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Investigating the possible roles of mutations in *axin1* and *axin2* genes in colorectal cancer

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ABSTRACT

Introduction: Colorectal cancer (CRC) is one of the leading cancers, following skin, breast, and stomach cancers. This study aimed to investigate the relationship between mutations in axin1 and axin2 in association with CRC.

Methods: Our study contains 147 fresh frozen samples from CRC patients, 25 normal samples, and 3 cell lines, including HT29, SW480, and CACO-2. The chosen SNPs from databases are placed in exon 5 of axin1, in exon 2 of axin1, and in exon 7 of axin2. By PCR-RFLP method, mutated samples were identified and sequenced.

Results: The results showed that mutations in the single-nucleotide polymorphism (SNP) in axin2 were observed in 1 out of 147 patient samples (0.68%). In the three sequences examined in axin2 (exon 7), mutations in SNP with rs79024445 at A2052C were observed. Statistical analysis of clinical and pathological data of patients showed a significant relationship between the tumor size factor and grade of cancer (P=0.016) as well as the degree of tumor diffusion to the lymph nodes factor with a grade of cancer (P=0.001).

Conclusion: The multi-factorial nature of cancer, the high genetic diversity of the Iranian population, and the limited statistical population could affect these outcomes. The observed mutations in each sample can also indicate the importance of personalized medicine in studying diseases.

Keywords:

Axin2, Axin1, SNPs, Colorectal Cancer, RFLP.

1. Introduction

Colorectal cancer (CRC) as one of the prominent healthcare issues, which is the development of cancer from the colon or rectum (parts of the large intestine). CRC is the third most prevalent diagnosed cancer in men and the second in women (1). Moreover, the second cancer with the highest mortality accounted for about 0.9 million deaths in 2020 (2-5). According to available statistics, the disease affects males and females equally, and its prevalence is often after age 50; usually, it occurs earlier in the case of heredity and family. Statistics in Iran indicate that half of the patients are under the age of 50. It is estimated that after age 50, 5-6% of people get this type of cancer during their lifetime (6). Different

biological activities take place through distinct intracellular signaling pathways. In the canonical Wnt pathway, the function of the Wnt proteins is performed by cell surface receptors and creates an extracellular signaling pathway to activate β-catenin (7). Phosphorylation, degradation, and β-catenin regulation are the basis of the signaling of the Wnt pathway (8). Wnt proteins are known to regulate stem cells self-replicating, apoptosis, and cellular mobility (9).

Axin1 is a scaffold protein forming the β-catenin destruction complex, so it could be considered a tumor suppressor gene. *Axin* participates in the destruction complex of β-catenin as a scaffold protein in coordination with GSK3-β, CK1, and APC, and could be considered as a tumor suppressor due to its ability to downregulate the β-catenin and Wnt pathway (10, 11). *Axin2* is a negative regulator of the Wnt/β-catenin pathway and targets the β-catenin gene; therefore, it creates negative feedback on the Wnt pathway (12). Mutation in these two genes is reported in several cancers such as colorectal, medulloblastomas, hepatocellular cancer, hepatoblastoma, endometrial, esophageal squamous cell carcinoma, endometrioid ovarian adenocarcinoma and adenoid cystic carcinoma (13).

In CRC which the mismatch repair system is inefficient, the mutation in the *axin2* gene leads to an increasing concentration of β-catenin (14). Fluorescence hybridization analysis in situ also shows that *axin2* in breast cancer, neuroblastoma, and other tumors often loses its heterozygosity (15). *Axin2* has been identified with multiple mutations in several patients with various polyps, although the association between these mutations is not completely clear (16). The somatic mutations that affected *axin1* and *axin2* genes have been observed in several cases of CRC (17). It has been reported that mutations in exons2 and 5 of *axin1* (18, 19), as well as exon 7 in *axin2* (20, 21), are more important because they form essential parts of the protein structure, and in many CRC cases, these areas have mutated. Exon 7 in *axin2* is the bonding site of PP2A phosphatase, and exon 5 in *axin1* is the bonding site of β-catenin.

Axin was found as a result of a classic mutation of a fused locus in mice for the first time, and its phenotype is axial duplication in mouse embryos (22). in humans, *axin1* and *axin2* are encoded by different genes located in 16p13.3 and 17q24.1, respectively. Both have two isoforms (a and b) and the b isoforms are shorter due to exon splicing. *Axin* has several partners and binds them through several domains. *Axin* interacts with tankyrase (TNKS) through its binding domain (1-80 aa), G proteins by its regulators of G protein signaling (RGS) domain (121-247 aa), β-catenin by its β-catenin binding domain (414-476 aa) and disheveled protein through its Dishevelled/*axin* homologous (DIX) domain (757-820 aa). The DIX domain is necessary for *axin* homodimerization and heterodimerization with disheveled protein. *Axin* also interacts with adenomatous polyposis coli (APC) protein (81-200 aa) and GSK3-β (372-413) (13, 23). The main objective of the present study was to investigate the two major SNPs in *axin1* and one SNP in *axin2* and their mutations in CRC. As detail, the SNPs in exon 2 and 5 in *axin1* gene as well as exon 7 in *axin2* gene were studied by RFLP and sanger sequencing methods on tissues of patients with CRC.

2. Material and Methods

2.1. Bioinformatics studies

Initially, the analysis of *axin1* and *axin2* genes and their proteins was done by bioinformatics. The NCBI database (https://www.ncbi.nlm.nih.gov) was searched for these proteins' nucleotides and

amino acids. Using the Ensembl genomic tool, we examined the desired gene nucleotides; hereafter, the locations of *axin1* and *axin2* exons were investigated. Consequently, among the various SNPs, missense SNPs were examined in the PolyPhen-2 (an automatic tool for prediction of the possible impact of an amino acid substitution on the structure and function of a human protein) tool based on the damaging score for each SNP (https://bio.tools/polyphen-2) (24). Afterward, we used the UCSF Chimera software (https://www.cgl.ucsf.edu/chimera/) (25) which is an extensible program for interactive visualization and analysis of molecular structures and related data and gives high-quality images or movies.

Post-mutation changes were reviewed with the aid of Swiss-PdbViewer software (https://spdbv.unil.ch/) (26). The proteins can be superimposed in order to deduce structural alignments and compare them with other relevant parts. Also, amino acid mutations, bonds, angles, and distances between atoms are obtained for the intuitive graphic. Subsequently, suitable restriction enzymes were found on the NEBcutter website (https://nc3.neb.com/NEBcutter/) (27).

2.2. Participants and collecting samples

One hundred seventy-two tissue samples (147 cancerous and 25 normal adjacent tissue) were used in this project from patients with CRC referred to Imam Khomeini Hospital, Tehran, Iran during 2020-2021. In addition, the extracted DNA from 3 cell lines of colorectal cancer such as HT29, SW480, and CACO-2 were provided by Pasteur Institute, Iran. All the patients filled out a questionnaire and consent form. Furthermore, this study was approved by the ethical code of IR.NIGEB.EC.1400.12.10.G by the National Institute of Genetic Engineering and Biotechnology (NIGEB) research ethics committee. The inclusion and exclusion criteria for the study are: Not being related to patients with colorectal cancer, Sampling of patients before using any type of medication or treatments, not having any type of cancer other than colorectal (tumor cecum, ascending, transverse and descending colon, rectosigmoid), and no family history of colorectal cancer in the patients' families. Patients' demographic data is shown in Table 1.

Table 1. Clinico-Pathological characteristics of the CRC patients.

Parameters		**Number (%) (n = 147)**
Sex	Male	80 (59.7)
	Female	54 (40.3)
Age	<50	29 (21.8)
	>50	104 (78.2)
Smoking status	No	43 (95.6)
	Yes	2 (4.4)
Grade	1	27 (19.3)
	2	29 (27.9)
	3	35 (25)
	4	28 (20)
	Unknown	18(7.8)
TNM	T*	
	T2	19 (13.6)
	T3	79 (56.4)
	T4	25 (17.9)
	Unknown	24(12.1)
	N**	
	N0	43 (30.7)
	N1	45 (32.1)
	N2	37 (26.4)
	Unknown	22(11)
	M***	
	M0	31 (22.1)
	M1	77 (55)
	Unknown	39(22.9)

*T: Tumor size, **N: Invasion to lymph nodes, ***M: Metastasis. The meaning of the numbers 0 to 4 for TNM: The higher the number, the larger the tumor size for T, the more lymph node invasion (N). 0 for metastasis means no metastasis and 1 means detection the metastasis in patient.

2.3. DNA extraction

The DNA was extracted from tumor tissues and cancer cell lines by the phenol-chloroform method. Also, the quality and the quantity of the extracted DNA were evaluated by horizontal electrophoresis and NanoDrop, respectively.

2.4. PCR

To amplify *axin1* (exon 2 and 5) and *axin2* (exon 7) genes, primers designed by oligo7 (28) and gene runner software, considering that the sequences made by primers have an enzyme with a particular restriction site (Thermo Fisher Scientific, USA). For this purpose, the NEBcutter website was used. For each gene, 10µL of master mix (Ampliqon, Denmark), 2µl from each primer with 10 pM concentration, 5µl deionized water, and 1µl DNA of each sample (50ng/µL average concentration of DNA) was used. Each gene was amplified for 35 cycles. The annealing temperature of primers and their sequences are provided in Table 2.

2.5. RFLP

To investigate any variation of exon 5 of *axin1* and digestion with Mva-1 enzyme, 5µl PCR products, 1 µl R buffer with of 10X concentration, 1 µl Mva-1 enzyme, with a concentration of 10 u/µl and 9 µl deionized water were used. For digestion of exon 2 of *axin1*, 5 µl of PCR product, 1 µl O buffer in 10X concentration, 1 µl AflII enzyme in 10 u/µl concentration and 9 µl deionized water were used. For digestion of exon 7 of axin2, 5 µl PCR products, 1 µl tango buffer in 10X concentration, 0.5 µl Ava-1 enzyme, with 10 u/µl concentration and 9 µl deionized water were used. The time required for optimal activity of the Mva-1 and AflII (and Ava-1, too) enzymes was 2 and 1 hour, respectively. These enzymes cut wild type sequences and mutant DNA don't digest affected by these restriction enzymes. After enzymatic digestion, cutting or non-cutting samples were determined by using horizontal electrophoresis. In this study, the wild type DNA is cut at the digested SNP but the mutant DNA remains intact.

2.6. Sequencing

In this study, the RFLP method was used to investigate the presence of mutations in exon 5 and 2 of *axin1* and exon 7 of *axin2* in 147 patients and 3 cell lines including HT29, SW480, and CACO-2. Selected samples (randomly selected 30% of normal and tumor samples, all the positive samples, and the cell lines samples) were sent for sequencing by PISHGAM Company (Tehran, Iran) for each gene. The results of sequencing were analyzed via Chromas software.

2.7. Data analysis

SPSS 16.0 software and the ANOVA test were used to evaluate the frequency of clinical and demographic variables and investigate the relationship between types of expressed variables and cancer grades. The P-value≤ 0.05 was considered as statistically significant.

Table 2. description of the primers used for amplification of exons of *axin1* and *axin2* genes (F forward, R reverse).

Exon No	Primer sequence	Annealing temperature (° C)	product size (bp)
Exon2 *axin1*	F 5′AAAGGTGAGACTTCGACGGC3′ R 5′CTCTGCCTTCGCTGTACCG3′	63	715
Exon5 *axin1*	F 5′CTCAGAAGTTCGCGGAGGAG3′ R 5′CATGGCCTCAAGGAACCAA3′	60	606
Exon7 *axin2*	F 5′CAAGGCTCCGGAAACCATG 3′ R 5′ATGGGGCTTGGGCTTGCTC3′	60	489

3. Results

3.1. Demographic and clinical results

In the present study, 59.7% of the patients with CRC were men, and 40.3% of them were female. Also, 78.2% of the patient group were more than 50 years old, while 21.8% of them were less than 50 years old. The mean age of patients was 55.6±18 years, and they were between 15 to 83 years old. In the study of cancer grades, 27 cases (19.3%) were in the early stages, and 63 (45%) were in the advanced stages of CRC. Regarding the TNM classification, 56.4% were local tumor growth (T3), 32.1% were distribution of tumor to lymph nodes (N1), and 55% showed distant metastasis (M1). Moreover, there was a significant difference between the factors of T, size of the tumor (P = 0.016), N, invasion to the lymph nodes (P = 0.001), and the grade of CRC in patients.

3.2. Bioinformatic results

The principal reason for selecting specific SNP positions was based on bioinformatic results and clinical value of the Poly-Phen database. In *axin1*, SNP with rs34440193 was selected, nucleotide C in the Ctg/Atg codon with the amino acid position L396M (C1558A) in *axin1* (exon 5), and its restriction enzyme is BstN1 with the CC ^ W_GG restriction site. In this project, the isoschizomer of the Mva1 enzyme has been used. Also, SNP with rs35289539 nucleotide A in aAg/aUg codon with the amino acid position K203M (A79013T) was selected in *axin1* (exon 2), and its specific enzyme is AflII with the restriction position of C ^ TTAA_G. In *axin2*, SNP was selected on the nucleotide C gene in the cCc/cTc codon with the amino acid position P609L (C2140T). Its specific enzyme is BsoB1 with the restriction position C ^ YCGR_G. However, the isoschizomer of the Ava-1 enzyme was selected.

The results of the mutation study with Swiss-PdbViewer showed that the proteins could be superimposed in order to deduce structural alignments and compare them with other relevant parts. Amino acid mutations, bonds, angles, and distances between atoms are obtained from the intuitive graphic. Therefore, by using this software, we were able to get appropriate information which indicated that the mutation in leucine 396 in exon 5 and lysine in exon 2 can reduce *axin1* protein stability and negatively affect its function (Table 3). Previous studies showed that the protein structure of *axin2* in the exon 7 region has not been predicted; therefore, changes after mutation in the amino acid position P609L (C2140T) couldn't be checked.

3.3. Experimental results

RFLP results specified that no mutation was observed in *axin1* in all samples and cell lines, but in *axin2* (exon 7), only one sample out of 147 patients (0.68%) was mutated in C2140T (Fig 1). The positive sample was sent for sequencing of both *axin1* and *2* genes. In addition to patient code No.1, 30% of samples of patients and normal subjects were randomly selected and sent for sequencing.

Table 3. Results of axin1 status prior- and post-mutation at positions 203 in exon 2 and 396 in exon5 by Chimera. Changes in structural energy are due to changes in bands, angles, electrostatic energies, and etc.

Residue	Total Energy	
	Prior- Mut	**Post- Mut**
position *203 in exon 2*		
Leu 202	-36.260	-36.757
Lys/Met 203	-15.479	-14.750
Ser 204	-34.354	-34.172
Total (Kj/mol)	-5993.308	-5991.417
position *396 in exon 5*		
Arg 395	-271.206	-274.652
Leu/Met 396	-36.220	-26.264
Glu 397	-35.723	-31.439
Total (Kj/mol)	-15800.676	-15787.013

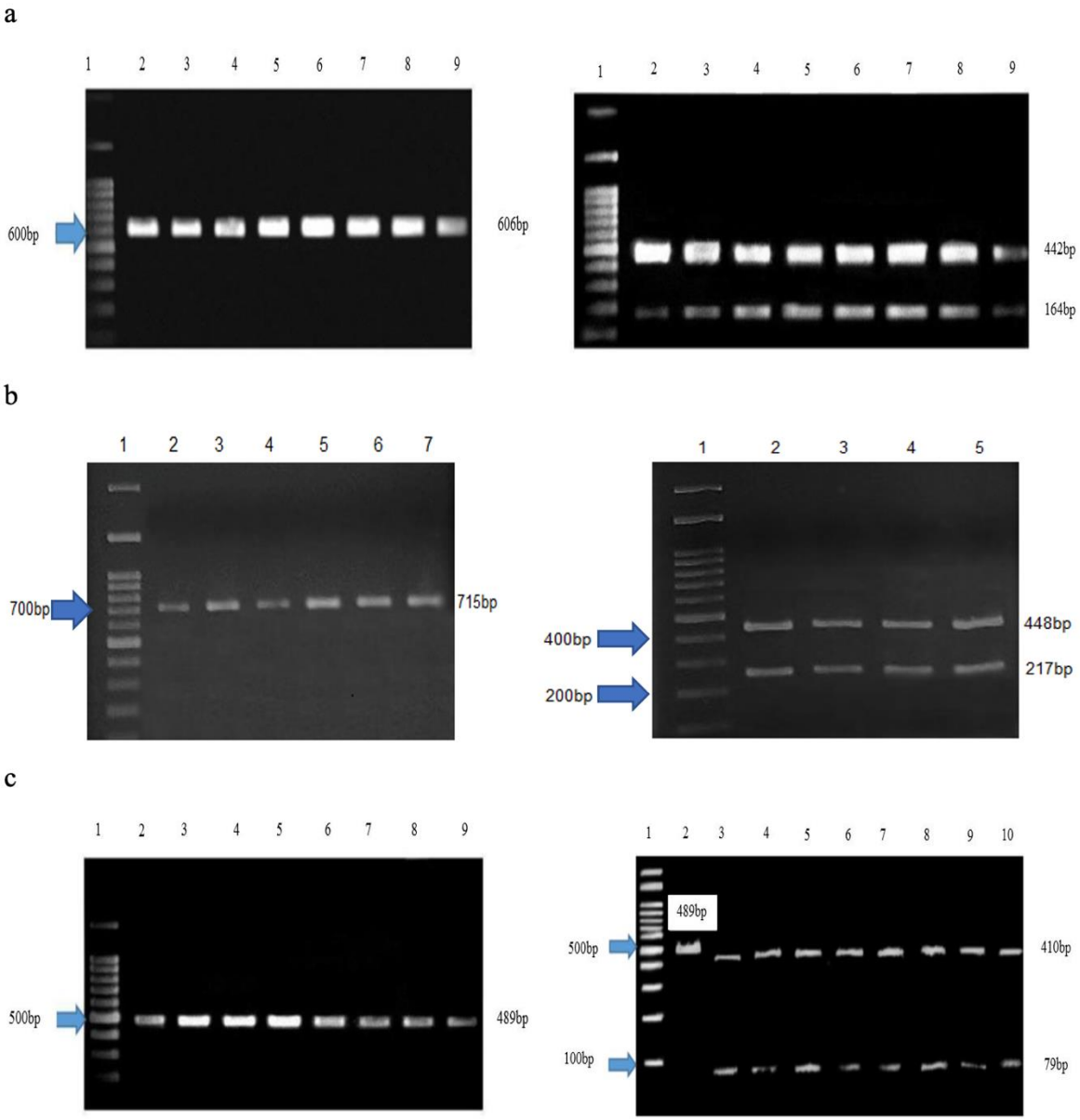

Fig 1. RFLP results; a) PCR products/606bp (left) and digested DNAs/164,442bp (right) for exon 5 of axin1; b) PCR products/715bp (left) and digested DNAs/217,448bp (right) for exon 2 of axin1; c) PCR products/489bp (left) and digested DNAs/79,410bp (right) for exon 7 of axin2).

The analysis of sequencing results showed several mutations in other nucleotides of these exons, indicating the importance of exons that were examined in CRC. The following main mutations are explained:

In the case of the patient with code No.1, two unexpected mutations were detected in exon 5 of the *axin1* gene including deletion at C1493 (rs538987269) and missense mutation A1553T (rs779671114) (Fig 2). Despite of C2140A (rs370618491) mutation, the SNP was selected for this study. And in other patient samples five other missense mutations were observed in exon 7 of the *axin2* gene including mutations G2041C (rs764205632), A2052C (rs79024445) and A2070G (rs747878470) (Fig 3) and mutations in G2109A (rs1060502129) and G2121C (rs145353986) (Fig 4).

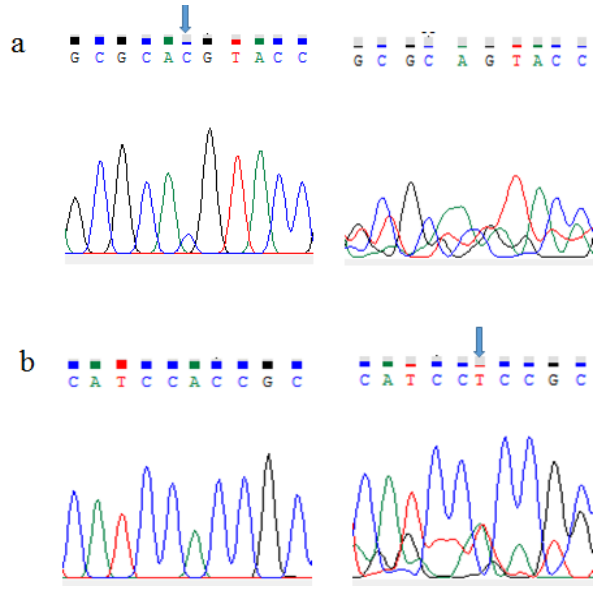

Fig 2. Sequencing results in sample 184 two mutations were observed in *axin1* (exon 5). As detail: a-Partial nucleotide sequence of the normal (left) and nucleotide removal of C1493 (frameshift mutation) in rs538987269 in exon 5 of *axin1* gene. B-Partial nucleotide sequence of the normal (left) and Missense mutation A1553T in rs779671114 in exon 5 of *axin1* gene. The blue arrow points toward base change in mutants with respect to normal sequences.

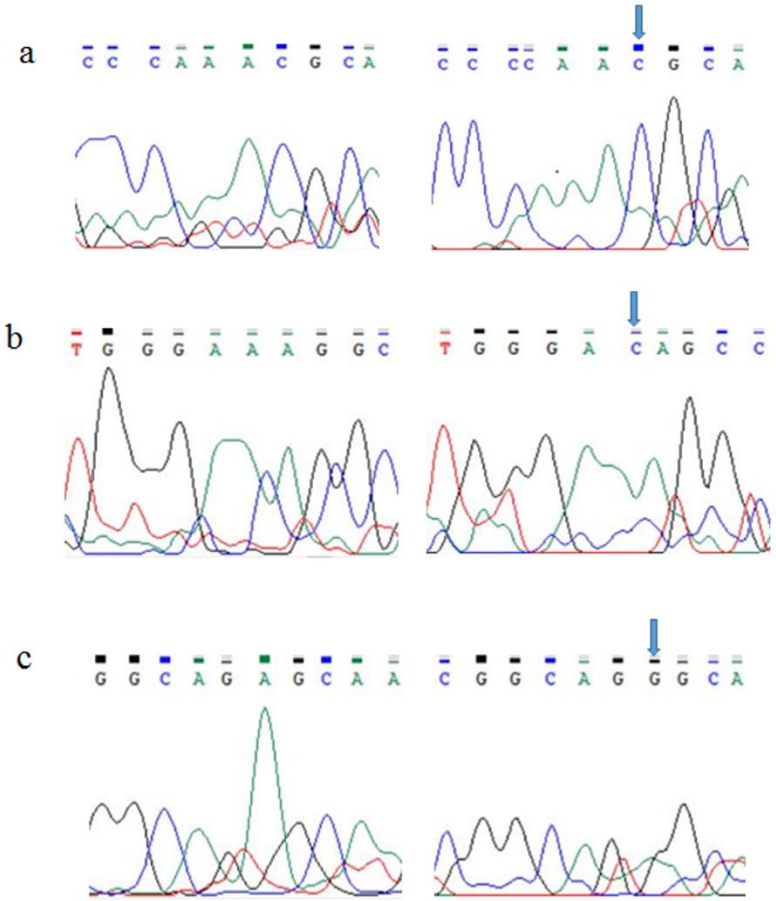

Fig 3. Sequencing results in the CACO2 cell line in exon 7 of *axin1* gene. Three new missense mutations were observed as detailed: a-Partial nucleotide sequence of the normal (left) and mutation A2052C in rs79024445, b-Partial nucleotide sequence of the normal (left) and mutation A2065C in rs769404267, and c- Partial nucleotide sequence of the normal (left) and mutation A2202G in rs763312277 in exon 7 of *axin1* gene. The blue arrow points toward base change in mutants with respect to normal sequences.

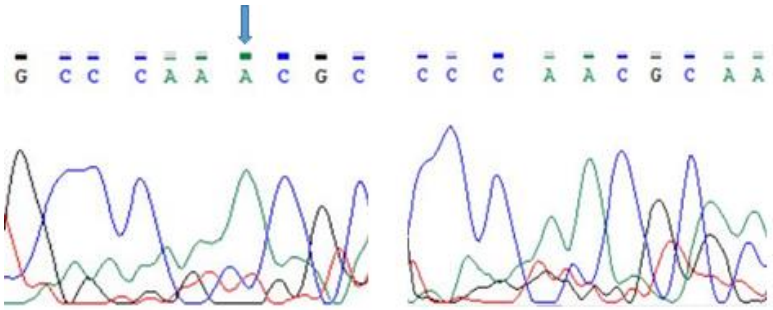

Fig 4. Sequencing results in the cell line SW480, in exon 7 of the *axin2* gene. The removal of nucleotide A2052 was observed in rs79024445. Partial nucleotide sequence of the normal (left) and mutant (right) in exon 7 of *axin2* gene. The blue arrow points toward base change in mutants with respect to normal sequences.

In the CACO2 cell line, five unexpected mutations were spotted as detailed: two mutations in the exon 2 of the *axin1* gene, including T-C (rs774448016) and C-T (rs765934562) (fig 5A), and three mutations in exon 7 of the *axin2* gene including A2052C (rs79024445), A2065C (rs769404267) and A2202G (rs763312277) (Fig 5B). In the SW480 cell line, the deletion mutation at A2052 (rs79024445) was observed in exon 7 of the *axin2* gene (Fig 6). There was no significant relationship between the results of experimental tests and bioinformatics search in the point of frequency of SNPs. However, in terms of relevance or possible role in causing cancer, a case of a new mutation is mentioned in the discussion.

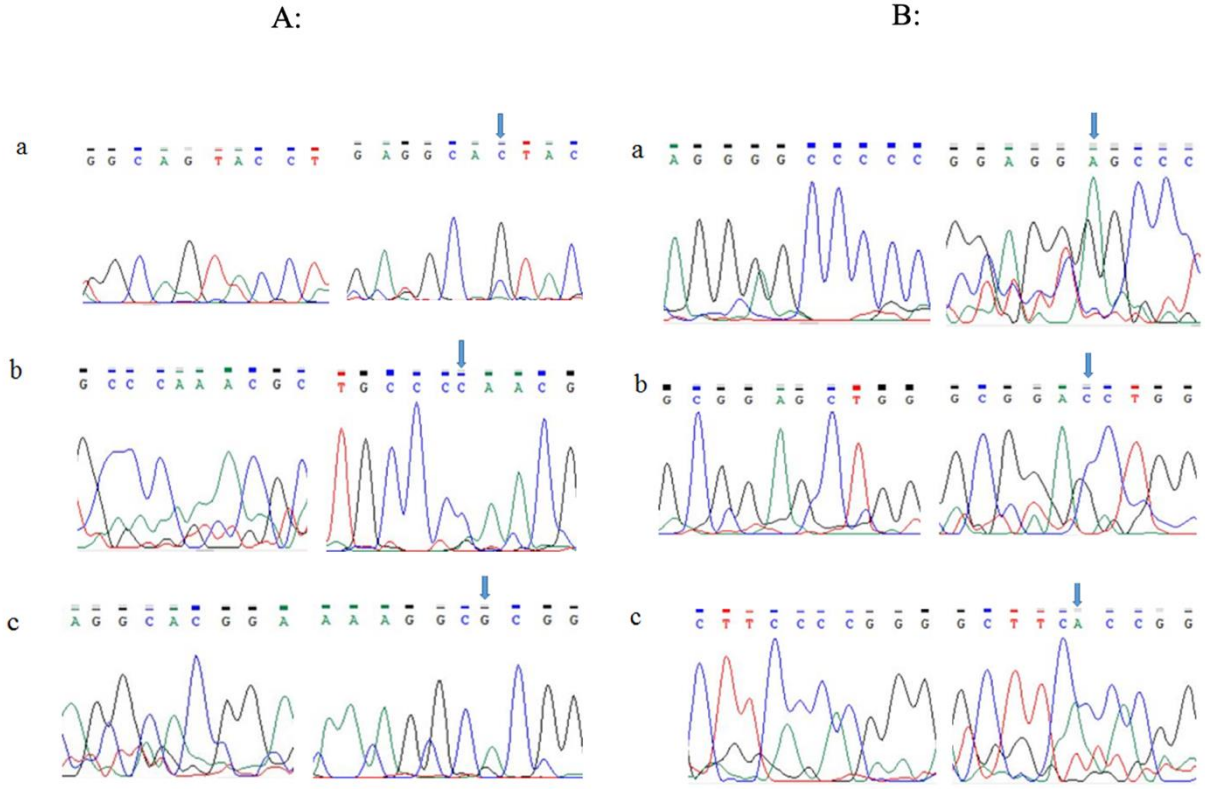

Fig 5. A: Sequencing results in sample 116. Three new mutations exon 7 of *axin2* gene were observed as detailed: A a-Partial nucleotide sequence of the normal (left) and mutation G2041C in rs764205632; Partial nucleotide sequence of the normal (left) and mutation A2052C in rs79024445 (b), and partial nucleotide sequence of the normal (left) and mutation A2070G in rs747878470 in exon 7 of axin2 gene (c). B: Sequencing results in sample 109. Three new mutations in exon 7 of *axin2* gene were observed as detailed: Partial nucleotide sequence of the normal (left) and mutation G2109A in rs1060502129 (a); partial nucleotide sequence of the normal (left) and mutation C2140A in rs370618491(SNP in *axin2* that was investigated in this project) (b), and partial nucleotide sequence of the normal (left) and mutation G2121C in rs145353986 in exon 7 of *axin2* gene (c). The blue arrow points toward base change in mutants with respect to normal sequences.

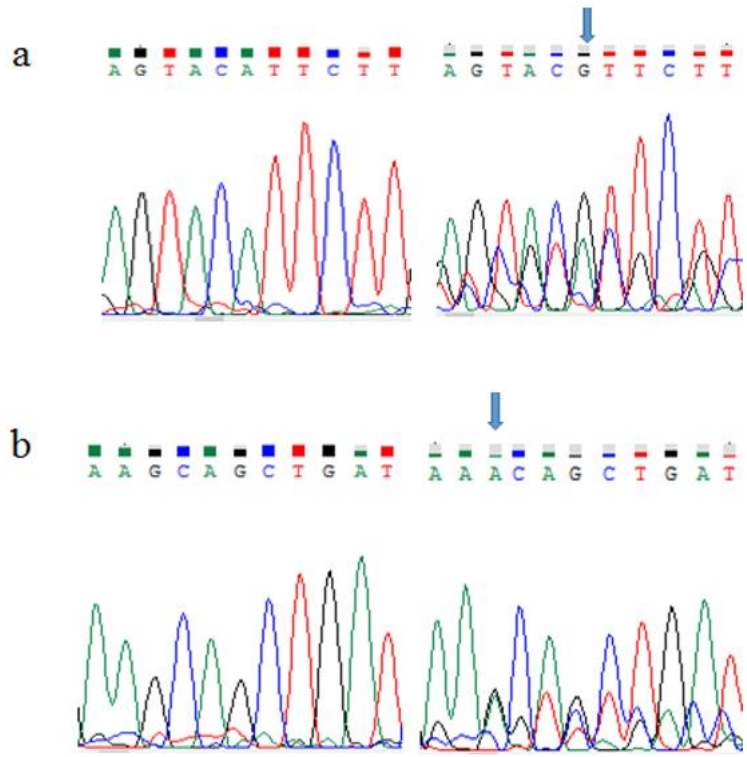

Fig 6. Sequencing results in the CACO2 cell line. Two new missense mutations in exon 2 of axin1 gene were observed as detailed: Partial nucleotide sequence of the normal (left) and mutation T-C in rs774448016 (a), partial nucleotide sequence of the normal (left) and mutation C-T in rs765934562 in exon 7 of axin2 gene (b). The blue arrow points toward base change in mutants with respect to normal sequences.

4. Discussion

In this study, we intended to do a survey on *Axin* 1 and 2 genes in case of potential roles in CRC by bioinformatic tools and databases and validate them through experimental investigations. *Axin* is a scaffold protein for the formation of a β-catenin degradation complex. It comprises several domains that regulate various pathways by connecting or cooperating with them, including Wnt/β-catenin, SAPK/JNK, TGF β, and P53 pathways (29). Somatic mutations, which affect *axin1* and *axin2* genes, have been observed in several CRC patients, but the functional effects of these mutations are unclear. In this present study, *Axin2* (exon 7) was observed in frameshift and missense mutations. Liu *et al.* observed a frameshift mutation in *axin2* (exon 7) in 11 of 45 cases of CRC, which resulted in a stop codon. It was stated in this study that mutation in this exon by activating the pathway of β-catenin/TCF results in ineffective mismatch repair; therefore, it plays a role in CRC (30). Contrarily, Li-Hua Jin et al. found an individual out of 54 Chinese patients who had a mutation in rs35289539 (31), which suggests that the importance of this SNP in other populations, such as the Chinese and due to higher the number of people studied in this study, these SNPs are probably less important in Iranian population. Khan et al. described that 3 samples (cell lines SW480, CACO2, and sample number 116) had a mutation in rs79024445 A2052C; the frequency of this mutation in this population was 2% (3 out of 147). Also, they observed mutation G>T (Gct> Tct) in *axin2* (exon 7) in codon G695T with 6% frequency, which could indicate the importance of this exon in the *axin2* gene and cancers (32). Despite our results which indicated L396M was evaluated in *axin1* (exon 5) and no mutation was observed in our studied samples, Webster et al. observed L396M point mutation in 3 cell lines, including HCT-8, HCT-15, and DLD-1. The significance of this region is due to its association with GSK-3, and the change significantly improves the ability of *axin1* to interact with two activators of TCF and

disheveled transcription so that it can lead cells to carcinogenic pathways (33). The main reason for diversity among bioinformatic and experimental results could be accounted for by multiple explanations, such as cancer risk factors high variety, genetic diversity in the Iranian population, limited sample population, and more importantly, a mutation occurrence rate which is less than 0.5% (34).

Peterlongo *et al*.'s study results were in line with previous reports of the role of *axin2* in the development of CRC. For analysis of 82 cases with familial inherited CRC, 20 variants of *axin2* in 19 samples showed that the role of *axin2* mutations in germ cells did not support the risk of CRC, as well as a mutation in G2121C in a patient's germ cell with CRC and control samples (35). In our study, a mutation in this region in one patient's sample was observed, but its significance cannot be verified due to the low abundance of both populations.

All unexpected mutations detected by sequencing in this study were checked with the protein *axin1* structure through a protein data bank (PDB), and none of them were in the interacting domains of *axin1* with other proteins except one. The rs779671114 with position A1553T and conversion of histidine to arginine (H394R) occurs in approximately 19 amino acid regions in the middle of the *axin1*, which is the area that interacts with GSK3-β. The crystallography structure of GSK3-β in complex with a 19 amino acid segment of *axin1* determined by Dajani, R et al. demonstrated that in addition to hydrophobic forces between the amino acids of two proteins, only one side chain-side chain hydrogen bond forms between the histidine 395 of *axin1* and the aspartate 264 of GSK3-β (36). The missense mutation that converts the H394 (close to the position involved in hydrogen bond) to arginine and also reported by Jin, L, and colleagues in a study on 54 CRC patients (31), results in the elimination of the single hydrogen bond that exist in the interaction space of *axin1* and GSK3-β analyzed by hex-8.0.0 and Chimera 1.10.2 software and PIC server (Fig 7). This mutation could affect the downstream events such as hyperphosphorylation of β-catenin resulting in ubiquitination and proteolysis. According to *in-silico* results mentioned above, there is an expectation of a reduction in the interaction affinity of *axin1* and GSK3-β which may lead to more stabilization of β-catenin and more activation of Wnt/β-catenin signaling pathway indeed. Since the mentioned mutation is observed only in one sample that was randomly sent for sequencing, and there is a possibility for the existence of this SNP in more samples, it should be specifically investigated in the Iranian population or other populations by several detection methods to estimate its frequency and clarify its potential role in Wnt signaling activation and tumorigenesis.

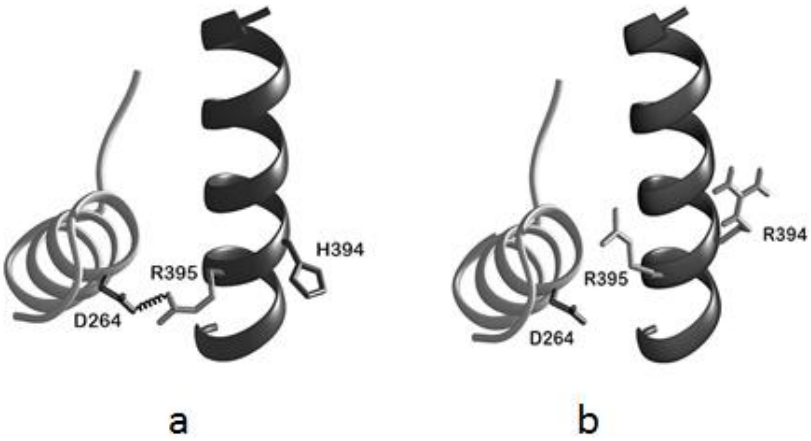

Fig 7. The region involved in the H-bond between GSK3-β and axin1 in normal and mutant form. R395 in axin1 (black chain) interacts with D264 in GSK3-β (gray chain) by H-bond in 1- normal and b- mutant. The H-bond between R395 in axin1 and D264 in GSK3-β eliminates after mutation of H394 to arginine.

In this project, only 147 patient tissues were collected due to constraints such as shortage of time, and difficulties in management for sample collection, so for characterization of a specific mutation and clarifying its potential role in a pathway, it is necessary to consider a larger sample size with more precise methods such as high-performance liquid chromatography (HPLC), in addition to sequencing. Also, in further studies, considering the potential changes made due to mutations like H394R in binding the GSK3-β to *axin1* by immunohistochemistry (IHC) could increase understanding in this matter. Other mutations related to exon 7 of *axin2* detected by sequencing were also considered through the protein structure database, and none were in the interacting domains. Further studies need to execute homology modeling for *axin2* due to the absence of a crystalized structure in the protein data bank. Furthermore, according to the experimental findings, the obtained *Axin* 2 variants from sequencing could be a potential case of study. Finally, according to the observed heterogeneity of mutations in *axin1* and *axin2*, it seems reasonable for studies to a propensity to personalized medicine and considering the genome of individuals supplemented by comprehensive and accurate demographic information of each patient.

5. Conclusion

Statistical analysis of patients' data in this study demonstrated that the prevalence of CRC is higher in men than in women; The increased risk of developing this disease with age, the importance of paying attention to a sharp decrease in weight without a cause, the relationship between the increase in cancer grade and the size of the tumor and the relationship between the degree of cancer and the degree of tumor's diffusion to the lymph nodes of patients. Sequencing results showed no mutation in the studied SNPs. Still, significant mutations were found in some other locations, which can affect the function of *axin1* and *axin2* as well as the Wnt pathway, therefore according to previous studies, these results suggest that exon 7 of *axin2* and exon 5 of *axin1* can be crucial in CRC. Given the importance of studying CRC, because of the increasing prevalence and high costs that this disease can impose on the individual and the community, the study of the factors influencing the prognosis of this disease is of particular importance. Instead, with recent studies, the importance of individual medicine and the role of rare variants have been proven in complex diseases and traits. The analysis of gene expression and the effect of *axin1* and *axin2* in future studies may determine the role of these proteins in CRC.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Authors contribution

LS& AJ: conducting the experimental study; RM: writing, and editing the manuscript; ShM: sample gathering; MB: writing the draft; MKh: Scientific and literary editing; ShMG: PI and supervision of the project. All authors read and approved the final manuscript.

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