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Title: Contribution of APC gene in rs1255190244 mutations on the colon cancer patients in Tehran Province.

Abstract

Mutations on the adenomatous polyposis coli (APC) gene can cause colon cancer. Colon cancer is a kind of cancer that starts in the large intestine (colon). cancer colon begins with overgrown cells in a body.
Objectives: the point of this studying field is analyzing APC gene in rs1255190244 mutations on the colon cancer patients and in Tehran Province.

Methods: From 1397 to 1398, one hundred samples of 6 to 10 ml per patient were collected from patients with colorectal cancer who were definitively diagnosed. Then the DNA of each sample was extracted and after checking the quality of the DNAs by electrophoresis method, the required primers were designed that all primers were designed at almost the same annealing temperature. After performing all PCR steps, the information was surveying and Conclusions were made.

Results: the results which obtained in this study, it shows that the incidence of rs1255190244 in the patient group compared with the control group with P value = 0.003 was significantly associated with the incidence of colon cancer.
Conclusions: From the results of this study, it can be concluded that the polymorphism rs1255190244 can be used as a marker to prevent colon cancer, but more research is needed to further investigate other polymorphisms..

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Introduction

Colorectal cancer is one of the most frequent cancers in humans. Unfortunately, the symptoms of bowel cancer are not detectable at the beginning, but knowing the symptoms of this disease, one can get seen by a doctor and perform the necessary tests to further investigate and diagnose this type of cancer. Colorectal cancer is more common in the elderly but can occur at any age. Colorectal cancer usually starts as a small non-cancerous mass called a polyp that forms inside the gut. (1) Over time, some of these polyps become cancerous According to GLOBOCAN 2018 data, clone cancer is the fourth most common cancer in the world, while rectal cancer is the eighth. Together, CRC is the third most common form of cancer diagnosis worldwide, accounting for 11% of all cancer diagnoses. (2) At least four sequential genetic changes need to occur to ensure colorectal cancer evolution. One oncogene (KRAS) and three tumor suppressor genes adenomatous polyposis coli (APC), SMAD4, and TP53 are the main targets of these genetic changes. (3)

Most colon cancers are caused by lifestyle factors and aging, and several cases are due to genetic disorders. The most Risk factors include being overweight, diet, smoking, and lack of physical activity. Diet-related factors that increase the risk of this disease include red meat and processed meats, as well as excessive alcohol consumption. Some of the inherited conditions that cause colon cancer include hereditary familial adenomatous polyps and hereditary non-polyp colon cancer, But these account for less than five percent of cases. (4)

The process used to find out if cancer has spread within the colon cancer to other parts of the body is called staging. In stage (0), abnormal cells are found The innermost layer covering the colon. These abnormal cells may turn into cancer and spread to nearby normal tissues. In stage one (I), cancer forms and spreads from the innermost layer of the colon wall tissue to the middle layer. In stage (II), cancer has spread to the top of the colon wall but has not affected the lymph nodes. The second stage is colon cancer. In stage (3), cancer has spread to the colon and affected the lymph nodes, but cancer has not yet affected other organs in the body. (5)

Intestinal cancer tumors that are affected by genetic inheritance are divided into two categories: Lynch syndrome and familial adenomatous polyposis (FAP). Unlike familial adenomatous polyposis, Lynch syndrome usually has a small number of polyps in the bowel that may become cancerous over time. FAP syndrome is caused by inherited changes in the APC gene. The APC gene is a tumor suppressor gene that naturally helps cells grow. In people with inherited changes in the APC gene, cell growth stops and it has a braking state which causes hundreds of polyps in the large intestine. (6)(8) Over time, cancer develops in almost one or more of these polyps. Mutation of the adenomatous tumor suppressor gene of polyposis coli (APC) initiates most colorectal cancers (CRC). After mutation in the APC gene, the Importin-11 protein binds

to βcatenin and accompanies it to the nucleus of colorectal cancer cells. (7)(9)
One of the mutations in the APC gene is RS1255190244, which is a single nucleotide polymorphism(SNP) is a change in the DNA sequence that differs in a nucleotide in the genome between individuals of the same biological species or between a pair of chromosomes in an individual. SNPs are considered a major genetic source of phenotypic variation within a species and are considered an important and good genetic marker. SNPs have many usages, including molecular markers in genetic research and drug modification and genomics, genetic mapping, and more. (10) RS1255190244 variation type is SNV(Single nucleotide variation) in chromosome 5.
This study aimed to evaluate mutations in the rs1255190244 point gene at the APC gene in the Iranian population at different ages. Contributed significantly to the treatment of patients with colorectal cancer.

Sampling: This study is descriptive-analytical and the study population is Iranians with colorectal cancer. A total of 100 patients in the patient group and 100 people in the control group were studied. 100 blood samples of patients with colorectal cancer were collected from Hazrat Rasool Akram Hospital in 1396-98.
For each patient group member, demographic information including age, sex, history of cancer, history of surgery, history of smoking, and stage of cancer was recorded, and for the control group members, age, sex, history of surgery, and history of smoking were recorded too.
After consent, 10-6ml of blood sample was taken. Blood samples were stored in tubes containing anticoagulants and DNA samples were diluted to a concentration of 10ng/ml and stored in DNase-free Eppendorf tubes in a freezer-20.

Extracting DNA from blood: DNA extraction was performed by using the Yekta Company kit and DNA quality was evaluated by electrophoresis. We designed the requested primers by using the NCBI site and after designing, they were ordered by Pishgam Company. All primers were designed at almost the same annealing temperature.
SAMPLE PREPARATION
Blood: Whole blood must be collected in EDTA(1mg/ml)- to prevent clotting and DNA degradation. DNA extracted from heparinized blood cannot be used for PCR.
Typically 100µl of fresh blood is used for DNA isolation with a yield of 1.0-5.0 µg. If the blood is to be stored for later use it can be left at 2-4°C for (no longer than) 2 weeks. For long-term storage, the samples should be aliquoted in 100µl portions and kept at -20°C.

Note:
1. Transcriptionally active tissue and bacterial culture contain high levels of RNA, which can copurify with genomic DNA. RNA may inhibit some downstream enzymatic reactions, but not the PCR itself. If RNA-free genomic DNA is required, add Ribonuclease A (final concentration 0.2mg/ml, Cat. No. PR891628 to the sample during protease incubation.
2. For whole blood, DNA yield depends on the number of leukocyte cells and the storage duration and condition of the sample.
3. Since the DNA quantity is too small, Viral DNA from sera samples is invisible in agarose gel, and only host nucleic acids from lysed leucocytes may be monitored.
4. Use 1-10µl of DNA solution for each 50µl of PCR mixture. In case of high background PCR product, extracted template DNA may dilute 1/100 and repeat PCR reaction.

Investigation of RS1255190244 polymorphism by Tetra Arm PCR method: ARMS PCR (Amplification Refractory Mutation System) is a standard, easy, fast, and reliable method for detecting point mutations and polymorphisms. This method is used to differentiate the allele of a gene that differs by as little as one base pair.
Materials required for PCR: 1. DDW: Used to provide a liquid environment and bring the final volume.2. Template DNA: PCR is a technique by which a large number of DNA strands

can be obtained from a single strand of DNA, provided that the two ends of the DNA strand that we intend to amplify are well known. The DNA fragment can be the product of genomic DNA extraction, plasmid DNA, or even another PCR product.3. dNTPs: The four nucleotides that make up a DNA are the building blocks used by DNA polymerase to make new DNA strands and must be put together to form a complementary strand. The required concentration required of each nucleotide is the same for the PCR reaction.4.forward and reverse primer: Primers are small polynucleotide fragments designed and synthesized in the laboratory to have complementary nucleotide sequences with region 3 in one of the desired DNA strands. Because the DNA polymerase enzyme binds to the primer fragments, only the target DNA molecule is replicated and amplified. Because DNA is double-stranded, two types of primers are required in PCR. Primers do two things; First, they determine the location of the gene to be amplified, and second, they determine the size of the amplifying fragments. PCR primers were designed to be completely specific and complementary to the target region of the target DNA.5. 5. Taq DNA polymerase: DNA polymerase is an enzyme that can replicate using nucleotides, a patterned strand, and a primer fragment. 6. buffer: The buffer creates suitable environmental conditions in terms of pH and different ions to ensure the stability of DNA polymerase and its optimal activity.7. Magnesium ion: Both DNA and the primer have a negative charge, so using the magnesium ion, the positive charge created binds the DNA and the primer. It acts as a cofactor, is used as a substrate for the Taq enzyme, and accelerates the process of the enzyme.

Suitable primers for rs1255190244 polymorphism A>T

primer	Sequence(5' →3')	length	TM	GC
FO	CTGAAATGACTTCATGTGAGGG	22	63	45
RO	CATCCCTAGTCCAAAGTAGAGTG	23	64	48
RI	GTTGAGTGAATAGGTGAAGGAG	26	65	42
FI	GTCTAAAAATGAACCAAAAAATCATATTAGCTTC	32	63	26

In this method, one of the internal primers is specific for the A allele, which produces 311 bp, and the other internal primer is specific for the T allele, producing 193 bp. An external primer produces a common product of 503 bp, which creates both alleles if present.

Electrophoresis: Electrophoresis is a method that electrically charged samples are passed through a porous network using an electric field. Molecules can pass through this network at different speeds, depending on their size, the type of molecules, and the electrical charge. In these devices, the Agarose gel is placed between two buffer chambers and serves as the only intermediary for the passage of electricity between these chambers. in electrophoresis, when DNA is deposited on an agarose gel in the presence of a buffer and under the influence of an electric field, it is separated based on its molecular charge and mass. Because the amount of fluorescent light is proportional to the total mass of DNA. Finally, the amount of DNA in the sample can be estimated with fluorescent light emitted by a ladder with some quantitative standards.

Results:

In this study, DNA extraction samples were amplified using the TETRA ARMS PCR technique, and PCR products were taken on agarose gel and the result was observed as bands with lengths of 311, 193, and 503 for rs1255190244. In order to evaluate the quality of DNA, 3 µl of the sample was examined by horizontal electrophoresis on 1.5% gel agarose.

After reviewing the results of Tetra Arms PCR products on 1.5%agarose gel, the results for rs 1255190244showed that out of 100patients, 24people had the AA genotype, 42 people had the AT genotype and 34people had the TT genotype.

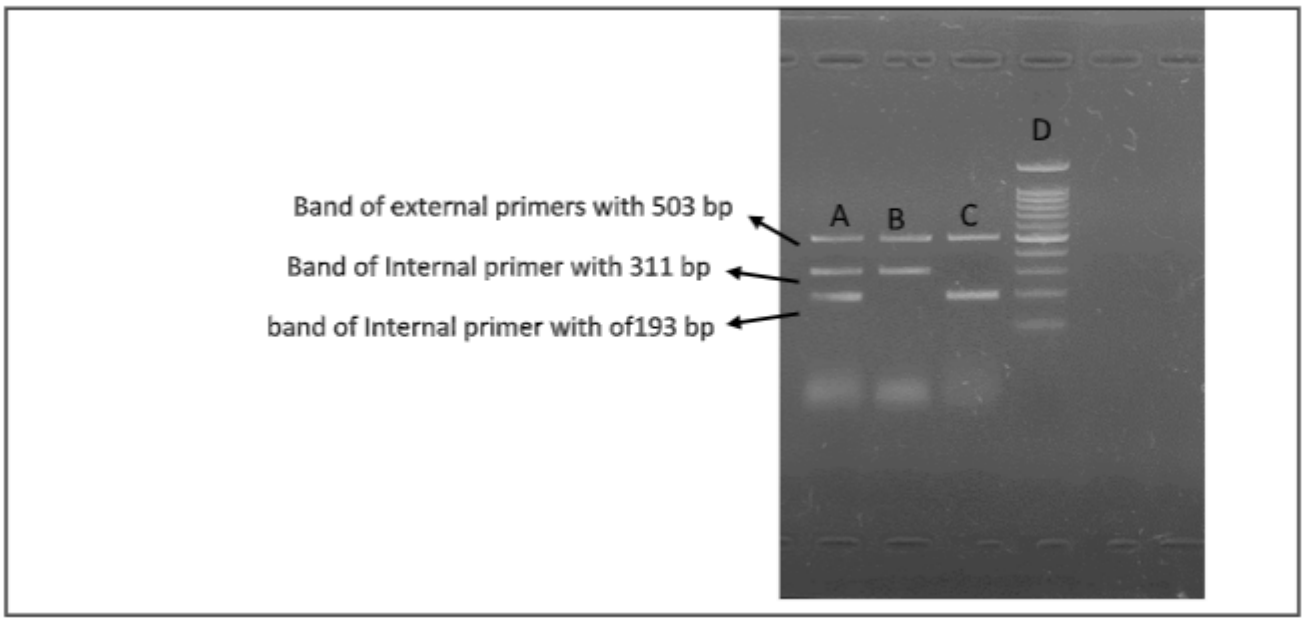
A>T	genotype	Patients case	Control case

		number	Percent	number	**Percent**
rs125519024	AA	24	24	23	**23**
	AT	42	42	73	**73**
	TT	34	34	4	**4**

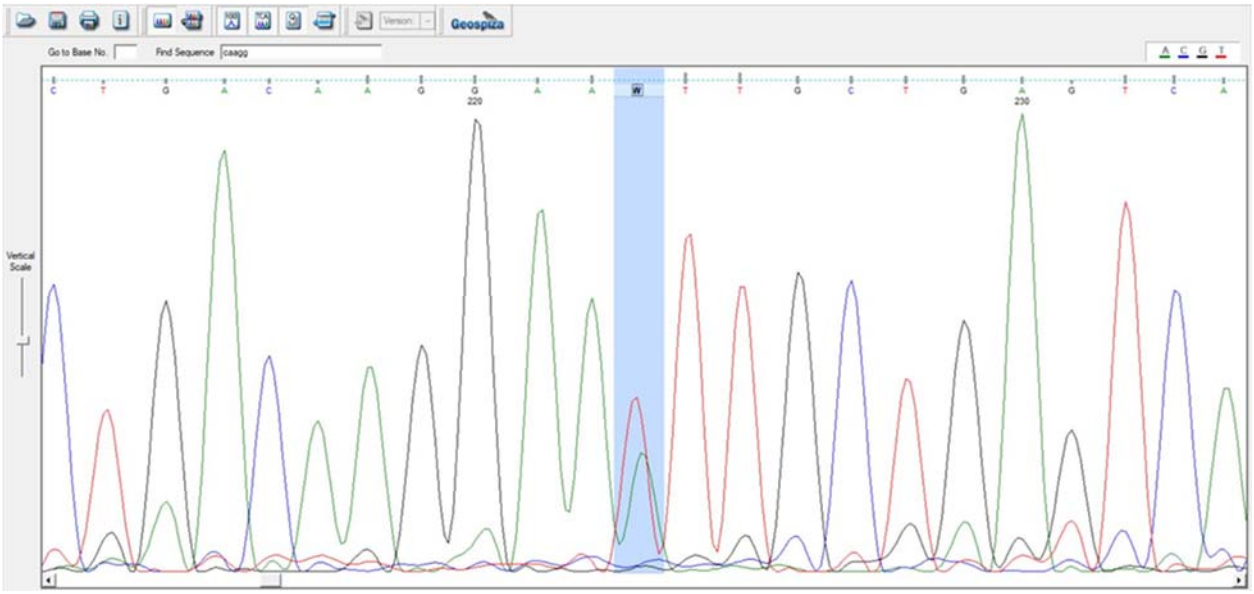
Table 1: General statistics on the percentage of genotypic frequency in rs125519024.

Out of 200 patient and control samples, 52% were male, 48% were female, 59% had a history of surgery, 69% had a history of smoking, the mean age was 61 years and the main weight was 78 kg.

in the Tetra ARMS-PCR method for rs1255190244, one of the internal primers is specific for the A allele, which produces 311 Base pairs, and the other internal primer is specific for the T allele, which produces 193 Base pairs, and an external primer, which is a 503 Base pair, which creates both alleles in the manner of existence. (Figure 1)



(figure1): A case has AT genotype, the case has the TT genotype and the C case has the AA genotype. In figure 1, the first hole from the left(A) represents a person with a heterozygous genotype with both normal internal bands and mutants and external bands. The second hole (B) represents a person with a mutant genotype with an internal band of mutants, the third hole (C) represents a person with a normal genotype with a normal internal band, and in the fourth well (D) a ladder is loaded.



In the next step, a homozygous sample was sequenced to confirm the genotype.

Figure 2: The sequencing results in the figure above show the homozygous genotype.

Mutate allele

CTCAAGACAG CCACACCTGA GACCTCATCA CTCTTCCTTC AACCACCTCA GCCCTAAGTA
ATTTGTTGAT CTAGGTAACA TTACACTTTT AATGAAACCT GTAGCTCCAC TGTAGAAAAT
ACTTGCTTCC TGGGATATCC AATAATAGAC ATCTCCTTTA ATAGTTTGAC TCAAGGTGCT
ACCACCTCTG GAGTCCCTGA CAAGGAA
Y
TTGCTGAGTC AAACCTGCAA ACTAAGACTA GAGGAATCCA CCCCAGAGAG TCATACCACA
TACACAATAT GGTGAAAGCT CTCTAAACAC CAGAGGTAAG TGATTTGTGC CAATAAAACT
ATCTTACACT ACTTGTCCAT CTGTTCCAAG GCTTCACACA CAGAATTTAC TTCCTTAGAG
TGTTCAGGGT ATAGTCTCTG

The following sequence represents the rs1255190244 region in the APC gene, in which in this sequence the Y point represents the A or T allele. If the allele is A, the genotype is normal, but if the allele is t, the genotype was mutated in rs1255190244.

As you can see in the figure above, according to the obtained results from patients, 42% of them have AT genotype and are heterozygous.24% of patients with colorectal cancer have AA genotype and 34% of patients have TT and homozygous genotype.

Statistical study of the relationship between colorectal cancer and environmental and genetic factors in rs1255190244 polymorphism

Statistical study of smoking history and the possibility of colorectal cancer in the patient group:

MODEL	**Genotype**	**OR**	**P value**
Codominant	Mutant	0.00	0.047
	heterozygous	2.71 (-13.41 – 18.83)	
	normal	1.19 (0.72-1.98)	
Dominant	Mutant	0.00	0.087
	heterozygous- normal	1.16 (0.74-1.83)	
Recessive	heterozygous- Mutant	0.00	0.023
	normal	0.80 (0.58-1.12)	
Over dominant	normal -Mutant	0.00	0.034
	heterozygous	0.91 (0.60-1.37)	

Table2: Statistical study of smoking history and the possibility of colorectal cancer in the patient group.

Statistical analysis of smoking history and the risk of colorectal cancer in the patient group shows that in the recessive model, p-value =0.023 so the risk of cancer is higher in smokers because the risk of mutation in the rs1255190244 gene point is higher. Also, statistical analysis of smoking history and the risk of colorectal cancer in the patient group shows that in the Dominant model with p value=0.087, smokers are less likely to develop cancer, because the probability of mutation in the rs1255190244 gene point is lower.

Statistical study of Having a history of surgery and the possibility of colorectal cancer in the patient group:

MODEL	Genotype	OR	P value
Codominant	Mutant	0.00	0.047
	heterozygous	0.79 (0.57-1.12)	
	normal	1.51 (1.08-2.11)	
Dominant	Mutant	0.00	0.087
	heterozygous -Mutant	1.07 (0.77-1.48)	
Recessive	heterozygous -Mutant	0.00	0.023
	normal	0.72 (0.51-1.01)	
Over dominant	normal - Mutant	0.00	0.034
	heterozygous	1.10 (0.79-1.54)	

Table 3: Statistical study of Having a history of surgery and the possibility of colorectal cancer in the patient group.

People with no history of surgery and the dominant model are less chance to develop colorectal cancer with p value=0.087 because they are more likely to have a mutation in the rs1255190244 gene point. Sample No. 12 weighs 102 kg and is 75 years old, with a history of smoking and having surgery with TT genotype in rs1255190244 with colorectal cancer.

Statistical study of patient weight and the possibility of colorectal cancer in the patient group:

MODEL	Genotype	OR	P value
Codominant	mutant	0.00	0.047
	heterozygous	1.20 (0.84-1.71)	
	normal	1.09 (0.79-1.52)	
Dominant	normal	0.00	0.087
	heterozygous normal	0.83 (0.61-1.12)	
Recessive	heterozygous -Mutant	0.00	0.023
	normal	1.11 (0.81-1.50)	
Over dominant	mutant - normal	0.00	0.034
	heterozygous	1.25 (0.88-1.77)	

Table 4: Statistical study of patient's weight and the possibility of colorectal cancer in the patient group.

Concerning patients' weight with the risk of colorectal cancer in the patient group, the higher the weight of the patient, the greater the risk of colorectal cancer, because the probability of mutation in the rs1255190244 gene point is higher. Regarding the weight of patients with the risk of colorectal cancer in the patient group, the lower the weight of the patient, the lower the risk of colorectal cancer, because the probability of mutation in the rs1255190244 gene point is lower. Sample No. 12 weighs 102 kg and is 75 years old, with a history of smoking and having surgery with TT genotype in rs1255190244 with colorectal cancer.

Statistical study of age and the possibility of colorectal cancer in the patient group:

MODEL	Genotype	OR	P value
Codominant	mutant	0.00	0.047
	heterozygous	1.02 (0.75-1.39)	
	normal	1.50 (1.10-2.04)	
Dominant	mutant	0.00	0.087
	heterozygous- normal	1.55 (1.11-2.16)	
Recessive	heterozygous - mutant	0.00	0.023
	normal	1.44 (1.06-1.94)	
Over dominant	mutant - normal	0.00	0.034
	heterozygous	1.45 (1.03-2.05)	

Table 5: Regarding the age of patients and the risk of colorectal cancer in the patient group, the older the person, the higher the risk of colorectal cancer, because the risk of mutation in the rs1255190244 gene point is higher. Sample No. 97 weighs 102 kg with the age of 78 years, with a history of smoking and without surgery with TT genotype in rs1255190244 with colorectal cancer.

Discussion:

Malignant cells and abnormal growth of cells in the area of the colon, and rectum (rectum) cause colorectal cancer. The disease begins with a benign adenoma polyp, later it progresses to an advanced adenoma with high-intensity dysplasia, and then develops into invasive cancer. The American Joint Committee on Cancer (AJCC) represent that Invasive cancers can be treated as long as they are confined to the colon wall (stage I and II), but if they are left untreated, they can metastasize to peripheral lymph nodes (stage III) and then to distant tissues and organs (stage IV). Stage I and II tumors can be treated with surgery and more than 73% of stage III patients can be treated with surgery with chemotherapy. But stage IV patients are generally incurable. Due to these cases and low prognosis about the symptoms and disease process in different communities and the identification of most patients after metastasis, colorectal cancer mortality, and high treatment costs that are incurred by the disease, all indicate the importance of research into colorectal cancer, faster diagnosis, and identification of risk factors for the disease. Some environmental factors that cause colorectal cancer, such as age;

Our review of the latest SEER data confirms that older people, which means people over 50, are more likely to develop colon cancer, the average age of people based on this information is

68 years. And family history; The sentinel account of a hereditary colorectal family was by Dr. Aldred Warthin, who first suspected the disorder in the family of an affected woman (who subsequently died of endometrial cancer) over 100 years ago, which is uncontrollable.

CRC is a disease in which both environmental and genetic factors influence its development. (11) Controlling other environmental factors, such as smoking, poor diet, low physical activity, and even reduced alcohol consumption, can help reduce the risk of colon cancer. (12) Based on a 2007 study by Randall Burt, Family history studies have shown that the disease has a significant genetic background and 15% of colon cancers also have an inherited background. However, known syndromes such as hereditary non-polyposis colorectal cancer and Mendelian-inherited intestinal polyposis adenomatosis are allocated for less than 5% of colorectal cancers. As a result, less influential polymorphisms may be presented as an important part of the genetic risk of this disease. Our studies and research are also based on the effects of environmental factors such as smoking, obesity, surgical history, and weight, as well as genetic factors such as mutations in polymorphism in the APC gene in colorectal cancer.

Studies by the Human and Clinical Genetics Group, LUMC, and the Dutch Sylvia Laboratories showed that Mutations in the APC gene lead to a decrease in the expression of this gene as a tumor suppressor, meaning that if the cell division deviates from its normal cycle and causes a tumor, the APC gene will not be able to suppress tumor development due to the mutation. (13)

Genome-wide association studies (GWAS) estimated that there are about 31 million SNPs in the human genome. They conducted a systematic search for GxE interactions using extensive genomic data from the colon cancer family registry. X. Huang in a 2012 study of Various single nucleotide polymorphisms (SNPs) in the APC gene have been observed in CRC patients. (14) In a case-control study performed in the Chinese Han population, genotyping ten single nucleotide polymorphisms (SNPs) were assessed that indicates SNP has a role in predisposing to CRC cancer results acquired.

rs1255190244 is one of the SNP in the APC gene that the presence of mutations in rs1255190244 and other environmental factors such as smoking, obesity, age, and history of surgery affect colorectal cancer.

Based on Kelvin Tsoi's study in relation to Cigarette Smoking and the Risk of Colorectal Cancer, Smoking was associated with a significantly increased risk of CRC. (15) And Makoto Okamoto's study showed a direct link between aging and colorectal disease. and our results obtained from the statistical analysis of the history of smoking and the risk of colorectal cancer in the patient group show that in the recessive model, p = 0.023 therefore the risk of cancer is higher in smokers because the risk of mutation in the rs1255190244 gene is higher, so all three pieces of research results are the same.

Also, People with no history of surgery and the dominant model are less chance to develop colorectal cancer with p value=0.087 because they are more likely to have a mutation in the rs1255190244 gene point, and Rentsch M's research on the history of surgery and colorectal cancer also showed similar results to our research.

Carmen Jochem and Michael Leitzmann's 2016 study on Obesity and Colorectal Cancer shows a significant association between colorectal cancer and obesity. (16) Also, N Shimizu's research in April 2003 showed that they performed all statistical analyzes using PC-SAS, similar to our conclusion that weight gain is one of the risk factors for colorectal cancer. Besides, based on patients' weight with the risk of colorectal cancer in the patient group, the higher the weight of the patient, the greater the risk of colorectal cancer, because the probability of mutation in the rs1255190244 gene point is higher Regarding the age of patients and the risk of colorectal cancer in the patient group, the older the person, the higher the risk of colorectal cancer because the risk of mutation in the rs1255190244 gene point is higher.

From the results of this study, it can be concluded that the rs1255190244 polymorphism can be used as a marker to prevent colorectal cancer, but more research is needed to further investigate other polymorphisms. A more comprehensive study can be obtained by increasing the number of samples in the control and patient groups, carefully examining other APC gene polymorphisms, examining other SNPs in the APC gene, and finally examining the expression between APC gene expression and genotype.

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