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The Evaluation Of rs11776042 Polymorphism Effect On Colorectal Cancer Risk In The Iranian Population: A Case-Control Study

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ABSTRACT

Background: Recently, it has been shown that, piwi-interacting RNAs (piRNAs) as a new class of non-coding RNAs (ncRNAs), play crucial roles in germline development and carcinogenesis. Despite this, the study on the effects of piRNAs polymorphism (piR-SNP) on colorectal cancer (CRC) risk is scarce. We evaluate the impact of rs11776042 in piRNA 015551 on CRC initiation and development in the Iranian population for the first time.

Methods: The association of novel polymorphisms rs11776042 in piRNA 015551 gene with CRC risk using a case-control study on the Iranian population was estimated. In this project 284 CRC patients and 389 non-cancerous controls were evaluated by TETRA primer-Amplification refractory mutation system polymerase chain reaction (TP-ARMS- PCR assay).

Results: The genotypes frequency was 27%, 68% and 0.05% for C/C, C/T and T/T in controls and 31%, 65% and 0.04% in CRC patients respectively. The frequency of the C allele was 63% in patients versus 61% in controls and, T allele frequency was 37% in patients versus 39% in controls.

Conclusion: No significant difference was found in genotype and allele frequencies between the cases and controls for rs11776042 polymorphism in piRNA 015551 in our population.

Keywords: Colorectal Neoplasm (Colorectal Cancer), piR-SNPs , rs 11776042, piR_015551

INTRODUCTION:

CRC is the third common cancer in men and the second common cancer in women [1], and also, the third cause of cancer-related death worldwide [2]. Previous studies indicated that numerous risk factors such as meat consumption, inflammatory bowel disease, Diabetes mellitus (DM), smoking, physical inactivity, genetic factors, inadequate fiber intake, heavy alcohol consumption, dietary patterns, obesity, epigenetic and genetic are the factors responsible for CRC susceptibility [3,4]. But the mechanism, which is to initiate CRC formation and development, is not clearly detected yet [5].

The prevalence of CRC will arise in the last decades [6], so it is necessary to pay serious attention to this common deadly cancer. In 2020, there were about 104,610 newly diagnosedCRC and 43,340 rectal cancers in the US [7]. Approximately 53,200 cases were died from CRC in 2020, including 3,640 men and women younger than age 50 [7]. Despite advances in different treatment methods such as resection surgery, chemotherapy and, radiotherapy, the survival rate of CRC patients is low and early detection of CRC is crucial to reduce the rate of morbidity of the population. Detection of CRC is so hard in the first stage of cancer because the symptoms are rare in early-stage, So, about 61% of patient have metastasis when CRC diagnosed. Their survival rate is about 90% for 5-years according to data released by the National Cancer Institute (2006–2012) [8].

According to recent research, the elementary cause of death is metastasis and recurrence in CRC patients [9]. Previously, a new class of non-coding RNAs (ncRNAs) named (piRNAs) or Piwi-interacting RNAs, which are explain by a 3′-terminal 2 ′ -O-methylation,have been discovered [10,11]. piRNAs known by this name because of their characteristics of specific relation with the Piwi proteins, but not the Ago subfamily [12,13]. PIWI-interacting RNAs (piRNAs) are a new class of small non-coding RNAs that has 24-31 nucleotides long.PIWI proteins bind to piRNAs to produce piRNA/piwi complex [14]. The process of post-transcriptional regulation occurs in the cytoplasm. The complex which, named piRISC (piRNA-induced silencing complex), protects genome integrity through binding to transposable elements as well as lncRNAs and mRNAs [15]. Initially, the researchers have been thinking that piRNAs only are in germ cells, but ongoing studies indicated that piRNAs are in different human cells and tissues, such as brain tissues and cardiac progenitor cells [16,17]. Furthermore, found that piRNAs are abnormally express in tumor cells [18,19]. So, it proposed that piRNAs play an important role in the forming of tumours, proliferation, and cell migration [20,21].

There are more than 30,000 piRNA isoforms described in the human genome, and it is presumed that the most variant regulatory subgroup of noncoding RNAs are piRNAs [22-24]. Although for several years the functions of piRNAs were focused only on the genome integrity and development of germinal stem cells [25-27], the tasks of piRNAs and PIWI proteins as epigenetic regulators have started to emerge and observed that a subset of piRNAs also implicated in the regulation of protein-coding genes [28-31].

Many researchers found that the piRNAs are express in germline cells in a wide range [32]. Newly, high throughput sequencing shows that the signalling pathway of piRNAs is active in somatic cells either, particularly in human cancers [33-36]. Hence, these findings proposed that piRNAs could participate in carcinogenesis.

PiRNAs have been related to mechanisms leading to cancer, included encourage proliferative signalling, avoiding growth suppressors, invasion activation, and metastasis [37-40].

Chu et al. based on previous findings [41,42], searched on all known human piRNAs which were available in the public piRNA Bank database, and they have chosen seven piRNAs according to the Hap Map database and 1000 Genome project. They found that rs11776042 in piR-015551 had a decreasing effect on CRC risk in an additive model significantly. In addition, they suggested that rs11776042 from piR-015551 has a direct effect on CRC development[43].

In accordance with previous findings [43], we hypothesize if rs11706042 in piR-015551 could affect CRC risk in the Iranian population either, so in the present study, we

have done genotyping on rs11776042 from piR-015551 in CRC patients and non-cancerous individuals to determine the relationship between this piR-SNP and risk of CRC in our selected Iranian population.

2. MATERIALS AND METHODS

2.1 Ethics statement

This study conduct under the ethics committee of Shahid Beheshti University of Medical Science (SBMU) and Taleghani General Hospital (Code IR.SBMU.RIGLD.REC.1396.182). Written consent took from the person who was take part as a case or control in our project.

2.2 Study subjects:

A total of 284 CRC patients and 389 non-cancerous controls were selected randomly in our case-control study. The cases choose from patients referred to Taleghani General Hospital, Tehran, Iran, between 2013 and 2016. For DNA extraction,peripheral blood(5ml) obtained from patients and non-cancerous controls were matched for sex and age ($p>.05$) and have been collected in ethylene-diamine tetra acetic acid (EDTA) tubes.

All participants were initially being Iranian. The person who used to smoking daily about one year or more in their life considered as ever smoker, and the others never named smokers and who considered as drinker used to consume one or more alcoholic drinks in a week for about 1 year, the other participants were considered as non-drinkers.

The epidemiological characteristics of controls and CRC patients, including sex, age, literacy, marital status, grade, principal occupation, ethnicity, stage, and familial cancer history of participants gathered by the consulter and are summarized in table 1. There were not any apparent differences between CRC cases and controls in epidemiological characteristics.

For validation of CRC, colonoscopy and histopathology tests done on the biopsy tissues. Also, the patients who had radiation or chemotherapy in the past were excluded from our study. Clinical information composed of tumor size, the stage of cancer, metastasis, and differ-

Table 1. Demographic data and clinical characteristics of case and controls for CRC risk

	CRC patients(n=389)	Non-cancer controls(n=284)
Median age, years	53.1+-14.6	50.2+-15.2
Sex		
Male	202(52.07%)	159(56%)
Female	187(47.93%)	125(44%)
Primary tumor location		
Colon	68%	
Rectum	21%	
Cecum	11%	
Differentiation status		
Well-differentiated	42%	
Moderator	24%	
Differentiated	4%	
Poorly differentiated	30%	
Not differentiated	0	
Clinical stages, TNM		
I	11%	
II	54%	
IIIC	26%	
IV	9%	

Abbreviation: CRC, colorectal cancer

entiation status have been gathered. It is important to emphasize that control group subject should have no evidence of cancer in their family history or inflammatory diseases such as Crohn s colitis or ulcerative colitis. The control group's and cases had the same age, gender, ethnicity, and area of residence.

2.3 DNA Extraction

Genomic DNA has been extracted from the peripheral blood of each subject using the salting-out protocol [44].

2.4 Primer

Primer1 online software (available from http://primer1.soton.ac.uk/primer1.html) [45] was used to design TETRA-PRIMER ARMS PCR assay primers. We used Oligo7 software (Molecular Biology Insights, Inc., DBA Oligo, Inc.) for analyzing the designed primers to diminishing any duplexes, hairpins, or primer-chimers. The sequences of our TP-ARMS PCR primers were as follows: Forward Outer primer:5 TGACAAGTTGGAGAATGTCAACTGTCCA 3, Reverse Outer primer:5 TCACAAGAATAGCACAGGAAAGACCCAG 3, Inner Forward primer:5 ATCAGAGAGGACGAGATGGCTCAG 3, Inner reverse primer :5 TCTTCCTGTCTTCAAGGCTACACACAGG 3.

To amplification of rs11776042 T, C alleles, Inner Forward and Inner Reverse primers were used. The size of products for the C allele, T allele was 145bp and 195bp , and two outer primers were 284 bp exactly.

2.5 Genotyping rs11776042 by Tetra-PRIMER-ARMS PCR

piR-015551 polymorphism (rs11776042) genotyping was determined using the TETRA-PRIMER ARMS PCR method (tetra primer amplification refractory mutation systems polymerase chain reaction system) or (TP-ARMS-PCR) [46].

Tetra-ARMS PCR reactions were set up in a final volume of 25µl which containing 12.5 µl Taq polymerase 2x Master Mix Red (Amplicon, Odense, Denmark),7.5 µl PCR grade water,1 µl of each designed primers(10p-mol),1 µl genomic extracted DNA (100-200ng DNA). For optimizing the multiplex-PCR reaction circumstance, multiple annealing temperatures were used in gradient (from 64 to 66.8).

The amplification has been done by Gene Touch thermocycler (Eppendorf). The best PCR program (the cycling condition) for amplification was used as bellow: Initial denaturation at 95C for 4 minutes, Denaturation at 95C for 30sec, repeated 32 cycles and followed by annealing temperature at 65.5C for 30sec and extension at 72 C for 30sec and the elongation step was set up for 4min in 72 C. The PCR product was separated by 1-1.5% agarose gel electrophoresis and observed by staining with ethidium bromide.

We used distilled water as negative control and two blinded duplicates in the Tetra-ARMS PCR assay for quality control of our genotyping. In continuous for validation of the accuracy of our results, all genotyping was performed blindly without any information about the kind of sample status case or control. We also repeated 30% of genotyping by random selection and, the rate of accuracy was 100%, and SNP had a minor allele frequency greater than 10%.

2.6 Statistical analysis

In this study, we used chi-square statistics derived from the Student t-test to evaluate differences in the distributions of selected demographic variables between CRC cases and cancer-free controls. Hardy-Weinberg equilibrium in the control group was tested using a goodness-of-fit chi-square test.

In addition, genotype and allele frequencies polymorphism (rs11776042) and the Hardy-Weinberg equilibrium were calculated by Chi-squared test by using SNPstats online software available from http://bioinfo.iconcologia.net/SNPstats and MEDCALC online software available from https://www.medcalc.org/calc/odds_ratio.php. A probability level of P-value less than 0.05 was considered to be statistically significant.

3. RESULTS:

3.1 Characteristics of the Study Population

Total of 673 persons (377 males and 296 females), which contain 389 (57.8%) noncancerous cases and 284 (42.2%) CRC patients who were referred to Taleghani General Hospital, Tehran, Iran, between 2013 and 2016 participated in this study. The average age of the CRC group was 53± 14.6 years. Furthermore, non-cancerous indi-

viduals with the mean age of 50.2±15.2 years who didn't have any history of malignant or hereditary disease participated in this project as a control group. All participants included cases, and controls had a similar ethnicity (Iranian population).

The distribution of target characteristics of the controls and the cases are provided in Table 1. In the CRC group, 68% of patients had a primary tumor in the colon, and 21% had a rectal tumor, whereas 11% of CRC group had a tumor in their cecum, And in clinical staging of patients, 11% of them has stage1 , 54% stage II, 26% stage IIIC and 9% of them had TNM stage IV.

3.2 rs11776042 was genotyped successfully by Tetra-ARMS PCR

According to technical detail which was described in section 2.4, we used four designed primers and the best circumstance for applying multiplex PCR.

The three different genotypes, including TT, CC, and TC, were genotyped well .The frequency of the C allele was 63% in patients versus 62% in controls and , T allele frequency was 37% in patients versus 39% in controls .The genotypes frequency was 29% ,67% and 0.04% for C/C , C/T and T/T in controls , while there were 30% ,66% and 0.04% in patients.

3.3 rs11776042 Polymorphism and Risk of CRC

We evaluated rs11776042 polymorphisms in piR-015551 and CRC risk in the Iranian population in recessive and dominant inheritance models.

We observed that rs11776042 C/T genotype (dominant model) didn't have any significant effect on the risk of CRC in the patients (p-value: 0.72, odds ratio (OR)=0.87(0.62-1.23), confidence interval (CI), 95%). Furthermore, the result suggested that there isn't any relation between recessive model (TT genotype) and CRC risk in participants (p-value: 0.81, odds ratio (OR)= 0.83 (0.37-1.87), confidence interval (CI), 95% (Table 2).

1-1.5% gel agarose electrophoresis image represent in figure 1. For validation of the accuracy of our results, all genotyping was performed blindly without any information about the kind of sample status case or control. We also repeated 30% of genotyping by random selection and the rate of accuracy was 100%.

At last our results suggested that there is a lack of association between rs11776042 (in piR-015551) genotypes and alleles with the CRC risk in the inheritance model in the Iranian population.

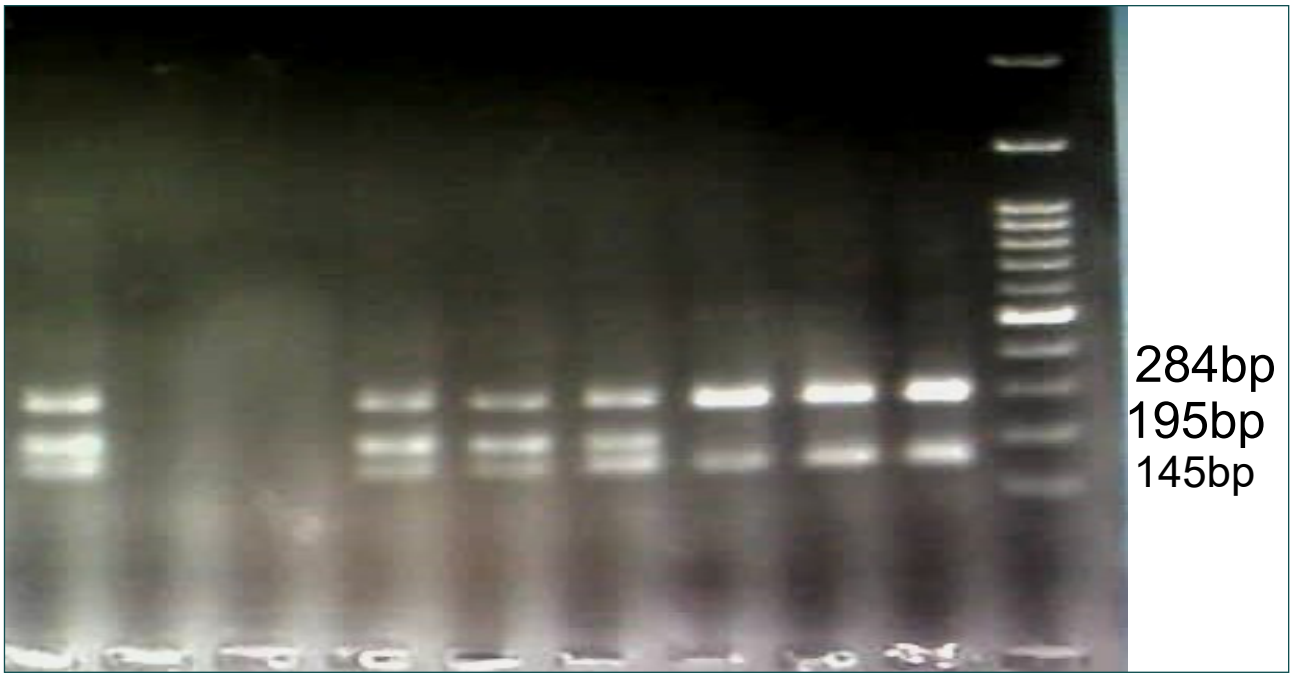

Figure.1. A representative 1% agarose gel electrophoresis for identification of the rs11776042 from piR-015551 gene. A:lane1:positive control,lane2&3:negative control,lane 4&5&6:heterozygote patients(CT genotype),lane7,8,9:homozygote patients(CC genotype),lane10:DNA Ladder100bp

Table 2. Allele and genotype frequencies of piR-015551 (rs11776042) in patients with CRC and controls

Model	Genotype	Control	Case	OR (95% CI)	P-Value
Codominant	C/C	107(27.5%)	85(29%)	1.00	0.72
	T/C	265(68.1%)	188(66.2%)	0.87(0.62-1.23)	
	T/T	17(4.4%)	11(3.9%)	0.83(0.37-1.87)	
Dominant	C/C	107(27.5%)	85(29%)	1.00	0.42
	T/C-T/T	282(72.5%)	199(70.1%)	0.87(0.62-1.22)	
Recessive	C/C-T/C	372(95.6%)	273(96.1%)	1.00	0.81
	T/T	17(4.4%)	11(3.9%)	0.91(0.42-1.98)	
Overdominant	C/C-T/T	124(31.9%)	96(33.8%)	1.00	0.5
	T/C	265(68.1%)	188(66.2%)	0.89(0.66-1.24)	

Abbreviation: CI, confidence interval; OR, odds ratio.

4. Discussion

Discussion about the relation between small non-coding RNAs has significantly been studied in the field of cancer biology, pointing to determine new biomarkers in tumor formation. Through the non-coding RNAs group, the piRNAs are a novel subgroup, which seems to read carefully to reveal the role of piRNAs in carcinogenesis [47-50]. piRNAs or PIWI-interacting RNAs is familiar to the association with keeping the integrity of the genome, and, for many years hypothesizes that piRNAs expression limited to stem cells only [51-54] and Reproductive cells in mammals [55-57].

piRNAs participate in gene expression regulation in a transcriptional and post-transcriptional situation through epigenetic mechanisms [58-61].

Cheng et al. suggested that the abnormal regulation of piRNAs in gastric cancer leads to cell cycle arrest in gastric tumor cells [62,63]. There are not types of research investigating piRNA expression in various cancers, especially in CRC. Found three piRNAs that affect on gastric cancer (piR-48966, piR-49145, and piR-31335) by using next-generation sequencing (NGS) data [64].

Observed that patients who had been shown high expression level of piR-1245 had shorter overall survival, and in continuous they hypothesized that piRNAs could also dysregulated in CRC, so they observed that piRNAs could involve in CRC progression and development [65]. piRNAs play a vital role in the progression of bladder cancer [66]. Previously reported that the piRNA piR651 is over-expressed in several types of human cancer tissue, including gastric, lung, colon, breast, and multiple myeloma cancer tissues compared with paired adjacent normal tissues [67,68,69]. Furthermore, piR651 expression levels in gastric cancer tissues are associated with the tumor - node - metastasis (TNM) stage [70,71].

Newly, some piRNAs have been found to be dysregulated in tumour tissues, and piRNAs which involved in alterations play a crucial role in cancer cell proliferation, apoptosis, and metastasis, and possibly they can mention as an excellent prognostic and diagnostic biomarkers in the process of cancer development [72,73]. In the research study on breast cancer the results have shown that some piRNAs indicated up and down regulation [74,75] (Table 3).

Recently, piR-823 (DQ571031) was found to be up-regulated in CRC tissues[78]. Interestingly, piR-823 was also observed to be deregulated not only in tissue, but also in blood serum of patients with renal cell carcinoma [79] and gastric cancer [80].

Currently, next-generation sequencing (NGS) is widely used to identify known as well as novel piRNAs with deregulated expression in cancer. In 2013, Huang and colleagues [81] demonstrated that a wide variety of RNA species, including piRNAs, are embed in the circulating vesicles. This observation was confirmed three years later when Freedman and colleagues [82] found 144 different piRNAs to be stably present in human plasma. It seems that up today, only one study analyzed theexpression of circulating piRNAs in CRC [83]. They found significant deregulation of piR-019825 (DQ597218) in plasma samples of patients compared with healthy donors.

In another study in the project on lung cancer piR-55490 was down-regulated, but piR-651 indicated up-regulation [84,85]. piR-55490 induced the proliferation of lung cancer cells [85]. Researchers have been found that piR-651 shown up-regulation in tumour tissues, which is related to the state of metastasis. Demonstrated that piR59056, piR-54878 and piR-62701 are highly expressed in CRC either, and are associated with recurrence-free survival [73].

Weng et alfound that piR-823 participated in tumorogenesis in CRC by arising the HSF1 transcriptional activity [65].law PT reported that piR-Hep-1 indicated up-regulation in 46% of hepatocellular carcinoma tissues by deep sequencing of hepatocellular carcinoma [87]. In 2015, Chu and colleagues identified 7 common single-nucleotide polymorphisms in 9 known piRNAs. Further, they revealed that piR-015551 (DQ591252) may be generated from long noncoding RNA LNC00964-3, which is significantly down-regulated in CRC tissues and may be involved in disease development. In addition, observed that rs11776042 polymorphism in piR-015551 was associated with a decreased risk of CRC. This year, piR-25447 (DQ558335), piR-23992 (DQ556880), and piR-1043 (DQ540931) were found to be over expressed in tumor tissue compared with adjacent mucosa, whereas piR-28876 (DQ598676) was significantly under expressed. Furthermore, 27 piRNAs were deferentially expressed between adjacent tissue and CRC metastases [87]. Chu et al. found that piR-015551 in LNC00964-3 is significantly lower in CRC tissues. In the other hand, piR-015551 expression level is associated with LNC00964-3 in positive manner, shows that piR-015551 possibly originates from LNC00964-3, and maybe taking part in the CRC development [88]. On the other hand, they didn't see any apparent difference between the additive model when they tested the false discovery rate (p=.140) and when they evaluated the association between rs11776042 in piR-015551 and clinicopathological characteristic of CRC in patients and case controls, they have seen that patients with colon cancer have low differentiated CRC. Haiyan Chu et al. demonstrated that the dominant model in rs11776042 in piR015551 has an noticeable effect on CRC development.

Unfortunately, there is a few studies on the role of piRNAs on CRC progression and development, but the recent studies have been shown that piRNAs and PIWI proteins could participate in CRC risk.

In our case-control study, we assess the association between rs11776042 polymorphism in piR-015551 and CRC risk in the Iranian population. Our results suggested that, rs11776042 in piR-015551 C/T and T/T genotype didn't have any noticeable effect on CRC risk in our population. In addition, we didn't observe any relation between the recessive model of this piR-SNP and the risk of CRC in our participants.

To our knowledge, this project is the first research which is investigating the association between rs11776042 from piR-015551 and CRC initiation and progression in the Iranian population. According to the last research piR-SNPs could be used as a promising biomarkers, in a different kinds of cancer prediction. In the other hand, research on piR-SNPs in CRC risk is in very primary steps. It seems that we need more research to investigate the role of piR-SNPs on CRC risk in Iranian population too. Our findings provide novel insight about the role of this piR-SNP in CRC risk in Iranian population. However, we must emphasize that there are many limitations in our study. First of all, our project was done in one case-control study, so the results need to validate with another large project. The next point is that the sample was small and collecting more DNA samples from CRC patient in our population led to better evaluation of the association between the risk of CRC and rs11776042 genotypes from piR-015551 in Iranian population. However, we did not use any molecular, and biological techniques to investigate the primary mechanism between piR-015551 and CRC risk in our population, so additional functional assays should be done to indicate the active role of this piR-SNP in Iranian CRC risk and development.

In conclusion, our finding suggested that this single nucleotide polymorphism (rs11776042) in piR-015551 didn't have any association with CRC risk in the Iranian population. Additional studies should be done to confirm our findings.

Disclosure:
The authors declare no conflict of interest.
Competing Interests:
The authors declare there are no competing interests.

Ethics Statement:
This study was approved by the ethics committee of Shahid Beheshti University of Medical Science (SBMU) and Taleghani General Hospital (Code IR.SBMU.RIGLD.REC.1396.182). Written informed consent was obtained from all the subjects who participated in this project.

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-Study Design, Data collection, Statistical Analysis, Manuscript writing, Manuscript revision
-Hamid Asadzadeh Aghdaei:
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All data generated or analyzed during this study are included in this published article

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