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Original Article

The fluctuation of APC gene in WNT signaling with adenine deletion of adenomatous polyposis coli, is associated in colorectal cancer

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ABSTRACT

Colorectal cancer is one of the most important malignancies in the classification of gastrointestinal cancers. One of the predisposing factors at molecular level for this cancer is via WNT signaling which is associated with the vast numbers of different genes. Thus, in this study, we aimed to investigate whether Adenomatous Polyposis Coli gene (APC) mutation of rs41115in two locations such as 132.002 and 131.989 acts as a trigger or cause of colorectal cancer. Relatively, 30 blood samples of colorectal cancer patients and 30 normal blood samples as control group after colonoscopy and also confirmation of pathology report at Rohani Hospital in Babol (Iran) were investigated. The primers were designed in order to be included the rs41115 to identify the particular polymorphisms of gene. The polymerase chain reaction (PCR direct sequencing method) was used. Conclusively, deletion of adenine in two specific locations such as 131.989 and 132.002 has been identified, but there was no relationship between rs41115 polymorphisms located in adenomatous polyposis coli gene and colorectal cancer.

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A flutuação do gene da APC na via de sinalização WNT com deleção de adenina na polipose adenomatosa do cólon está associada ao câncer colorretal

Palavras-chave:
Genético
Câncer colorretal
Gene da polipose adenomatosa do cólon
Polimorfismo

RESUMO

O câncer colorretal é uma das neoplasias malignas mais importantes na classificação dos cânceres gastrointestinais. Um dos fatores predisponentes no âmbito molecular para esse câncer é através da via de sinalização WNT, que está associada a um grande número de genes diferentes. Portanto, neste estudo, objetivamos investigar se a mutação rs41115 do gene da polipose adenomatosa do cólon (Adenomatous Polyposis Coli – APC) em dois locais como 132.002 e 131.989 atua como gatilho ou como causa do câncer colorretal. Relativamente, 30 amostras de sangue de pacientes com câncer colorretal e 30 amostras de sangue normal (grupo controle) foram analisadas após a colonoscopia, bem como a confirmação do laudo da patologia no Rohani Hospital em Babol (Irã). Os *primers* foram projetados de modo a incluir o rs41115 para identificar os polimorfismos particulares do gene. A reação em cadeia da polimerase (método de sequenciamento direto por PCR) foi utilizada. Conclusivamente, a deleção de adenina em dois locais específicos, como 131.989 e 132.002, foi identificada, mas não houve relação entre o polimorfismo rs41115 localizado no gene da polipose adenomatosa do cólon e o câncer colorretal.

Introduction

Colorectal Cancer (CRC) is one of the most important causes of cancer related deaths in the world.[1] Relatively, it is believed that fewer than 12% of CRC (adenomas) are believed to improve to adenocarcinomas, more than 70% of colon carcinomas are believed to be associated with preexisting sporadic precursors.[2] Meaningly, it is a multifactorial disorder that comprises environmental and also genetic factors.[3,4] For the genomic location of the APC gene (Adenomatous polyposis coli), we can say that it is located at 5q21–q22 and includes 15 exons and remarkably it has a key role in the early stages of human tumorigenesis in colorectal cancer.[5] Correspondingly, its tumor suppressing function is thought to be located on the activity and also regulating process of the intracellular β catenin within the transduction signaling pathway.[6] Considerably, a fluctuation of a mutation in this gene progresses the amounts of β catenin which may result in the cellular proliferation process in order to lead to the beginning stages of CRC.[7,8] In this account, many recent studies have recommended that various SNPs (single nucleotide polymorphisms) may be responsible for a main notable risk factor of CRC.[9,10] The most common place polymorphism in APC gene, which concludes in the different amino acid alterations, may have a practical importance, in order to lead to dys-regulation in different cancers especially in CRC. Conspicuously, many studies on the pathogenic germ-line mutations in this gene that are detected in FAP (familial adenomatous polyposis) and also the conclusions of routine allele variants in this gene are not so clear.[11,12] Noticeably, it is evident that the different SNAPs of APC gene have different performances. Although, there are at least 12 SNPs which 8 of them located in exon 15. Interestingly, many potential interactions between routine genetic variants in APC gene and environmental factors especially lifestyle with CRC (colorectal adenoma) have been even less investigated.

Anyway, more investigations, specially, studies of epigenetic factors like DNA methylation alongside with the expression of this gene is recommended.[13–18]

In this research, we investigated the fluctuations of the APC gene (WNT signaling pathway) with adenine deletion of adenomatous polyposis coli, in colorectal cancer.

Materials and methods

Sampling

In this study after screening with colonoscopy, we have selected 60 patients from Rohani Hospital and Omid clinic in Babol (Iran) after obtaining patient's consent. 30 patients with confirmation of pathology biopsy report with CRC and 30 patients without CRC were included as control group. For monitoring and comparison, the patients questionnaire survey was completed by the patients at time of enrollment, and 2 mL blood samples from each patient were collected, with added EDTA as anticoagulant and was used for DNA extraction. The proper handling and storage of blood samples were made for further studies.

DNA extraction

DNA extraction was performed using Takapoozist, Iran kit according to the manufacturer's instructions. In this way, whole peripheral blood was used. Then, in order to ensure the quality of the extracted DNA of all specimens, they were electrophoresed on 1% agarose gel. Relatively, their OD was

CGTCATGTGGATCAGCCTATTGATTATAGTTTAAAATATGCCACAGATATTCCTTCATCACAGAAACAGT
CATTTTCATTCTCAAAGAGTTCATCTGGACAAAGCAGTAAAACCGAACATATGTCTTCAAGCAGTGAGAA
TACGTCCACACCTTCATCTAATGCCAAGAGGCAGAATCAGCTCCATCCAAGTTCTGCACAGAGTAGAAGT
GGTCAGCCTCAAAAGGCTGCCACTTGCAAAGTTTCTTCTATTAACCAAGAAACAATACAGACTTATTGTG
TAGAAGATACTCCAATATGTTTTTCAAGATGTAGTTCATTATCATCTTTGTCATCAGCTGAAGATGAAAT
AGGATGTAATCAGACGACACAGGAAGCAGATTCTGCTAATACCCTGCAAATAGCAGAAATAAAGAAAAG
ATTGGAACTAGGTCAGCTGAAGATCCTGTGAGCGAAGTTCCAGCAGTGTCACAGCACCCTAGAACCAAAT
CCAGCAGACTGCAGGGTTCTAGTTTATCTTCAGAATCAGCCAGGCACAAAGCTGTTGAATTTTCTTCAGG
AGCGAAATCTCCCTCCAAAAGTGGTGCTCAGACACCCAAAAGTCCACCTGAACACTATGTTCAGGAGACC
CCACTCATGTTTAGCAGATGTACTTCTGTCAGTTCACTTGATAGTTTTGAGAGTCGTTCGATTGCCAGCT
CCGTTCAGAGTGAACCATGCAGTGGAATGGTAAGTGGCATTATAAGCCCCAGTGATCTTCCAGATAGCCC
TGGACAAACCATGCCACCAAGCAGAAGTAAAACACCTCCACCACCTCCTCAAACAGCTCAAACCAAGCGA
GAAGTACCTAAAAATAAAGCACCTACTGCTGAAAAGAGAGAGAGTGGACCTAAGCAAGCTGCAGTAAATG
CTGCAGTTCAGAGGGTCCAGGTTCTTCCAGATGCTGATACTTTATTACATTTTGCCACGGAAAGTACTCC
AGATGGATTTTCTTGTTCATCCAGCCTGAGTGCTCTGAGCCTCGATGAGCCATTTATACAGAAAGATGTG
GAATTAAGAATAATGCCTCCAGTTCAGGAAAATGACAATGGGAATGAAACAGAATCAGAGCAGCCTAAAG
AATCAAATGAAAACCAAGAGAAAGAGGCAGAAAAAACTATTGATTCTGAAAAGGACCTATTAGATGATTC

Fig. 1 – Designated primers for PCR reaction.

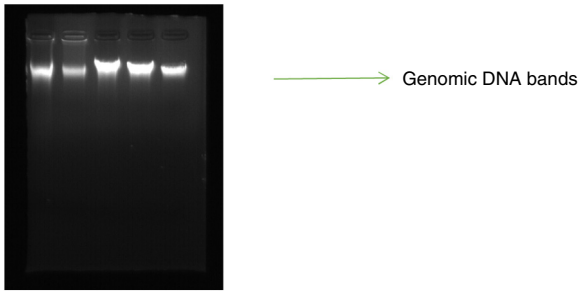

Fig. 2 – Qualitative analysis of DNA extraction Via Agarose gel electrophoresis. Sample of DNA extracted in 1.5% agarose gel electrophoresis. The bands of extracted DNA are noticeable.

measured by a spectrophotometer (Eppendorf Bio photometer, Germany) at 260–280 m wavelengths in order to investigate their quantity.

PCR

Polymerase Chain Reaction (PCR) was applied to extract DNA copies, with use of gel electrophoresis and more DNA copies were reproduced and identified. This method of DNA reproduction is similar to natural cells production.

Relatively, PCR was used to amplify DNA. This method provides options for selecting any DNA strands with repeated nucleotides. These nucleotides with the aid of primers, enzymes and heat are capable of producing more DNA strands; upon cooling down, the DNA stands will be attached to compliment DNA counterparts.

SNAP of rs41115 was searched in NCBI. The APC gene was received from Base Website Ensemble. The oligonucleotide seventh primers software was used for designing polymorphisms and was checked in primer blast section website. Table 3 shows primers related to rs41115 polymorphisms.

For application and production of designated polymorphisms (Fig. 1), first PCR technique was applied to separate and divide polymorphisms, and then thermal cycler was applied at temperature 95 °C. The thermal cycler separates double stranded DNA from each other. Then, temperature was reduced from 95 °C to approximately 50–60 °C. This temperature is suitable for gradual connection between base pairs (bp), at the same time and the temperature has to be high enough to prevent formation of unnecessary DNA strands. For this reason, 5 mL DNA genome was added to the final mixture with total volume of 45 mL. This mixture was added to 1.5 mL test tube for future use.

PCR product confirmation

In order to ensure the duplication of genes fragment at its special polymorphism site, the PCR products were electrophoresed on the agarose gel 1.5%.

Data analysis

For confirmation of final results of genotype after PCR extraction, the samples were sent to Takaposist Company in order to have the sequences of each PCR product and CLC software was used for reading the results. The results of this study were analyzed with the aid of CLC software. The Chi Square root (X^2) functioning and logistics regression were applied for statistical analysis and interpretation.

Results

Study population

This study includes 30 patients with CC and 30 patients without CC as control group. All these patients were natives of Mazandaran (Iran) who participated in this study at Rohani Hospital in Babol (Iran). The qualitative (Fig. 2) and quantitative (Fig. 3) results are investigated for each sample (Table 1).

Qualitative results of PCR products analysis

The specific DNA strand from PCR technique was confirmed by using 1.5% agarose gel electrophoresis. The obtained DNA strand and length were assessed. In the case of polymorphism of rs41115 in the APC gene, primer designing was used. The length of double strands of 155 bp was opened and was compared with available standard sample length in National Center for Biotechnology Information (NCBI) database for comparison and confirmation (Fig. 4).

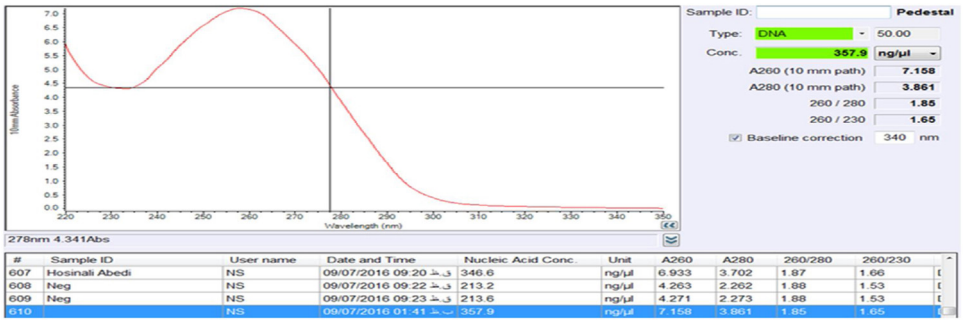

Fig. 3 – Quantitative results analysis of extracted DNA via Nano Drop Spectrophotometer. Sample of DNA, light absorption with the aid of Nano Drop Spectrophotometer.

Table 1 – Primers of rs41115 polymorphisms.

Type of Primer	Sequences (5'≥3')	Length	GC%	T_m
Forward primer	GAAATAGGATGTAATCAGACG	21	0.025	50.7
Reverse primer	AGTCTGCTGGATTTGGTTCTA	21	0.025	55,7

G, Guanine; A, Adenine; T, Thymine; C, Cytosine; T_m, Melting Temperature.

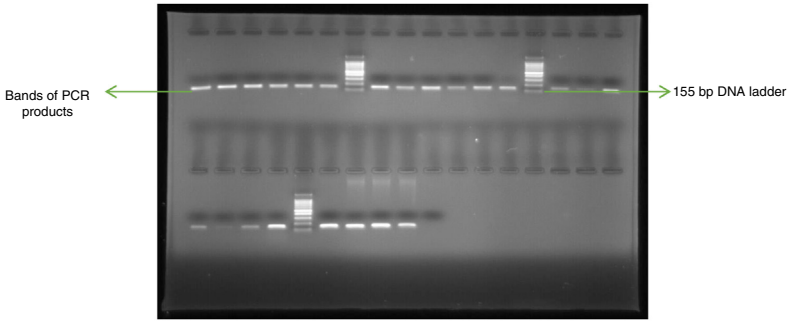

Fig. 4 – Qualitative results analysis of PCR of APC gene on 1.5% agarose gel electrophoresis, 100 bp ladder was used for purpose of comparison with 155 bp.

Table 2 – Patients' age is calculated as mean of 57.6 ± 14.02 years. Calculation of mean age of patients.

Age (years)	N° of patients	Mean	SD
20–80	30	57.6	14.02

SD, Standard Deviation.

Table 3 – Statistical analysis with logistic regression indicates correlation between patients' age and adenine base deletion in locations of 132.002 and 131.989.

Variable	Constant regression	Wald test	Sig
Age	−0.056	1.036	0.309^{ns}
Constant value	5.613	2.458	0.117^{ns}

Sig, Statistically Significant.
*Significant with probability of 5%; ns: not significant.

Results of PCR sequencing

All the samples both cancerous and normal were sequenced (Figs. 5–8).

The statistical correlation between patients' age and adenine base deletion in 132.002 and 131.989 locations in patients' population was assessed with use of Chi Square (X^2) test with 95% confidence (Table 2).

Furthermore, statistical calculation of patients' age was made with logistic regression. The results of this analysis are presented in Table 3 with respect to patients' age no relationship between adenine deletions in locations of 132.002 and 131.989 was found. The patient's age was between 20–80 years old with $p > 0.05$.

Correlation between patients' gender and adenine base deletion in 132.002 and 131.989 locations with use of Chi Square (X^2) test with 95% confidence

In this study, no statistical correlation found between gender and adenine base deletion in 132.002 and 131.989 locations. Table 4 shows this correlation.

We also assessed and compared adenine base deletion in both male and female patients. The adenine base deletions were seen in both male and female, but this deletion is more prominent in male.

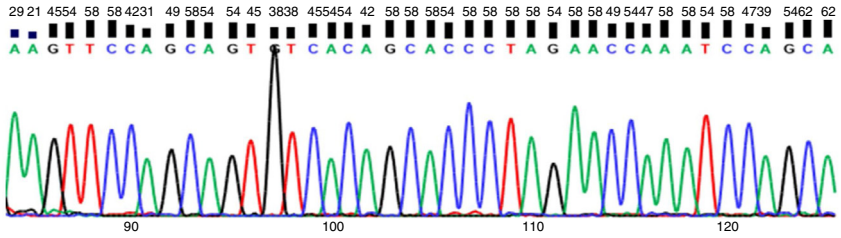

Fig. 5 – Results of sampling of bp and chromatogram of rs41115 polymorphism in APC gene of a patient with CRC.

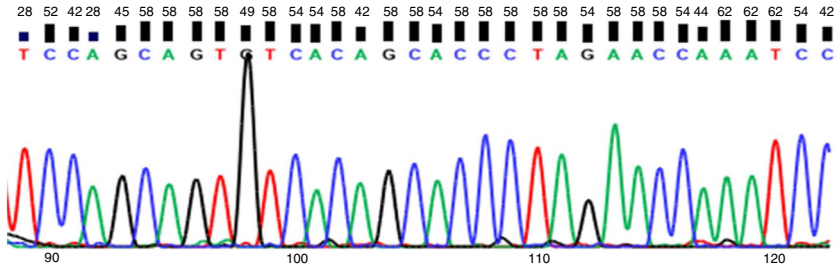

Fig. 6 – Results of sample of bp of rs41115 polymorphism in APC gene as compared to gene of a healthy individual.

Table 4 – Numbers of male and female, with respect to adenine base deletion in 132.002 and 131.989 locations.

Base deletion/gender	Male	Female
Negative	11	11
Positive	7	1

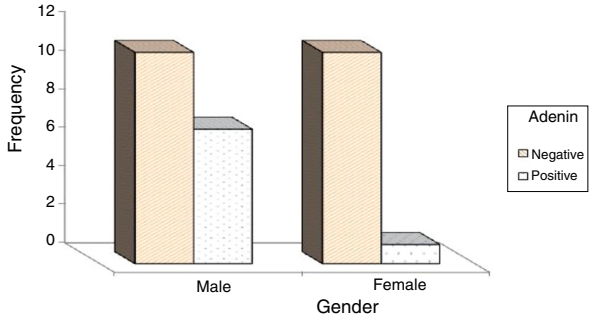

Fig. 7 – Comparison of adenine base deletions in 132.002 and 131.989 locations seen in both genders.

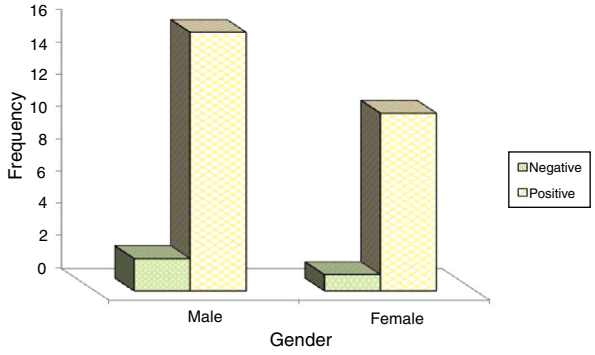

Fig. 8 – Mean comparison of genders; depend on base deletions in 131.989 and 132.002 locations.

Table 5 – Results of Chi square test and Fisher's exact test with respect with gender and adenine deletions in 132.002 and 131.989 locations.

Test	Ratio	Sig
Chi Square	3.438	0.064^{ns}
Fisher exact	–	0.099^{ns}

Sig, statistically significant.
*Significant with probability of 5%; ns, not significant.

The relationship of adenine base deletions in 132.002 and 131.989 locations was noticeable in both male and female. Further analysis was made with Chi Square test with Table 2×2. The results for accuracy reported as Fisher's exact test. Again Chi Square test and Fisher's exact test do not show significant statistical correlation between gender and adenine base deletions in 132.002 and 131.989 locations.

Correlation between adenine base deletions located in 132.002 and 131.989 in control group (healthy) populations with use of Chi Square (X^2) test with 95% confidence.

Tables 3–5 patient's age calculated as mean age of 57.6 ± 14.02 years. The age of patients ranges from the youngest of 20 years old to the oldest of 80 years old.

Logistic regression was used to assess patients' population and Mean age.

Gender differences were assessed in adenine base deletion in 131.989 and 132.002 locations and statistically analyzed by Chi Square (X^2) test with 95% confidence.

Chi Square (X^2) test with 95% confidence was used to compare male and female with respect to adenine base deletion in 131.989 and 132.002 locations. A further comparison was made between patients and healthy (control) populations. No significant correlation was found between patients' age and probability of base deletion ($p > 0.05$) (Tables 6–8).

Table 6 – Mean age of patient's population.

Age (years)	N° of patients	Mean	SD
20–80	30	57.6	14.2

SD, Standard Deviation.
*Significant with probability of 5%; ns, not significant.

Table 7 – Statistical comparisons between male and female with base deletion in 131.989 and 132.002 locations.

Base deletion/gender	Male	Female
Negative	2	1
Positive	16	11

Table 8 – Results of Chi-Square test and Fisher's exact test compared male and female patients with base deletions in 131.989 and 132.002 locations.

Test	Ratio	Sig
Chi square	0.072	0.804^{ns}
Fisher exact	–	1^{ns}

Sig, statistically significant.
*Significant with probability of 5% and ns, not significant.

This comparison is presented in Figure 4–2 between male and female with base deletions in 131.989 and 132.002 locations, and were seen in both genders, but more noticeable in male than female.

Gender differences and base deletion once again were assessed with Chi Square test and Fisher's exact test. No differences were found in these variables.

Discussion

Cancer is a genetic disease that can manifest itself in humans as a result of genes mutations. This phenomenon depends on genetic predisposition and environmental factors and may be triggered when normal body control mechanisms cease functioning properly. Therefore, causing the malfunction of cells cycle and uncontrolled cells proliferation; old cells do not die and new abnormal cells will be formed. DNA polymorphisms such as SNPs, located at specific positions in human genome has an important role in abnormal cell colony formation, genetic mapping and genetic coding; that can cause production of abnormal genes and therefore cancer cells growth.

APC gene is an autosomal recessive tumor gene capable of somatic mutations that can cause production of small, defective and non-functional proteins, these changes play an important role in CRC. The CRC manifestation mainly depends on loss of both copies of genes, and causes uncontrollable cells growth and therefore is the cause of cancer.

The first time gene mutation was observed when a study was conducted on patients with multiple colon polyps syndrome.[19] It was observed that the protein production via this gene mutation affects protein in beta catenin WNT pathway.[20] Thus, it stimulates excess of cell division and cancer cells production.

In this study first, we have aimed to identify the relationship between rs41115 polymorphisms in APC gene and CRC in North of Iran. Second, we looked at whether there exist significant differences in male and female with respect in abundant in these particular rs41115 polymorphisms in APC gene in North of Iran.

The results revealed that all of these patients had adenine base deletions; these findings may require further studies for confirmation.

In this way, Palacco[21] conducted a study in Colombia South America in Latino population under the title of Analysis and Interpretation of Genetic Data of APC, K-ras and TP53 in 30 patients with CRC. The APC gene mutation frequency was 15.3%, the sample of CRC mutation frequency was above 40.7%. The most common polymorphisms in CRC were found to be rs41115 polymorphisms in APC gene. In comparison with our results, there is no similarity and correlation.

Another study in order to determine whether 11,307 K polymorphisms APC gene is associated with CRC or not, was done.[22] After analysis and interpretation of extracted DNA Heteroduplex of APC gene of 240 colonic tumors, no significant 11,307 K mutations were found in these patients. Correspondingly, in this research, accidental somatic mutation with low frequency without 11,307 K-allele mutation was found. No mutations or alterations in 1307 codon around APC gene regions were noticed. This result of the above project confirms the obtained results of our study.

Jahang et al. 2013, reports two cases, the first one was a 23 year old male patient with over 100 adenomatous polyps in small and large intestines, identified with colonoscopy, and the other patient was 48 year old female who was the mother of the 23 year old patient. The 48 year old patient also had over 100 adenomatous polyps upon colonoscopy examination. Genetic analysis identifies new frameshift mutation of axon of gene 15 APC. Adenine-guanine deletion with thymine insertion in c.38333-3834 results in ending codon at1287. Both patients were treated with colectomy.[23]

In this account, another investigation studied the mutation of this gene.[24] Meaningly, genetic assessment in 12 patients was diagnosed with Familial Adenomatous Polyposis (FAP), and 11 patients of their first degree relatives were identified with mutation of axon on gene 15 APC. Seven patients and two individual relatives had mutation bands detected by Single-Stranded Conformation Polymorphism (SSCP) method. After gene sequencing, 5 patients between 24–35 years old had frameshift mutation, but in 2 patients and 2 relatives' despite having positive SSCP, no mutation was identified. From these results, we could conclude that 50% of FAP patients in this study have mutation of axon on gene 15 APC. Furthermore, in this study only small region of APC gene was evaluated and all the patients had equal mutation. In comparison with our projects result, there are some similarities.

Another study, with the aid of 3 methods such as PTT, direct genomic sequencing and SSCP identified population of 50 Swedish families with FAP which had gender cells mutations in APC genes, located in different codons such as 1061 in three families, 1602, 1309 in two families and 1334, 1414, 1450, 1465 were reported as well in other families.[25]

In 2006 another study in Colombia South America was conducted in Latino populations, under the title of Genetic

Analysis and Interpretation in APC, K-ras and T53 in patients with CRC.[21] After 30 samples were taken from these patients, the APC gene mutation was seen at 15.3%. The high frequency of CRC was higher than 40.7% and also high frequency of polymorphisms was observed in these patients. The most common polymorphisms are polymorphisms rs41115 in APC gene. The results of this above study had no correlation with our study's result.

In another study, 80 patients with CRC and 74 healthy individuals in their study to compare the different genotypes in entire length of APC gene were investigated.[26] This assessment was to find a meaningful relation between SNPs in APC gene and prevalent of CRC in Taiwanese population. Genetic analysis of AB3100 sequences was done, among 154 Taiwanese 3 new mutations were reported that were associated with an increase of CRC. This study identified 12 SNPs, SNP 8 located in axon 15, SNP 2 in axon 11, SNP 1 in axon 9 and 1 SNP in axon 13. One deletion in 490 codons resulted in frameshift mutation, in two cases meaningless mutations in P.V1125A and P.S1126R were identified. In addition to 3 new mutations associated with polymorphism; other single nucleotide and single genotype less harmful were identified in 1822 codon. This study confirms also our results.

Probability of mutations in APC gene in patients with adenomatous polyposis in FAP with the use of CSGE method was assessed.[27] These genetic signs were identified in colon and rectum. 5 families out of 150 families with CRC, were selected after identifying the known DNA piece and with direct sequencing; the genetic codes for the regions were identified. The results indicated genetic mutations in these patients.

In 1992 colorectal tumors for APC gene were studied by Meoshi et al., in 63 tumors including 16 adenoma tumors and 47 carcinoma tumors. Lack of heterozygote in 30 tumors and non-specific mutations in 43 tumors were found.[28] Also, point mutation in 21 tumors, none sense mutations in 16 cases, missense mutations in 2 cases, and mutations in intron region in 3 cases were observed. 22 cases had 1–31 bp deletion, 3 cases had 1 extra bp was added. On the other hand, 95% of these mutations were due to point mutation in APC. Overall 80% of the cases had at least one regional mutation and 60% had two regional mutations in APC gene. These results also acknowledged our study's results.

A study in relationship with 8 SNPs in APC gene mutations, with respect to environmental factors such as fatty diet and Hormone Replacement Therapy (HRT) was done.[29] 758 CRC patients were selected after screening with colonoscopy and biopsy confirmation and 767 healthy individuals were included as a control group. No relationship found between genotypes in SNPs of APC gene and aggressive distal cancer metastasis, but APC gene D1822 V and association with fatty diet need further study. By the way, the quality and quantity of extracted nucleic acids including DNA and RNA alongside compatible primers are of great importance.[30–33]

Conclusion

The first aim of this study was to recognize the relationship between rs41115 in APC gene and CRC in patients in North of Iran. Second, whether there is a relationship between gender and abundant of this polymorphism in population of North of Iran. Assessment of this study in polymorphism of APC gene of rs41115, which conducted in patients of colonoscopy department of Rohani Hospital and Omid clinic of Babol, Iran, indicates these polymorphisms in patients and control individuals are meaningless. The study of samples with one or two adenine base deletions determined the need for more investigation and larger number of patients.

Similar studies in the same gene of CRC with different rs have found various polymorphisms with higher percentage. With respect to rs41115, this study is new, and no polymorphism was identified in this region. The samples that were used in this study reveal either one or two adenine deletion in specific locations such as 131.989 and 132.002 of gene length.

Based on previous studies and findings of this study, we conclude that the genetic factors and environmental factors may have significant effects on the manifestation of prevalent of this kind of cancer in different geographical regions.

Authors' contributions

SEN, ZKK, RA, PR, SV, SG and RK collected data and accompanied in some parts of the project and also manuscript, **FAS** collected specimens, **SMTH** collected all the samples directly in his clinic and hospital by himself and also confirmed the clinical qualifications of all the patients as a gastroenterologist.

AAS controlled and confirmed the data quality, evaluated and optimized the informatics database, wrote the paper and edited it, some other essential functions containing study design, controlling the project and protocol development and also data analysis. All authors revised the article carefully, read and acknowledged the final version of the paper.

Conflicts of interest

The authors declare no conflicts of interest.

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