12 -0.68)	90%
AG	25 (32.9)	22 (30.1)	0.13	0.71	1.14 (0.54 -2.41)	6%
Alleles						
A	47 (30.9)	76 (52)				
G	105 (69.1)	70 (48)	13.72	0.0002**	0.41 (0.25 -0.68)	97%
ICOS(+1720C/T) Genotypes						
CC	27 (35.5)	31 (42.5)	0.75	0.38	0.75 (0.37 -1.52)	14%
TT	19 (25)	26 (35.6)	1.99	0.158	0.60 (0.28 -1.29)	29%
CT	30 (39.5)	16 (21.9)	5.38	0.020*	2.32 (1.07-5.1)	4%
Alleles						
C	84 (55.3)	78 (53.4)	0.1	0.75	1.08 (0.67 -1.74)	5%
T	68 (44.7)	68 (46.6)				
PD.1.1(-538G/A)						
Genotypes						
GG	60 (78.9)	18 (24.7)	44	0.0000**	11.46 (5 -26.73)	100%
AA	1 (1.3)	27 (37)	28.75	0.0000**	0.02 (0.0 -0.17)	100%
AG	15 (19.7)	28 (38.4)	6.29	0.012*	0.4 (0.18 -0.88)	73%
Alleles						
A	17 (11.2)	82 (56.2)	67.92	0.0000*	0.1 (0.05 -0.19)	100%
G	135 (88.8)	64 (43.8)				

*, Considered significant with P-value threshold of 0.05. In genotypes, each P-value is the result of comparing corresponding row with the sum of other rows; **, Considered significant after the Bonferroni correction (P-value threshold of 0.017); N, Absolute number; CI, Confidence interval; OR, odds ratio

Table 4. Most Common P.D.1 A/G, ICOS -693A/G , ICOS +1720C/T Single and Double Haplotype Distributions in Patients with Colon Cancer and Controls

Haplotypes	Patient Group N (%)	Control group N (%)	X^2	P value	OR (95%CI)	Study power (%)
Single haplotype						
GCG	45 (29.6)	26 (17.8)	5.71	0.016**	1.94 (1.08 -3.49)	94%
GTG	49 (32.2)	14 (9.6)	22.91	0.000017**	4.49 (2.25 -9.04)	100%
ACG	27 (17.8)	14 (9.6)	4.19	0.040*	2.04 (0.97 -4.3)	55%
ATG	15 (9.9)	29 (19.9)	5.91	0.015**	0.44 (0.21 -0.9)	68%
ACA	7 (4.6)	6 (4.1)	0.04	0.834	1.13 (0.33 -3.89)	4%
GCA	5 (3.3)	12 (8.2)	3.36	0.066	0.38 (0.11 -1.2)	44%
GTA	4 (2.6)	19 (13)	9.86	0.0017**	0.18 (0.05 -0.58)	92%
ATA	0 (0)	26 (17.8)	29.66	0.0000001**	0.00 (0.00 -0.15)	100%
Double haplotype						
GCG / GCG	10 (13.16)	4 (5.48)	1.76	0.185	2.61(0.71 -10.47)	22%
GTG / GTG	10 (13.16)	1 (1.37)	5.94	0.0147**	10.91(1.37 -233.9)	60%
GTG / GCG	9 (11.84)	0 (0)	7.23	0.0071**	Un defined	73%
ACG / GCG	5 (6.58)	2 (2.74)	0.52	0.471	2.5(0.41 -19.31)	13%
ACG / ACG	3 (3.95)	1 (1.37)	0.22	0.641	2.96(0.27-75.62)	11%
GCG /ACG	4 (5.26)	2 (2.74)	0.13	0.714	1.97 (0.30 -16.07)	8%
GCG / GTG	4 (5.26)	1 (1.37)	0.75	0.387	4.0 (0.41 -96.33)	17%
GCG / GCA	1 (1.315)	4 (5.48)	0.91	0.339	0.23 (0.01 -2.27)	15%
ATG / ATG	2 (2.63)	5 (6.85)	0.69	0.407	0.37 (0.05 -2.24)	12%
ATG /GTG	1 (1.315)	4 (5.48)	0.91	0.339	0.23 (0.01 -2.27)	15%
GTA / GTA	0 (0)	4 (5.48)	2.44	0.11	0.00 (0.00 -1.45)	25%

*, Each P value is the result of comparing corresponding row with the sum of other related rows; **, Each n<5 is the use of Yates Corrected instead of uncorrected; **, Considered significant after the Bonferroni correction (P-value threshold of 0.017); N, Absolute number; CI, Confidence interval; OR, Odds ratio
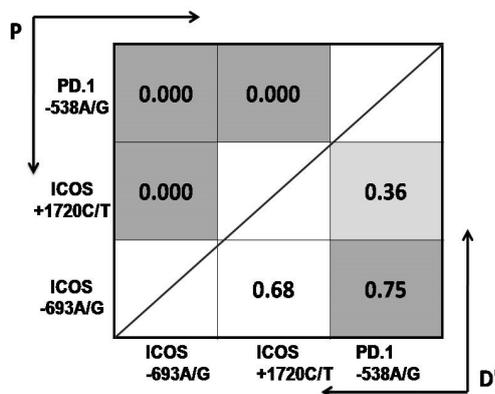

Figure 1. Linkage Disequilibrium Plot of co Stimulatory Molecules Polymorphisms in D′ Value. D′ values are multiplied by 100, for example, 100 in some boxes means a D′ of 1. Dark color boxes are representative of high linkage disequilibrium (LD) and light color boxes indicate low LD. The left upper boxes indicate the P values less than 0.05.

in patient groups compare to those of control group (P=0.0147 and P=0.0071, respectively) (Table 4).

Linkage disequilibrium determination: Strong LDs were detected between PD.1 (-538G/A), ICOS (-693A/G) and ICOS (+1720C/T) gene polymorphisms (P<0.001 for all comparisons). The LD measures, P-value and D′ are shown in Figure 1.

Discussion

Colon cancer as a multipath way disease, is one of the leading causes of cancer death worldwide (Bennet et al., 2006). In some of the studies it has been shown that tumor markers, VEGF and C3a can be beneficial in the assessment of early stage colorectal cancer patients (Mehrabani et al., 2014; Szajewski et al., 2014), While the negative and positive co-stimulatory molecules play a crucial role in immune responses.

It is shown that changes in the expression or function of these molecules may cause a trouble in the immune system and increase the risk of cancers occurrence (Cheng et al., 2006). PD.1 is an example of the negative co-stimulatory molecules which controls T cells activity (Gonzalo et al., 2001), while ICOS is a positive co-stimulatory molecule.

Certain genetic risk factors have been found to increase the risk of colorectal cancer occurrence and development. Recent genetic surveys have identified multiple SNPs within the PD.1 gene including two SNPs in exon 5 region which are PD-1.5 (7785C/T) (rs2227981) and PD-1.9 (7625T/C) (rs2227982), one SNP in intron region which is PD-1.3 (7146G/A) (rs11568821) and one SNP in promoter region which is PD-1.1 (-538G/A) (rs36084323) (Gardner, 1951; Kroner et al., 2005; Velazquez-Cruz et al., 2007; Wang et al., 2008). Since PD-1.1 SNP is located on the PD.1 promoter region, it seems that this polymorphism

can affect the PD.1 expression by interfering with the transcriptional regulation. Several studies have been carried out to find the correlation between PD.1 gene polymorphisms and various kinds of diseases (Gonzalo et al., 2001; Bouwhuis et al., 2010). In this regard, Yousefi et al., (2013) investigated the association of PD-1.1 gene polymorphism with colorectal cancer in Iranian patients. They showed that A and G alleles are associated with an increased risk of colorectal cancer. Mojtahedi et al., (2012) also showed that the genotype distribution of PD-1.5 polymorphism was significantly different between colon cancer patients with rectal cancer patients and healthy controls. Bennet et al., (2006) studied the risk for myocardial infarction (MI) in carriers of different variants of the PD.1. They did not find any association between PD-1.1 alleles and genotypes and MI. However, they observed a weak protective effect of PD-1.3A allele for myocardial infarction. According to our knowledge, there is no published article showing the relation between PD-1.1 (-538G/A) gene polymorphism and susceptibility to colon cancer. So, in this study we decided to investigate it. The result of our study showed a significantly higher frequency of GG genotype and G allele at position -538G/A of PD.1 in colon cancer patients compare to normal group. On the other side, our result indicated significantly lower frequency of AA genotype and A allele in colon cancer patients compare to normal group.

ICOS is a protein belonging to the same family as CTLA4 and CD28. This molecule has also been shown to be an important immune regulatory molecule that participates in regulation of T-cell mediated immunity (Odegard et al., 2009). The gene of this receptor is located on chromosome 2q33, contains 5 exons and 4 introns, spanning approximately 20 kb (Kristiansen et al., 2000; Yu et al., 2007; Odegard et al., 2009) . Association of some SNPs in ICOS gene with several autoimmune diseases like type-1 diabetes, SLE, autoimmune thyroid diseases and celiac disease has been shown(Kristiansen et al., 2000) Furthermore, the association of ICOS gene polymorphisms with various cancer types have been investigated (Cheng et al., 2006; Bouwhuis et al., 2010; Xu et al., 2011). In this regard, Bouwhuis et al., (2010) studied the role of ICOS gene polymorphisms and susceptibility to malignant melanoma. Furthermore, the influence of these gene polymorphisms on prognosis was determined in melanoma cases belonging to stage I or II of the disease. Their result showed no significant differences in allele or genotype frequencies between melanoma patients and controls. In another study, no association was found between ICOS gene polymorphism and CSCC (Cervical Squamous Cell Carcinoma) (Bouwhuis et al., 2010; Pawlak et al., 2010). On the contrary, in other studies, ICOS gene polymorphism was shown to be associated with chronic lymphoblastic leukemia (B-CLL), OSCC (Oral Squamous Cell Carcinoma) and CD (Coeliac Disease) (Haimila et al., 2004). Although the polymorphisms in ICOS gene have been extensively studied in various diseases (Cheng et al., 2006; Bouwhuis et al., 2010), according to our knowledge, association between this gene polymorphism and the risk of colon cancer is remained unclear. To determine the role of ICOS variants in colon cancer susceptibility, we genotyped two ICOS SNPs including ICOS (-693A/G) and ICOS (+1720C/T) in patients with colon cancer. Our result showed that the frequency of GG genotype and ICOS G allele at position -693 of ICOS gene were significantly higher in patient group compared to those of normal group. While, AA and AG genotypes at this position were significantly higher in control group compared to those of patient group. The difference of the frequency of genotypes and alleles at position +1720 of ICOS gene were not statistically significant.

Conclusively, our study clarified the role of PD-1 (-538G/A) and ICOS (-693A/G) gene polymorphisms in colon cancer susceptibility among Iranian patients, so we can suggest these polymorphisms as new potential risk factor for the development of colon cancer. However, it should be noted that the accuracy of the mentioned effect should be confirmed by repeated studies in various population.

Declaration of interest

The authors declare no conflict of interests related to this work.

Acknowledgments

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