


APC and AXIN2 Are Promising Biomarker Candidates for the Early Detection of Adenomas and Hyperplastic Polyps

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ABSTRACT: Aberrant activation of the WNT/CTNNB1 pathway is notorious in colorectal cancer (CRC). Here, we demonstrate that the expression of specific and crucial WNT signaling pathway genes is linked to disease progression in colonic adenomatous (AP) and hyperplastic (HP) polyps in an Iranian patient population. Thus, we highlight potential gene expression profiles as candidate novel biomarkers for the early detection of CRC. From a 12-month study (2016-2017), 44 biopsy samples were collected during colonoscopy from the patients with colorectal polyps and 10 healthy subjects for normalization. Clinical and demographic data were collected in all cases, and mRNA expression of APC, CTNNB1, CDH1, AXIN1, and AXIN2 genes was investigated using real-time polymerase chain reaction (PCR). CTNNB1 and CDH1 expression levels were unaltered in AP and HP subjects, whereas mRNA expression of APC was decreased in AP contrasted with HP subjects, with a significant association between APC downregulation and polyp size. Although AXIN1 showed no changes between AP and HP groups, a significant association between AXIN1 and dysplasia grade was found. Also, significant upregulation of AXIN2 in both AP and HP subjects was detected. In summary, we have shown increased expression of AXIN2 and decreased expression of APC correlating with grade of dysplasia and polyp size. Hence, AXIN2 and APC should be explored as biomarker candidates for early detection of AP and HP polyps in CRC.

KEYWORDS: Colorectal polyps, gene expression, WNT/CTNNB1 signaling, colorectal cancer, biomarker

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Introduction

A status report on the global burden of cancer worldwide estimated lung cancer is the most frequent cancer and the leading cause of cancer death among males, followed by prostate and colorectal cancer (CRC) for incidence. While among females, breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death, followed by CRC and lung cancer.¹⁻⁴ Colorectal cancer develops through 6 independent classification systems consisting of 4 consensus molecular subtypes (CMS) with distinguishing features: CMS1 (Microsatellite Immune, 14%), hypermutated, microsatellite unstable, strong immune activation; CMS2 (Canonical, 37%), epithelial, chromosomally unstable, marked WNT and MYC signaling activation; CMS3 (Metabolic, 13%), epithelial, evident metabolic dysregulation; and CMS4 (Mesenchymal, 23%), prominent

transforming growth factor β activation, stromal invasion, and angiogenesis.⁵⁻⁷

Most cases of CRC progress from precursors known as colorectal polyps; the 2 common types of polyps include hyperplastic (HP), which do not carry a risk of developing into cancer, and adenomatous polyps (AP). Adenomatous polyps further divided into 3 main subgroups: tubular adenoma (TP), tubovillous (TVP), and villus (VP). Recently, another type of polyp has been identified as sessile serrated polyps (SSA), which originate from HP and have been recognized as markers for synchronous and metachronous colorectal neoplasia and also premalignant lesions.⁸⁻¹⁰ The classic model that explains CRC development is the adenoma-carcinoma sequence correlated with activation of the WNT signaling pathway.¹¹⁻¹³



The human genome includes 19 WNT genes, falling into 12 conserved WNT subfamilies. WNT proteins regulate the proliferation of cells, and activation of the WNT signaling pathway as a result of genetic alterations of APC, AXIN1, and CTNNB1 has been found in various human cancers, including colon, liver, endometrium, ovary, prostate, and stomach cancers. The WNT signaling pathway is critical in the regulation of many biological processes and is one of the preliminary mechanisms which confer cell proliferation and cell polarity during tissue homeostasis and embryonic development.^{14,15} In this way, defects in WNT signaling are often associated with cancers, human birth defects, and other disorders.^{16,17} Recently, studies have demonstrated that abnormal activation of the WNT pathway plays critical roles in tumor cell differentiation and proliferation.¹⁸ Activation of the WNT pathway requires nuclear accumulation of β -catenin (CTNNB1) and the binding of CTNNB1 to T-cell factor 4 (TCF4).^{15,18,19} The most important act of WNT/CTNNB1 signaling is the maintenance of the stem cell-like character of crypt cells in the colon environment.²⁰⁻²² WNT has a lot of subtypes and it is indefinite which of them affect development of CRC.^{23,24} Thus, the investigation of WNT pathway genes in colorectal polyps as precursor lesions of CRC patient biopsies is essential for early malignant polyp detection. As there is limited information on the expression of WNT signaling pathway genes and different histological types of polyps, we aimed to focus on establishing mRNA expression levels of some critical WNT signaling pathway genes, including APC, CTNNB1, AXIN1, AXIN2, and E-cadherin (CDH1) in AP and HP cases of the Iranian population, aiming to identify novel biomarkers for the early detection of CRC.

Materials and Methods

Human sample collection

The present study was descriptive-analytical, and the investigated population was chosen from cases with colorectal polyps that had been referred to the Taleghani Hospital, Shahid Beheshti University of Medical Sciences, Tehran-Iran, from October 2016 to April 2017. In total, 44 biopsy samples were collected during colonoscopy from the patients with colorectal polyps and 10 healthy subjects for normalization. All experiments in this research were undertaken with the understanding and written consent of each subject, and that the study conforms with the Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the *British Medical Journal* (July 18, 1964). This research was reviewed and approved by the Research Institute for Gastroenterology and Liver Diseases (RIGLD) ethics committee at Shahid Beheshti University of Medical Sciences, Tehran, Iran (No. 1395.831).

Sample type selection was done randomly. Colorectal polyps were identified at colonoscopy and confirmed by pathology, and biopsies of 10 healthy subjects were collected for

normalization. Polyp-free controls were defined as those with no polyps identified during colonoscopy and no previous history of colorectal polyps. Exclusion criteria included any invasive medical intervention within the past 6 months, a past history of any cancer, the presence of other GI disorders, and inflammatory or infectious diseases of the intestine. Biopsies were immediately frozen and stored in RNA Later (Qiagen, Hilden, Germany) at 80°C.

Demographic and medical history assessment

Demographic information including age, sex, height, weight, family history, diabetes mellitus history, smoking habits, physical activity, GI disease history, and alcohol consumption was collected via questionnaire. Medical records including colonoscopy and pathology reports were collected directly from surgery and pathology departments, respectively.

RNA extraction, and quality control and complementary DNA synthesis

Total RNA was extracted from all samples by QIAamp RNA Mini Kit (Qiagen, Hilden, Germany). RNA concentration and purity ratios (OD260/280, OD260/230) were measured with NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The integrity of RNA was determined by electrophoresis on a denaturing 1.5% agarose gel. Total RNA was reverse-transcribed to first-strand complementary DNA (cDNA) according to the manufacturer's instructions (Thermo Scientific RevertAid Reverse Transcriptase 2 Step Kit, Thermo Fisher Scientific, Inc, Waltham, MA, USA). Briefly, 5 μ L RNA (100 ng/ μ L) was incubated with 1 μ L random hexamer primer (0.2 μ g/ μ L) and 6 μ L of nuclease-free water accordingly at 65°C for 5 minutes, then 8 μ L of the mixture was added to 12 μ L reaction solution, containing 4 μ L of 5 \times reaction buffer, 2 μ L of dNTPs (10 mM), 1 μ L of RiboLock RNase Inhibitor (20 U/ μ L), and 1 μ L of RevertAid RT (200 U/ μ L). The reaction was then performed at 25°C for 5 minutes, at 42°C for 60 minutes and at 70°C for 5 minutes using the Eppendorf Amp PCR System (Eppendorf AG 22331 Hamburg, Germany). The prepared cDNA was then stored at -70°C.

Oligonucleotide primers and polymerase chain reaction

Primer pairs were designed by using the primer express software to quantitative polymerase chain reaction (qPCR) recommendations (Applied Biosystems, CA, USA), then the different pair of primers was also tested for their specificity using the database similarity search program, nucleotide-nucleotide BLAST (Table 1). Optimal annealing temperatures of the primer pairs were established via PCR, and expected PCR product sizes were evaluated with gradient PCR. The

Table 1. Primers.

PRIMER	SEQUENCE	LENGTH (BP)	GC%	SIZE (BP)	ANNEALING TEMPERATURE (°C)
AXIN1 F	5'-TCACCCTGGGCCAGTTCAA-3'	19	57	85	61.8
AXIN1 R	5'-CAGTCAAACCTCGTCGCTCACTTTC-3'	24	50		62.34
AXIN2 F	5'-GTCTCTACCTCATTTCGCCGAGAAC-3'	24	50	88	60.44
AXIN2 R	5'-CGAGATCAGCTCAGCTGCAA-3'	20	55		60.46
APC F	5'-CCTCATCCAGCTTTTACATGGC-3'	22	50	78	59
APC R	5'-GCCCCGAGCCTCTTTACTGC-3'	19	63		60
β-catenin F	5'-GTGCTATCTGTCTGCTCTAGTA-3'	22	45.45	154	56
β-catenin R	5'-CTTCCTGTTTAGTTGCAGCATC-3'	22	45.45		57
E-cadherin F	F: 5'-TACACTGCCAGGAGCCAGA-3'	20	60	103	62
E-cadherin R	R: 5'-TGGCACCAGTGTCCGGATTA-3'	20	55		61

amplification reactions were carried out in a whole volume of 25 μ L, with 1 μ L of cDNA extract as a template. The PCR mixture consisted of 2.5 μ L incubation buffer (10 \times), 1 μ L MgCl₂ (50 mM), 0.5 μ L dNTPs (10 mM), 1 μ L each primer (10 pmol), and 0.25 μ L Taq polymerase (500 U/ μ L, Gene Fanavaran, Iran). The amplification reaction consisted of an initial denaturation step at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 45 seconds, primer annealing at 55°C to 65°C (based on primer types) for 45 seconds, and primer extension at 72°C for 30 seconds, with a final extension step at 72°C for 5 minutes.

Quantitative PCR

Real-time PCR was performed according to the manufacturer's instructions (SYBR Premix Ex Taq Kit, TaKaRa Bio, Inc, Otsu, Japan), using a final volume of 20 μ L, comprising 5 μ L cDNA and 1 μ L of 10 pmol forward and reverse primers. Thermal cycling conditions were as follows: an initial denaturation at 95°C for 30 seconds, followed by 37 cycles of 95°C for 5 seconds and 60°C annealing and extension for 30 seconds. The specificity of the PCR amplification product was confirmed by electrophoresis in a 2% agarose gel. Gene expression levels of tumor and normal tissue samples were calculated according to the $\Delta\Delta$ Ct method with Δ Ct = average of Ct target - average of Ct β -actin. β -ACTIN (ACTB) mRNA was utilized to calculate the relative abundance of mRNA transcripts. Each measurement was performed in triplicate. Fold change (FC) values indicate expression levels relative to normal tissue samples.

Statistical analysis

Data are expressed as the mean \pm SD, and the statistical analysis was executed using the Prism software (Version 5). The analysis

of continuous variables was performed with *t*-test and one-way analysis of variance (ANOVA), followed by an appropriate post hoc test. FCs of $P \leq .05$ were used as the criteria for the selection of significant differentially expressed genes.

Results

Demographic and clinical characteristics of samples

The demographic patient characterization—consisted of sex, age, alcohol consumption, smoking, rectal bleeding, diarrhea, constipation, abdominal pain, weight loss, and family history—is interpreted in Table 2. The clinical and pathological features of the polyps are outlined in Table 3. Pathology reports indicated that 68.2% of the lesions were adenomatous, including TP (43.3%), TVP (26.6%), and VP (30%); meanwhile, the HP and SSA comprised 64.3% and 35.7% of samples, respectively. The main pathological characteristics of study participants such as size, location, and comparison between AP and HP groups are shown in Table 4. Low-grade dysplasia was observed in 76.6% of AP and 85.7% of HP cases, while high-grade dysplasia was found in 23.3% of AP cases and only 14.2% of HP participants. Samples more than 5 mm in size were observed among 6.6% of AP and 28.6% of HP, while 93.3% of AP and 71.4% of HP subjects were \leq 5 mm. Most polyps were smaller than 5 mm with low-grade dysplasia. Moreover, about 80% of these AP and 64.2% of HP were situated in the colon. However, these differences were found to be not statistically significant (Table 4). Distribution of targeted genes based on Risk Quotient (RQ) between HP and AP is depicted in Figure 1.

CTNNB1 mRNA expression in the colorectal polyps

CTNNB1 mRNA expression in colorectal polyps showed no expression changes in both AP and HP groups compared with

Table 2. Demographic and clinical characteristics of the polyp cases in the present study.

CHARACTERISTICS	VARIABLE	FREQUENCY
Polyp types	Adenoma	30 (68.2%)
	Hyperplastic	14 (31.8%)
All polyp types	Serrated	5/14 (35.7%)
	Hyperplastic	9/14 (64.3%)
	Villus	9/30 (30%)
	Tubular adenoma	13/30 (43.3%)
	Tubule villus	8/30 (26.6%)
Dysplasia	Low	34 (77.2%)
	High	10 (22.7%)
Size (mm)	Valid \leq 5	38 (86.3%)
	Valid $>$ 5	6 (13.6%)
Location	Colon	39 (88.6%)
	Rectum	5 (11.3%)

Table 3. Pathological characteristics of the polyp cases in the present study.

CHARACTERISTICS	VARIABLE	FREQUENCY (%)
Sex	Male	22 (50)
	Female	22 (50)
Smoking	Yes	8 (18.2)
	No	36 (81.8)
Alcohol	Yes	5 (11.3)
	No	39 (88.6)
Age	$>$ 50	25 (56.8)
	\leq 50	19 (43.1)
Blood per rectum	Yes	5 (11.3)
	No	44 (88.6)
Diarrhea	Yes	4 (9)
	No	40 (90)
Constipation	Yes	6 (13.6)
	No	38 (86.3)
Abdominal pain	Yes	7 (15.9)
	No	37 (84.1)
Weight loss	Yes	4 (9)
	No	40 (90)
Family history	Yes	11 (25)
	No	33 (75)
Total		44 (100)

normal participants. Also, no significant differences were found in expression of this gene versus location, size, and grade of dysplasia (Table 5).

APC mRNA expression in the colorectal polyps

APC expression in polyps was significantly decreased in AP subjects compared with HP polyps ($P < .0001$). Moreover, a significant association was found between APC mRNA down-regulation and polyp size ($P < .0003$). No significant association was found between polyp grade and APC mRNA expression level (Table 5).

AXIN1 mRNA expression in the colorectal polyps

AXIN1 expression in polyps showed no significant changes between AP and HP compared with the control group. Also, no significant association existed between AXIN1 mRNA expression and location of the polyp, but association between AXIN1 expression level and grade of dysplasia was significant ($P < .004$, Table 5).

AXIN2 mRNA expression in the colorectal polyps

AXIN2 expression in polyps displayed significantly upregulation in both AP and HP colorectal polyps compared with normal participants ($P < .0001$). However, there was no association between AXIN2 upregulation and grade, location, and size of polyp (Table 5).

CDH1 mRNA expression in the colorectal polyps

CDH1 expression in CRC polyps showed no significant differences in CDH1 expression in both AP and HP groups compared with normal participants. However, a significant association between polyp location (colon/rectum) and CDH1 expression was observed ($P < .002$).

Discussion

To achieve early diagnosis in CRC and improve the management of patients, investigation into the underlying molecular events in different polyp subtypes is essential, and biomarker discovery for early malignant polyp detection is a critical step in CRC prevention.^{25,26} The WNT signaling cascade plays a vital role in embryogenesis and its deregulation is also implicated in carcinogenesis.^{27,28} Colorectal cancer cases may have alteration in one or more of the genes of the activated WNT signaling pathway during colorectal carcinogenesis.^{29,30}

In this study, we have quantified the expression levels of essential WNT signaling pathway genes including APC, CTNNB1, CDH1, AXIN1, and AXIN2 in colonic AP and HP cases compared with age-matched control subjects. A particular strength of our study is that every patient withstood a complete colonoscopy, full visualization of the colon and different types of polyp removed during colonoscopies were all reviewed and

Table 4. Selected characteristics of study participants by comparison between adenoma (HP, SSA) and hyperplastic (TP, TVP, and VP) groups.

CHARACTERISTICS		ADENOMA, N (%)	HYPERPLASTIC, N (%)	P-VALUE
Grade	Low	23 (76.6)	12 (85.7)	.38
	High	7 (23.3)	2 (14.2)	
Size (mm)	Valid ≤5	32 (93.3)	10 (71.4)	.06
	Valid >5	2 (6.6)	4 (28.6)	
Location	Colon	24 (80)	9 (64.2)	.58
	Rectum/sigmoid	6 (20)	5 (35.8)	

Abbreviations: HP, hyperplastic polyps; SSA, sessile serrated polyps; TP, tubular adenoma polyp; TVP, tubuovillous polyp; VP, villus polyp.

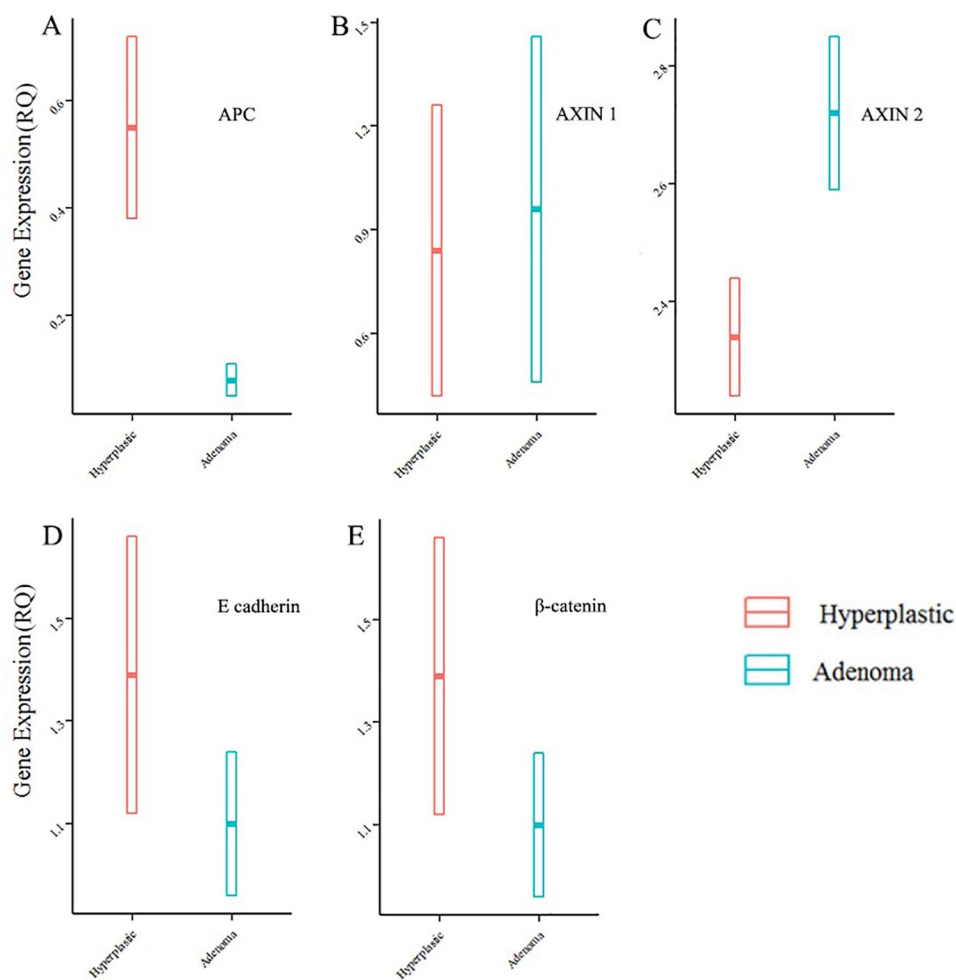


Figure 1. Expression of targeted genes (A) APC, (B) AXIN1, (C) AXIN2, (D) E-cadherin, and (E) β-catenin, in hyperplastic and adenomatous groups.

classified by the same pathologist. Critically, we demonstrate here that the expression level of AXIN2 was significantly increased in both types of AP and HP subjects ($P < .0001$). According to the relative expression levels of AXIN2 in both types of polyp, it could be assumed that dysregulation of AXIN2 may be occurring as a first step in changing intestinal tissue toward CRC initiation. Crucially, AXIN2 should be explored further as a biomarker for the characterization of AP and HP in the first step of CRC initiation. AXIN and conductin (also known as AXIN2) are

structurally related inhibitors of Wnt/β-catenin signaling that promote degradation of β-catenin. Whereas AXIN is constitutively expressed, conductin is a Wnt target gene implicated in Wnt negative feedback regulation. Therefore, AXIN2 participates in a negative feedback loop, which could serve to limit the duration or intensity of a Wnt-initiated signal.³¹⁻³⁴ Our results confirmed those of Schaal et al,³⁵ who also demonstrated AXIN2 mRNA level increases in CRC initiation and progression, although they found no association between heightened AXIN2

Table 5. β -catenin, APC, AXIN1, and AXIN2 mRNA expression level in adenomas and hyperplastic polyp cases of the present study.

CHARACTERISTICS	POLYP		LOCATION		SIZE		GRADE	
	ADENOMA	HYPERPLASTIC	COLON	RECTUM	≤ 5	> 5	HGD	LGD
	SD \pm MEAN OF RQ	SD \pm MEAN OF RQ	SD \pm MEAN OF RQ	SD \pm MEAN OF RQ	SD \pm MEAN OF RQ	SD \pm MEAN OF RQ	SD \pm MEAN OF RQ	SD \pm MEAN OF RQ
β -catenin	1.10 \pm 0.14 <i>P</i> -value: 0.6	1.39 \pm 0.27	1.25 \pm 0.20 <i>P</i> -value: 0.7	1.12 \pm 0.19	1.15 \pm 0.1 <i>P</i> -value: 0.7	1.59 \pm 0.4	1.38 \pm 0.51 <i>P</i> -value: 0.53	1.10 \pm 0.15
APC	0.08 \pm 0.03 <i>P</i> -value: <.0001	0.55 \pm 0.17	0.27 \pm 0.12 <i>P</i> -value: .58	0.38 \pm 0.10	0.10 \pm 0.03 <i>P</i> -value: .0003	0.81 \pm 0.27	0.51 \pm 0.31 <i>P</i> -value: .21	0.17 \pm 0.07
AXIN1	0.96 \pm 0.50 <i>P</i> -value: .75	0.84 \pm 0.42	0.95 \pm 0.05 <i>P</i> -value: .36	0.78 \pm 0.26	0.94 \pm 0.05 <i>P</i> -value: .81	0.89 \pm 0.09	0.70 \pm 0.05 <i>P</i> -value: .004	1.03 \pm 0.06
AXIN2	2.72 \pm 0.13 <i>P</i> -value: <.0001	2.34 \pm 0.10	2.58 \pm 0.11 <i>P</i> -value: .08	1.94 \pm 0.28	2.53 \pm 0.11 <i>P</i> -value: .44	2.73 \pm 0.13	2.26 \pm 0.12 <i>P</i> -value: .06	2.52 \pm 0.08
E-cadherin	1.43 \pm 0.38 <i>P</i> -value: .41	1.07 \pm 0.27	1 \pm 0.18 <i>P</i> -value: .002	2.18 \pm 0.31	1.41 \pm 0.21 <i>P</i> -value: .26	0.99 \pm 0.25	0.97 \pm 0.28 <i>P</i> -value: .30	1.47 \pm 0.33

expression and clinical parameters such as location, survival rate, and grade of dysplasia. However, we have observed direct and significant association between upregulation of this target gene and colon location compared with rectum. Hence, we conclude that increased AXIN2 in those polyp cases which located in the colon site may have a different molecular signature and progression of tumor formation compared with those located in rectum site. Also, increased AXIN2 in both HP and AP cases indicated that this gene may be involved in the process of deformation and malignancy of the large intestinal tissue.

Kim et al³⁶ showed that p53 regulates GSK-3 β nuclear localization via miR-34-mediated suppression of AXIN2 in CRC. Therefore, the causal link between loss of p53 function, increasing AXIN2, and tumorigenesis has been clearly demonstrated by their group and ours.³⁷ Also, Wei et al³⁸ demonstrated increased levels of AXIN2 and CTNNB1 and their important role in the tumorigenesis and progression of ameloblastoma.³⁷ They declared that increasing expression level of CTNNB1 causes its entry into the nucleus, and combining with TCF, it activates AXIN2 and enables AXIN2 to transcribe, causing abnormal expression and creating negative feedback inhibition in the Wnt signaling pathway.³⁷ Also, based on previous studies, nuclear CTNNB1 mRNA expression accumulated and could be another candidate biomarker associated with invasion, metastasis, and poor prognosis of CRC.^{38,39} We achieved the same upregulation of AXIN2 in HP and AP cases, and in activation of the WNT signaling pathway. This upregulation of AXIN2 expression was significantly higher in cases over 50 years old ($P < .0005$). Hence, age could also be considered as an important risk factor for precursor of CRC. Neither Wei et al,³⁸ nor our own previous study on CRC cases observe any expression changes for CTNNB1 in both AP and HP groups compared with normal participants, and there was no significant difference among the expression of this gene and location, size, and grade of dysplasia data.

This may have been due to differences in study populations, sample collection, and evaluating technique. We did not find any alterations in AXIN1 mRNA expression between AP and HP compared with normal control samples, which is in contrast to previous studies regarding reduction of AXIN1 in malignant behavior of lung cancers, meningeal brain tumors, oral squamous cell carcinoma carcinogenesis, metastasis, and esophageal squamous cell carcinoma.³⁹⁻⁴³ It may be concluded that this gene is ineffective during early steps of the changes in the colon tissue and CRC initiation, while other studies mentioned above focused on the expression of this target gene after tumor formation and metastasis.

Crucially, we have observed significantly reduced APC mRNA expression levels in adenomas compared with hyperplastic participants ($P < .0001$). Moreover, significant associations existed between the APC mRNA downregulation and the size of the polyps ($P < .0003$). Inactivation of the tumor-suppressor gene APC, a key regulator of the WNT signaling pathway, has been proven as one of the earliest transforming events observed

in colorectal tumorigenesis by previous studies.⁴⁴⁻⁴⁷ Thus, other signaling pathways seem to play more important roles in the growth and development of precancerous lesions toward CRC, and it may be that APC gene alteration occurs in early stage of carcinogenesis and not in the process of polyp formation. APC may be altered by DNA sequence changes and/or by promoter hypermethylation in most colorectal carcinomas.

Finally, Jurčić et al,⁴⁸ reported no significant differences regarding the expression of E-cadherin in the primary tumor of CRC. Indeed, these results are similar to our own findings in that we did not observe significant differences in CDH1 expression in both AP and HP groups compared with normal participants. Due to the small sample size and the inconclusive results obtained, further research is required to implement these parameters as prognostic factors.

Conclusions

This study demonstrates that CTNNB1 and CDH1 expression levels were unaltered in AP and HP subjects, whereas mRNA expression of APC was decreased in AP contrasted with HP subjects, with a significant association between APC downregulation and polyp size. Although AXIN1 showed no changes between AP and HP groups, a significant association between AXIN1 and dysplasia grade was found. Also, significant upregulation of AXIN2 in both AP and HP subjects was detected. Overall, we propose that this panel of genes, particularly AXIN2 and APC, may be of significant usefulness as biomarker candidates for the early detection of AP and HP in CRC patients. Due to the small sample size and some negative results obtained, further research is required to implement these parameters as prognostic factors.

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Author Contributions

SR, ENM, and MAB conceptualized the study, conducted the formal analysis and investigation, and wrote the original draft of the article. ENM and MAB supervised the study. And all authors developed the study methodology and reviewed and edited the article.

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