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Evaluation of rs37464444 Polymorphism of miR-499 Gene in Patients with Colon Cancer Compared with Healthy Subjects

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Abstract

Background: Single nucleotide polymorphisms (SNPs) have been introduced as a new genomic source for cancer. Therefore, it was decided to conduct a study to evaluate the rs3746444 polymorphism of miR-499 in patients with colon cancer in comparison with healthy subjects.

Methods: This case-control study was conducted to investigate rs3746444 polymorphism of miR-499 in blood samples of case and control groups. Patients with a confirmed diagnosis of cancer based on pathologic report were enrolled in the study as the case group and compared with healthy subjects. The level of significance was considered at $p< 0.05$.

Result: The mean of DNA count in samples was 63.17± 23.51 that was significantly higher in the case group. The rs3746444 polymorphism of miR-499 was significantly higher in patients with cancer compared to the healthy subjects ($P < 0.05$).

Conclusion: In this study, rs3746444 polymorphism of miR-499 was significantly higher in patients with colon cancer, which indicated that people with this polymorphism had a higher risk for malignancy.

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Introduction

Nowadays, the increasing number of people with cancer in the world and Iran has made this disease a worldwide health issue. Cancer is predicted as the first and most important cause of human death by 2030 (1).

Colon and rectal cancer is the third most common cancer in the world with an estimate of over 1200000 new cases per year

(2). Since 1975, the number of new cases (500000 new cases per year) has increased continuously (3).

In addition to the known environmental factors for colon cancer, genetic factors can also play a significant role in developing this cancer (4). In the recent years, numerous efforts have been made to reduce the mortality rates of colon cancer. Over the last three decades, the molecular genetics methods based on analysis of stool proteins, DNA and RNA have been developed (5). One of the most recent researches in this field is small non-coding endogenous RNA (miRNA).

Small RNAs by attaching the 3'UTRmRNA region of the target gene could act as the gene expression regulators. This discovery brought the Nobel Prize in medicine to Craig Mello and Andrew Fire. For the first time, miRNA was discovered in 1993 (6). Studies have suggested that up to 30% of the genes encoding proteins are controlled by miRNAs (7).

Currently, miRNAs are among the most important genes in regulating gene expression in the post-transcriptional phase. The miRNAs are transcribed on a path based on the miRNA genes. Initially, the Pri-miRNA is first transcribed from the gene and subsequently transformed into nucleotide Pre-miRNA70 by an enzyme called Drosha (8) and finally becomes a 22 nucleotide RNA with the Dicer enzyme.

Then, the mature miRNA will be capable of binding to the target mRNA by finding complementary sequences (9). Studies have already shown that miRNAs are involved in various biological processes including chromosomal structure, cell division, apoptosis, lipid metabolism, embryonic development and stem cell care (10). For the first time, the role of miRNA in cancer was identified as definitive in chronic lymphocytic lymphoma (11).

Single nucleotide polymorphisms (SNPs) provide powerful tools for genetic studies in the medical field (12). SNPs in miRNA sequences may affect binding to target mRNAs and pre-miRNA evolution (13). Recently, various studies have been conducted on the association between rs3746444 SNP (located in the pre-miRNAmiR-499 region) and gastrointestinal diseases (14). One of these studies has shown that the AG genotype in this SNP is significantly associated with the risk of ulcerative colitis (15).

The miRNAs can serve as biomarkers for cancer and unlike other RNAs, they are stable under *in vitro* and *in vivo* conditions (16). On the other hand, although the association between miR-499 and gastrointestinal disease has been reported, its association with colon cancer has not been investigated. Therefore, it was decided to investigate the association between the miRNA polymorphism and colon cancer.

Single nucleotide polymorphisms should be highly considered; for example, two SNPs including rs11614913 in hsa-miR-196a2 and rs2910164 in hsa-miR-146a had been the cause of many cancers according to the review articles (17).

As the rs3746444 polymorphism of miR-499 is one of the most recognized risk factors for cancer in different societies and there is no complete information on the role of the rs3746444 polymorphism of miR-499 in gastrointestinal cancers in Iran, it was decided to investigate the incidence of this polymorphism in gastric cancer patients.

Materials and Method

This study was a case-control research performed at Kerman University of Medical Sciences. The case group included 54 patients with colon cancer in Kerman/ Iran and 100 healthy individuals with the same age, gender and residence

were selected as the control group. The exclusion criteria included weight loss, anemia, history of gastrointestinal diseases such as bowel obstruction, cancer or polyp, family history of colon cancer or polyp. Informed consent was received from all patients before starting the work and the study had been approved by the ethics committee of Kerman University of Medical Sciences (Code: IR.KMU.REC.1394.631).

Sampling and genotyping

After obtaining written consent from all participants in the study, 2 ml of their peripheral blood were collected for DNA extraction. To prevent blood clotting, the samples were kept in tubes containing 1 ml EDTA with 1M concentration. The DNA was extracted from blood leukocyte cells by the saturated salt method and then its quantity and quality were analyzed by the spectrophotometer (16).

The rs3746444 polymorphism of miRNA-499 was determined by tetra-Primer ARMS PCR. This method requires two external primers and two internal and external primers to determine the single nucleotide polymorphism genotype. Since this method uses four primers in one reaction, it is possible to examine both marker alleles simultaneously (18).

The primers used in this study were designed through the tetra-primer ARMS PCR-SearchFrame database.

hsa-miR-499; rs3746444 T>C		
FO	GAGTGACCAGGCCCCTTGTCTCTATTAG	422
RO	TTGCTCTTTCACTCTCATTCTGGTGATG	
FI (C allele)	ATGTTTAACTCCTCTCCACGTGACCG	206
RI (T allele)	GGGAAGCAGCACAGACTTGCTGTTAT	268

The PCR reaction solution was prepared in the final volume of 25 µl as follows: In each standard PCR reaction, 10 pMole of each internal and external primer were used together with Taq DNA Polymerase with dNTPmix and buffer.

PCR reactions were performed on genomic DNA samples with a thermocycler (Ependorff, Germany) under the following optimized conditions. Five minutes initial denaturation at 95°C was followed by 30 consecutive cycle denaturation at 94°C (30 sec), the primer binding temperature at 58°C (50 sec) and the amplification temperature at 72°C (50 sec). Finally, 72°C temperature was applied for 5 minutes to complete the process of amplification of the pieces.

The amplified product was incubated on agarose gel 2% for 45 min at 80W. The gels were stained with ethidium bromide and the relevant bands were analyzed under the UV light using Gel documentation.

The case-control study method was used for statistical analysis. Genotype and allele frequencies of the two groups were determined and the correlation between disease and genotype was calculated with the probability ratio. Data analysis was done through SPSS software.

Results

This study was a case-control study with the corresponding samples and the results are as follows:

In general, all patients in the case group had adenocarcinoma based on the in the pathological report of whom, 5.6% (n = 3) were at stage 1, 31.5% (n = 17) at stage 2, 29.6% (n = 16) at stage 3, 18.5% (n = 10) at stage 4 and 14.8% (n = 8) were unknown (Table 1).

Table 1. The frequency distribution of patients based on the disease stage

Frequency / Disease stage	No.	%
I	3	5.6
II	17	31.5
III	16	29.6
IV	10	18.5
Unknown	8	14.8

In whole, 3.7% (n = 2) of the studied samples were at the poorly differentiated stage, 35.2% (n = 19) were at the moderately differentiated stage, 18.5% (n = 10) were at the well-differentiated stage and 42.6% (n = 23) were unknown (Table 2).

Table 2. The frequency distribution of patients based on the degree of tumor differentiation

Frequency / Degree of tumor differentiation	No.	%
Poorly differentiated	2	3.7
Moderately differentiated	19	35.2
Well differentiated	10	18.5
unknown	23	42.6

The involved area was colon in 16.7% (n = 9), cecum in 14.8% (n = 8), rectum in 25.9% (n = 14), anus in16.7% (n=9), sigmoid in 18.5% (n=10) and ileum in 7.4% (n=4) of the patients (Table 3).

Table 3. The Frequency distribution of patients based on the tumor area

	F	%
Colon	9	16.7
Cecum	8	14.8
Rectum	14	25.9
Anal	9	16.7
sigmoid	10	18.5
Ileum	4	7.4

In terms of metastasis, 24.1% (n=13) had distant metastases, 64.8% (n=35) had no metastasis and 11.1% (n=6) were unknown (Table 4).

Table 4. Frequency distribution of patients based on the tumor metastasis

Frequency / metastasis	No.	%
Distant	13	24.1
no metastasis	35	64.8
unknown	6	11.1

The mean number of DNA in the samples was 63.17±23.51. The frequency of polymorphism in the case group was significantly higher than that in the control group (Table 5).

Table 5. Frequency of polymorphism alleles in the two groups

Polymorphism \ Group	Case	Control	Total	p.v
TT	30 (55.6)	24 (24)	54 (35.1)	
TC	16 (29.6)	28 (28)	44 (28.6)	0.001>
CC	8 (14.8)	48 (48)	56 (36.4)	

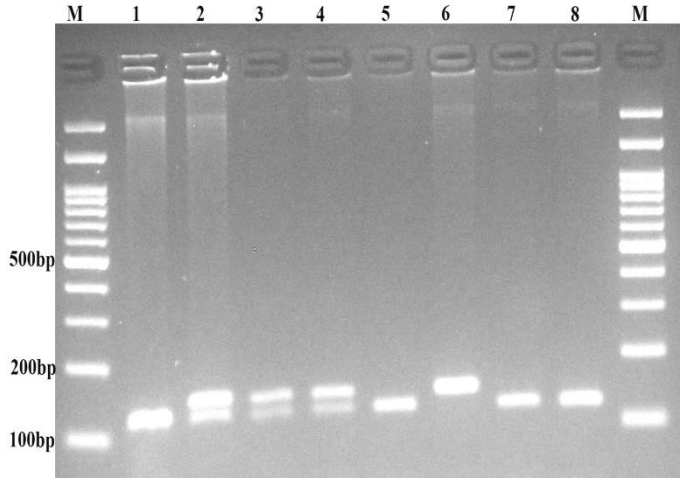

Figure 1. PCR sample performed on the studied samples

Discussion

The translocation of A instead of C in a known polymorphism of the miR-499 gene, which is found in the mature part of the miR-499 gene, changes the binding of A: U instead of G: U in the pre core stem area of the miR-499 (A instead of G at 3p and T instead of C at 5p) and it has recently been identified as a potent downstream pathway in rs3746444 of hsa-miR-499 as a pro core for the breast, lung and hepatocellular carcinoma and other cancers (19).

The results of the present study showed that the rs3746444 T> C polymorphism of miR-499 was significantly higher in patients with gastrointestinal cancers than in healthy individuals.

Tao et al (2014) investigated the prognostic value of the expression of 5 micro-RNAs genes in colon cancer. Their study showed that the elevated levels of miR-221-3p, miR-343-3p, and miR-491-5p were associated with colon cancer, and finding traces of these genes could be helpful in disease prognosis and treatment (19).

Single nucleotide polymorphisms (SNPs) should be emphasized because for example, two SNPs including rs11614913 in hsa-miR-196a2 and rs2910164 in hsa-miR-146a are the causes of many cancers (20).

The results of these studies indicate that the incidence of SNPs plays a significant role in the development of cancers and multiples the value of discovering these SNPs. In this study, the

incidence of rs3746444 T> C polymorphism of Mir-499 was clearly demonstrated in patients with cancer.

Hassani *et al.* (2014) investigated polymorphisms in the miR-146a and miR-499 genes and their association with the risk of childhood acute lymphoblastic leukemia. In this case-control study, 75 children with ALL and 115 age-matched healthy controls were selected as the case and control groups and rs2910164 G> C of hsa-miR-146a gene significantly increased the risk of ALL (21).

Omrani *et al.* showed that miR-499 rs3746444 polymorphism increases the risk of breast cancer (22). These studies show that different polymorphisms can cause various cancers in the Iranian race and it is proved in the present study that the rs3746444 polymorphism of miR-499 is an oncogene in patients with gastrointestinal cancer in Iranian population (Kerman).

In 2002, a study showed that being the G allele carrier in the Asian hsa-miR-499 rs3746444 gene might increase the risk of malignancy; however, this was not seen in Caucasian gene indicating a genetic and environmental background for the incidence of cancer (23).

A review and meta-analysis of 17 case-control studies on the existence of rs3746444 of miR-499 in different cancers showed that the rs3746444 polymorphism of miR-499 is a strong risk factor for the incidence of different cancers. However, the incidence of this polymorphism is different in different races and nationalities. Moreover, it is higher in people who are hospitalized for a known problem compared to the healthy people in the community, which indicates the certainty of the incidence of cancer by the rs3746444 polymorphism of miR-499 (24). The results of this study are consistent with the present study, which claims that the oncogene of rs3746444 T> C polymorphism of Mir-499 plays a major role in the development of various cancers, and the present study confirmed the role of this polymorphism in colon cancer.

The association between rs3746444 polymorphism and types of cancer has been investigated in various studies (25, 26). Several studies have investigated the impact of the polymorphism of various genes on colorectal cancer (27-29). Asadi et al. studied the effect of rs1042522 polymorphism of the TP53 gene on colorectal cancer in the Iranian Azeri population and reported no association between this polymorphism and colorectal cancer (27). In some studies, colorectal cancer was associated with the Pro72Arg polymorphism of TP53, but such association did not exist in other studies (28, 29). Du *et al.* addressed the association of single nucleotide polymorphisms of miR-146a, miR-196a, miR-149, and miR-499 genes with colorectal cancer potential. The results showed that the rs11614913 and rs2292832 polymorphisms of miR-196a2 and miR 149 might be involved in colorectal cancer (30).

Conclusion

In this study, the incidence of rs3746444 T> C polymorphism of Mir-499 was significantly higher in patients with colon cancer, which indicated that people with this polymorphism have a higher risk for this malignancy.

Limitations

The small number of patients with colon cancer and the time limitation of this study were the main limiting factors, which made it impossible to perform acceptable analyses between polymorphism and other variables such as distant metastasis, differentiation grade and tumor stage. Moreover,

the selection of an acceptable control group was another limitation of this study because usually people referring to hospitals have medical and non-medical problems, which may interfere with the study results.

Suggestions

Based on the results of this study, it is recommended to conduct studies to find this polymorphism in community in order to implement screening programs.

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