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The Interplay among *Wnt*/β-*Catenin* Family Members in Colorectal Adenomas and Surrounding Tissues

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Abstract: Background: Colorectal adenoma undergoes neoplastic progression via the normal epithelium-adenoma-adenocarcinoma sequence as reported in the Vogelgram. The risk of developing cancer is strongly associated with the number and size of adenoma and with the subtype. Currently, adenomatous polyps can be distinguished on the basis of histology: the prevalence are tubular, 5-15% are villous and tubular/villous. Considering the increased risk for malignant transformation described for tubular/villous adenomas, patients diagnosed with adenomatous polyposis are at increased risk of developing CRC. The Wnt/β-catenin pathway plays a key role in the onset of colorectal adenoma, in particular intestinal cells first acquire loss-of-function mutations in *APC* gene that induce the formation of adenomas. Methods: Wnt/β-catenin pathway *APC, Wnt3a, Wnt5a, LEF1, BCL9* genes and protein expression analyses were conducted by qRT-PCR and western blot in 68 colonic samples (polyps and adjacent mucosa) from 41 patients, of which 17 affected by FAP. Ten normal colonic mucosal samples were collected from 10 healthy donors. Results: In this study both *APC* gene and protein resulted less expressed in colon tumor compared to the adjacent colonic mucosa. Conversely, activated β-catenin was more expressed in polyps than in the adjacent mucosa. All results confirmed literature data on carcinomas. A statistically significant correlation between *Wnt3a* and *BCL9* both in polyps and in the adjacent mucosa underlies that the canonical Wnt pathway is activated in early colon carcinogenesis and that the adjacent mucosa is already altered. Conclusion: This is the first study analyzing the difference in expression of Wnt/β-catenin pathway in human colorectal adenomas. Understanding the progression from adenomas to colorectal carcinomas is essential for the development of new therapeutic strategies and improving clinical outcomes.

Keywords: CRA; colorectal adenoma; *APC; Wnt3a; Wnt5a; LEF1; BCL9;* polyps; early carcinogenesis; Wnt/β-catenin

1. Introduction

The prevalence of colorectal adenomas (CRA) increases with age, mainly in Western populations, and it is of 30-40% in the people over 50 years, predominantly in men [1,2]. The annual rate of adenoma progression to colorectal cancer (CRC) is ~0.25% [3].

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CRA is associated with colorectal cancer (CRC), and at least 80% of CRC undergo neoplastic progression via the normal epithelium-adenoma-adenocarcinoma sequence as reported in the Vogelgram model [4,5]. The incidence of cancer after a negative colonoscopy is significant because adenomas may be missed during colonoscopy or biological changes in tumor growth rate may occur [6]. Screening and surveillance programs can help identify precursor lesions and prevent death from CRC [7]. Thus, it is important to understand the progression from CRA to carcinomas to facilitate the development of novel treatment strategies and improve clinical outcomes.

The malignancy of adenomas is highly correlated with the occurrence of colon cancer and its depending on the subtype [8]. In addition, the risk of developing cancer is strongly associated with the number and size of (previously) encountered polyps [9]. Therefore, the development of multiple colonic polyps with malignant potential will result in an increased lifetime risk of developing CRC. At least three subtypes of polyps can be distinguished on the basis of histology and the underlying molecular pathway: adenomatous, serrated or hyperplastic (non-neoplastic) [10–12]. The first are characterized by an adenomatous histotype, whereas both sessile/traditional serrated adenomas and hyperplastic polyps have a serrated histotype [13]. The adenomatous can be tubular (more than 80%), villous (5-15%) and tubular/villous (5-15%). Hyperplastic polyps are very common and have a very low malignant potential [8]. Even though the different types of polyps may be disseminated in all the large bowel, adenomatous and hyperplastic polyps are prevalently located in distal colon [14–16] and sessile serrated polyps are often found in proximal colon [17–20]. Owing to the malignant potential of tubular/villous adenomas, patients diagnosed with adenomatous polyposis, i.e. the constitutive development of multiple colorectal adenomas, are at increased risk of developing CRC.

Most studies investigating the carcinogenesis of *CRA* have focused on villous and familial adenomatous polyps, which have the highest rates of carcinogenesis [21,22]. Whereas, few studies have investigated sporadic tubular adenoma, which has the highest clinical incidence [23,24].

Adenomas are the main precursors of colorectal cancer in high-risk family groups with a history of polyposis syndrome and in the general population. Indeed, genetic events such as gain or loss of function of molecules required for intestinal cell functional homeostasis can lead to the development of polyps [25].

The Wnt/β-catenin signaling deregulation is an early event in the onset of colorectal adenoma [26]. Its up-regulation is mainly due to the altered functions of the adenomatous polyposis coli (APC) protein, which reduces differentiation of intestinal epithelial cells (IEC), leading to the onset of adenoma and CRC progression. Furthermore, mutations and LOH of *APC* alter the quantitative regulation of the β-catenin protein, which accumulates in the nucleus, favoring the activity of transcription factors for cell proliferation genes expression and reducing differentiation [27]. Less than 1% of CRC cases belong to Familial adenomatous polyposis (FAP), an autosomal dominant inherited CRC syndrome as result from a germline mutation in the *APC* gene. Most patients (~70%) have a family history of colorectal polyps and cancer. FAP is characterized by the development of many tens to thousands of adenomas in the rectum and colon during the second and third decade of life. APC is essential for IEC homeostasis and its inactivation facilitates tumorigenesis. Indeed, *APC* somatic truncation mutations are observed in more than 90% of human colon cancers [28,29]. Wnt ligands may activate the canonical (β-catenin dependent) and the non-canonical (β-catenin independent) pathways. They are in concert to maintain renewal, defense and metabolic homeostasis of colon epithelia [30,31].

Most of the cellular β-catenin is confined to the adherens junctions on the plasma membrane. Cytosolic β-catenin associates in a complex with APC and AXIN1 proteins, which mediate the N-terminal phosphorylation of β-catenin. This event conducts to the ubiquitination of β-catenin by the E3 ubiquitin ligase β-transducin repeat-containing protein (β-TRCP) following proteasomal degradation. When Wnt ligands bind to the Frizzled receptors, Dvl/Dsh is phosphorylated and, in turn, recruits AXIN1 and GSK3β adjacent to the plasma membrane, therefore preventing the building of the degradation complex. As a consequence, unphosphorylated β-catenin eludes recognition by β-TRCP and translocates into the nucleus, where it binds to the T cell factor (TCF) and lymphoid enhancer-binding protein family (LEF) transcription factors.

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The activated β-catenin/TCF/LEF complex induces the transcription of genes controlling cell proliferation and survival. In normal cells two LEF1 isoforms regulate Wnt-dependent pathways as apoptosis, motility and gene transcription and its expression in human colon tissue gradually increased from normal colon, low-grade adenoma, high-grade adenoma to adenocarcinoma [32]. Then, β-catenin accumulates in the cytoplasm and in the nucleus [33].

B-cell CLL/lymphoma 9 (BCL9) protein is a novel co-factor of canonical Wnt/β-catenin signaling [34–36]. It forms a complex with β-catenin-LEF/TCF to activate transcription of Wnt target genes, after hyper-activation of canonical Wnt signaling [37]. In CRC tissues Wnt3 is highly expressed to sustain autocrine Wnt activity and CRC progression by EMT and is indicative of advanced stages with poor prognoses [38]. Inhibiting Wnt3 secretion inhibits colon cancer cells proliferation [39]. Wnt3a expression was also increased and associated with EMT, which is indicative of advanced stages with poor prognoses [40]. Also, Wnt3a was overexpressed in CRC primary tissues than in metastatic areas, suggesting that Wnt3a was expressed early in cancer rather than appearing as it progressed. A more recent study discovered that Wnt3a inhibits the ability of human colon myofibroblasts to proliferate and migrate [41]. Thus, various CRC subgroups have distinct molecular and cellular properties contributing to Wnt3a's context-dependent nature.

Wnt5a is one of the most effective non-canonical Wnt ligands which can significantly antagonize and eventually suppress canonical Wnt ligand functions [42]. Recent findings demonstrate that Wnt5a non-canonical ligand, as tumor suppressor and oncogenic agent, can promote and inhibit tumor processes through canonical and non-canonical Wnt signaling [43,44]. The exact role of *Wnt5A* in CRC is contradictory [45].

As described above, Wnt/β-catenin signaling is well known in human CRC, but less studied in the early stages of colon tumorigenesis. The main aim of this study was to investigate the relationships of some key elements of Wnt/β-catenin signaling in the early stage of colorectal adenoma carcinogenesis. Based on what is reported in the current literature, there are no studies analyzing these aspects on human adenomas. In this study, the analysis of the gene and protein expression of some components of the Wnt/β-catenin pathway was conducted on pathological samples (polyps and related adjacent mucosa) derived from patients both with sporadic adenomas and suffering from FAP.

2. Materials and Methods

2.1. Tissues

Pathological and control tissues were recruited from patients with sporadic adenomas undergoing endoscopic biopsy at the Digestive Endoscopy and Gastroenterology Unit, SS. Annunziata Hospital of Chieti, while those from patients belonging to families with FAP were recruited at the Surgery Unit, Careggi University Hospital in Florence. The collection of each pathological sample was accompanied by the normal-appearing tissues sample obtained from areas that were at least 5 centimeters away from the margins of the primary lesion. Finally, normal colonic mucosal samples were collected from healthy donors without inflammatory bowel disease and family history of cancer, who had undergone follow-up colonoscopy at the Digestive Endoscopy and Gastroenterology Unit, "SS. Annunziata" of Chieti (UOD of Digestive Endoscopy and Gastroenterology). After the surgical removal, the tissue fragments were stored in RNAlater™ solution (Thermo Fisher Scientific, Waltham, MA, USA) to stabilize the RNA and to preserve proteins; subsequently stored at - 80°C. The study protocol was approved by the local Ethics Committee and each participant tissue donor provided written informed consent. The study was conducted according to the declaration of Helsinki and approval was granted from the Institutional Review Board (Prot. Id RICH1KHE). Adenoma tissues were classified according to conventional histopathological criteria, as defined by WHO (World Health Organization). Patient characteristics and polyps histotype are shown in Table 1 [46].

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Table 1. Clinical and histopathological characteristics of 41 patients with FAP and sporadic adenomas analyzed.

Patients with FAP polyps						
Case	**age**	**sex**	**Phenotype**	**Site and size of polyps**	**Dysplasia (L or H)**	**n.of polyps**
5FI	25	F	Adenomatous	Diffuse or "carpet", <1cm	HGD	1060
6FI [a,e]	58	M	Adenomatous	Diffuse	HGD	25
7FI [a,e]	28	F	Adenomatous	Diffuse	HGD	375
8FI [b,e]	18	F	Adenomatous (Tubular-villous)	Diffuse	HGD	415
9FI [c,e]	15	F	Adenomatous	Diffuse	LGD	375
16FI	n.a	F	Adenomatous	Diffuse	LGD	n.a
25FI	n.a.	M	Adenomatous	Diffuse	LGD	n.a.
26FI	46	F	Adenomatous	Diffuse	LGD	835
31FI		M	Adenomatous	Diffuse		
33FI	49	F	Adenomatous	Diffuse	LGD	97
35FI [c,e]	31	M	Adenomatous	Diffuse	LGD	550
36FI	42	F	Adenomatous	Diffuse	LGD	250
39FI	42	M	Adenomatous	Diffuse	LGD	430
40FI [a,d,e]	61	F	Adenomatous	Diffuse	HGD	730
41FI	49	M	Adenomatous	Diffuse	LGD	1025
42FI	42	F	Adenomatous and amartomatous	Diffuse	LGD	210
43FI	36	M	Adenomatous	Diffuse	LGD?	n.a
Patients with sporadic polyps						
case	**age**	**sex**	**Phenotype**	**Site and size of polyps**	**Dysplasia (L or H)**	**Morphology**
1CH	50	M	Hyperplastic	Sigma, 6mm	LGD	Spl
2CH	67	M	Tubular	Sigma, 10mm	LGD	Spl
3CH	49	M	Hyperplastic	Sigma, 4mm	LGD	Spl
9CH	47	M	Tubular-villous	Retto, 15mm	LGD	Ppl
11CH	57	F	Hyperplastic-adenomatous	Descending, 4mm	LGD	Spl

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13CH	83	F	Tubular	Descending, 15mm	LGD	Spl
15CH	37	F	Villous	Sigma, 50mm	HGD	Ppl
16CH	60	M	Tubular	Cecum, 15mm	LGD	Ppl
17CH	66	M	Tubular-villous	Sigma, 15mm	LGD	Ppl
18CH	64	M	Tubular-villous	Descending, 8mm	LGD	Spl
21CH	78	M	Tubular-villous	Sigma, 10mm	LGD	Spl
22CH	67	M	Tubular-villous	Rectum, 10mm	LGD	Spl
23CH	68	F	Tubular-villous	Sigma, 10mm	LGD	Ppl
24CH	59	M	Tubular-villous	Ascending	LGD	Ppl
25CH	77	M	Tubular-villous	Descending	HGD	Ppl
26CH	69	M	Tubular	Splenic flexure, 10mm	LGD	Spl
27CH	61	F	Tubular-villous	Sigma, 15mm	LGD	Ppl
28CH	77	M	Tubular-villous	Hepatic flexure, 5mm	LGD	Spl
29CH	47	M	Hyperplastic-adenomatous	Descending, 20mm	Not atypical	Ppl
30CH	53	M	Hyperplastic-adenomatous	Retto-sigma, 7mm	Not atypical	Spl
31CH	76	M	Tubular	Ascending, 5mm	LGD	Spl
32CH	51	M	Tubular-villous	Ascending, 45mm	LGD	Spl
33CH	68	F	Tubular-villous	Colon, 40mm	LGD	LST-G
34CH	67	M	Tubular	Colon sx, 7mm	LGD	Ppl

[a] Presence of rectal cancer; [b] APC mutation: c.4666_4665ins(p.Thr1556fs);[c]APC mutation: c.2805 C>(p.Tyr935X); [d] APCmutation: c.3927_3931del(p.Glu1309_Asp.fsx1312); [e]Ficari F, Cama A, et all., Br J Cancer. 2000 Jan;82(2):348-53. Ppl: Pedunculated polypoid lesion; Spl: Sessile polypoid lesion; LST-G: Laterally Spreading Tumor (LST)-granular shape (G); HGD: High Grade Dysplasia; LGD: Low grade Dysplasia.

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Tissues were selected based on RNA availability. Samples with insufficient quantity of target in the cDNA template or protein were not included in the gene and protein expression analyses. The study included 68 colonic samples. 58 biopsies (33 polyps and 25 adjacent mucosa) belonged to 41 patients, of which 17 affected by FAP. Ten normal colonic mucosal samples were collected from 10 healthy donors.

2.2. Gene Expression by Real-Time Quantitative PCR Analysis (qRT-PCR)

Total RNA was isolated from colon tissues homogenized in liquid nitrogen with a mortar and pestle, using TRIzol® Reagent (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions at RNase free environment. The synthesis of complementary DNA (cDNA) were performed as previously described [47]. The mRNA levels were evaluated by SYBR Green and TaqMan assay by quantitative real-time PCR (qRT-PCR) analysis using StepOne™ 2.0 (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Data were analyzed by the comparative Ct method and were graphically indicated as $2^{-\Delta Ct} \pm SD$. In accordance with the method, the mRNA amounts of the target genes were normalized by the ratio on the median value of the endogenous housekeeping gene (GUSB). Primers sequences are available in Catalano et al 2021 [47] The cycling conditions were performed as follow, 10 min at 95 °C and 40 cycles of 15 s at 95 °C followed by 1 min at 60 °C and final elongation of 15 s at 95°C. All data were validated in a second analysis.

2.3. Western Blotting

Total proteins were isolated from pathological and control tissues using RIPA lysis buffer (Cell Signaling Technology, Beverly, MA, USA). Protein were quantified by Bradford Assay (Thermo Fisher Scientific, Waltham, CA, USA) and analyzed by western blot, using the primary antibodies: Active-β-Catenin and APC (Merck-Millipore, Burlington, MA, USA). The β-actin primary antibody (Sigma-Aldrich, St. Louis, MI, USA) was used as a protein loading control. Secondary antibodies were HRP-conjugated anti-rabbit or anti-mouse (Bethyl Laboratories, Montgomery, TX, USA). The immune complexes were visualized using the ECL Western blot detection system (EuroClone) by using AllianceLD2 hardware (UVItec Limited, Cambridge, UK).

2.4. Statistical Analysis

All measurements were made after three independent experiments, and for each data is shown a representative value of all experiments plus standard deviation *(SD)*. Statistical analyses were performed using the t-test or one-way analysis of variance (ANOVA) as appropriate. All p-values are two-sided and a p-value ≤ 0.05 was considered as significant. All analyses were performed using SPSS software. Kruskal-Wallis non parametric test for independent samples was used to compare the expression of genes in polyps, adjacent mucosa and normal tissue. To assess for a possible correlation between the five genes, Spearman's ϱ correlation coefficient was evaluated. All p-value are twotailes and a p-value of <0.05 was considered as significant. Statistical analyses and descriptive statistics were carried out using GraphPad Prism version 9.0 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

We analyzed the expression of five genes of Wnt/β-catenin family in all available tissues, grouping them into three categories: colorectal adenomas, adjacent mucosa and normal tissues. To evaluate any alterations in the protein expression of APC and β-catenin in the mucosa adjacent to the adenoma transition, we performed protein expression in the cases in which matched adenoma and adjacent mucosal tissues were available.

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3.1. Gene Expression of APC, Wnt3A, Wnt5A, BCL9 and LEF1 in FAP and Tubular-Villous Adenomas

Gene expression of five Wnt/β-catenin signaling genes (*APC, BCL9, LEF1, Wnt3a, Wnt5a*) was conducted on 52 colonic samples. 42 biopsies (25 polyps and 17 adjacent mucosa) belonged to 33 patients, of which 15 affected by FAP, and 10 normal colonic mucosal samples from 10 healthy donors.

The expressions of *APC, Wnt3A, Wnt5A, BCL9* and *LEF1* in polyps, adjacent mucosa and normal tissue were compared by Kruskal-Wallis non parametric test for independent samples (**Figure 1**). A statistically significance difference was detected in *APC* expression in adjacent mucosa compared to normal tissue (**P=0.01**). Statistically significance differences were also detected between *Wnt5a* expression in polyps compared to normal tissue (**P=0.004**) and in adjacent mucosa compared to normal tissue (**P=0.01**).

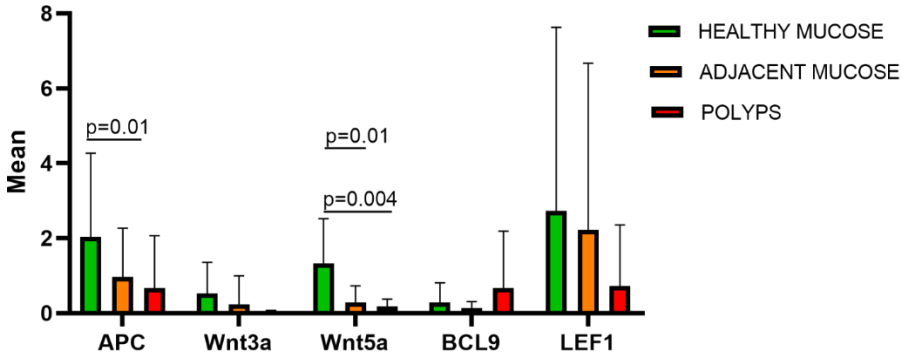

Figure 1. The figure shows the five Wnt/β-catenin pathway genes (*APC, Wnt3a, Wnt5a, BCL9, LEF1*) expression means in healthy colorectal mucosa, adjacent mucosa and polyps, compared by Kruskal-Wallis non parametric test for independent samples. Statistically significance differences were detected in *APC* and *Wnt5a* expressions in adjacent mucosa compared to normal tissue (**P=0.01**), and, for *Wnt5a* expression, also in polyps compared to normal tissue (**P=0.004**).

Furthermore, the Spearman's ϱ analysis revealed a statistically significance correlation between *Wnt3a* and *BCL9* (**P= 0.0294**) when polyps and adjacent mucosa were considered in the same category (Table 2). A statistically significance correlation between *Wnt5a* and *LEF1* (**P= 0.025097**) was detected in healthy tissues (Table 2).

Table 2. Correlation among *APC, Wnt3a, Wnt5a, BCL9, LEF1* gene expression in 52 colon tissue samples. *Correlation is significant at the 0.05 level (2-tailed).*

		Wnt3a		**BCL9**	
Group		**Spearman's rho** (2-tailed)	**P-value**	**Spearman's rho** (2-tailed)	**P-value**
Polyps and adjacent mucosa	n. 42				
	Wnt3a			0,336376	**0,0294**
	BCL9	0,336376	**0,0294**		
		Wnt5a		**LEF1**	

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Group		Spearman's rho (2-tailed)	P-value	Spearman's rho (2-tailed)	P-value
Normal colonic mucosa	n. 10				
	Wnt5a			0,69697	**0,025097**
	LEF1	0,69697	**0,025097**		

Gene expression results were graphically represented using a heat map. *APC, Wnt3a, Wnt5a, BCL9, LEF1,* tend to reduce from healthy to pathological tissue (**Figure 2**). *APC, Wnt5a, LEF1* are the most expressed genes in healthy mucosa (**Figure 2).**

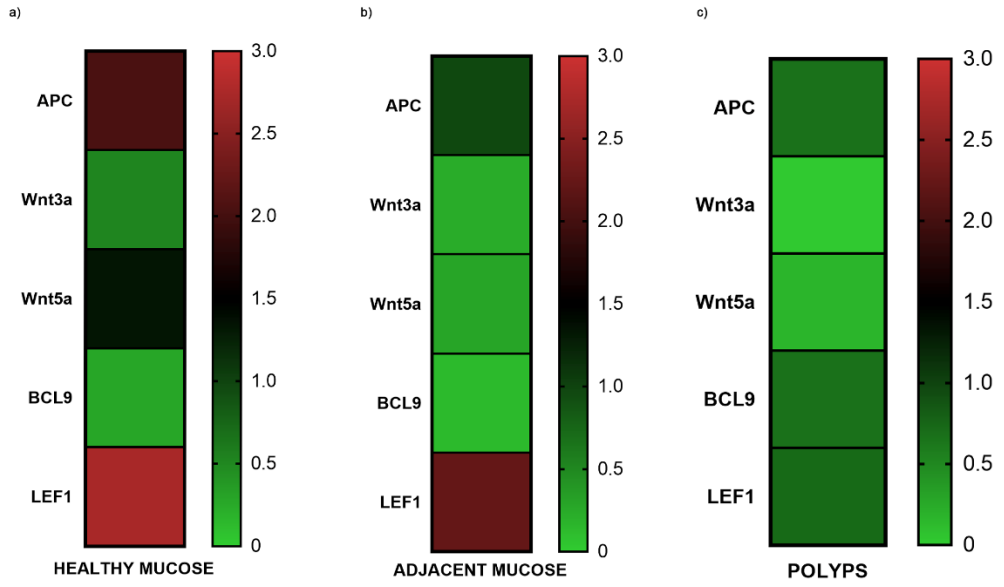

Figure 2. The figure represents an heat map of the genes expression means of *APC, Wnt3a, Wnt5a, BCL9, LEF1,* in healthy mucosa (a), adjacent mucosa (b) and polyps (c). It is shown how gene expression tends to reduce from healthy to pathological tissue. *APC, Wnt5a, LEF1* are the most expressed genes in healthy mucosa.

3.2.1. Protein Expression Assay by Western Blotting of APC and β-Catenin

A 50% reduction of APC gene expression in the adjacent mucosa compared to the healthy mucosa, and an more accentuated reduction in polyps, have been detected (Figure 1). Therefore we wanted to investigate the levels of APC and β-catenin proteins, in still available tissues. Then a Western blot analysis of matched polyp-adjacent mucosal tissues was performed. Matched adenoma and adjacent mucosal tissues were available for 6 FAP patients and for 7 patients with sporadic adenomas. The analyses revealed the expression of the full-length APC protein (300 kDa band) in all matched adenoma-adjacent mucosa samples (**Figures 3a and 4a**). A decrease in APC expression is visible in all the adenomas, both familial than sporadic, compared to adjacent mucosa. As we expected, as regards β-catenin expression, its active form appeared increased in all the familial and sporadic adenomas compared to adjacent mucosa (**Figures 3a and 4a**). Sample 42FI-P does not show

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a decrease in APC expression and intriguingly it shows a reduced active β-catenin expression, also visible in matched adjacent mucosa. Also for sample 43FI, a reduced expression of active β-catenin was noted, apparently not associated with APC modulation. Relative expression quantification of the selected proteins (APC, active β-catenin) detected by WB in available matched adenoma and adjacent mucosal tissues are shown in **Figures 3b,c and 4b,c.**

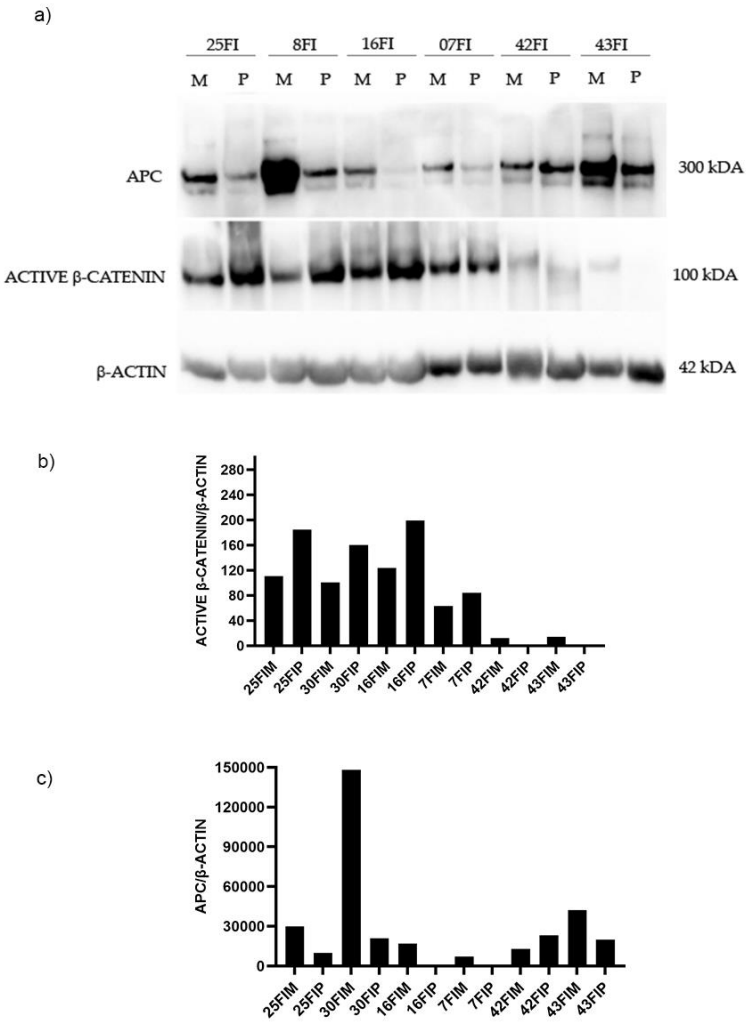

Figure 3. Western blotting analysis in familial adenomas determining the protein expression levels of APC and β-Catenin in polyp (P) vs. adjacent mucosa (M). Data shows are representative of three independent experiments. The average expression levels of panel "a" were determined by densitometric analysis (panel b and c) and calculated in relation to the β-Actin level. kD: Kilodalton as protein molecular weight unit.

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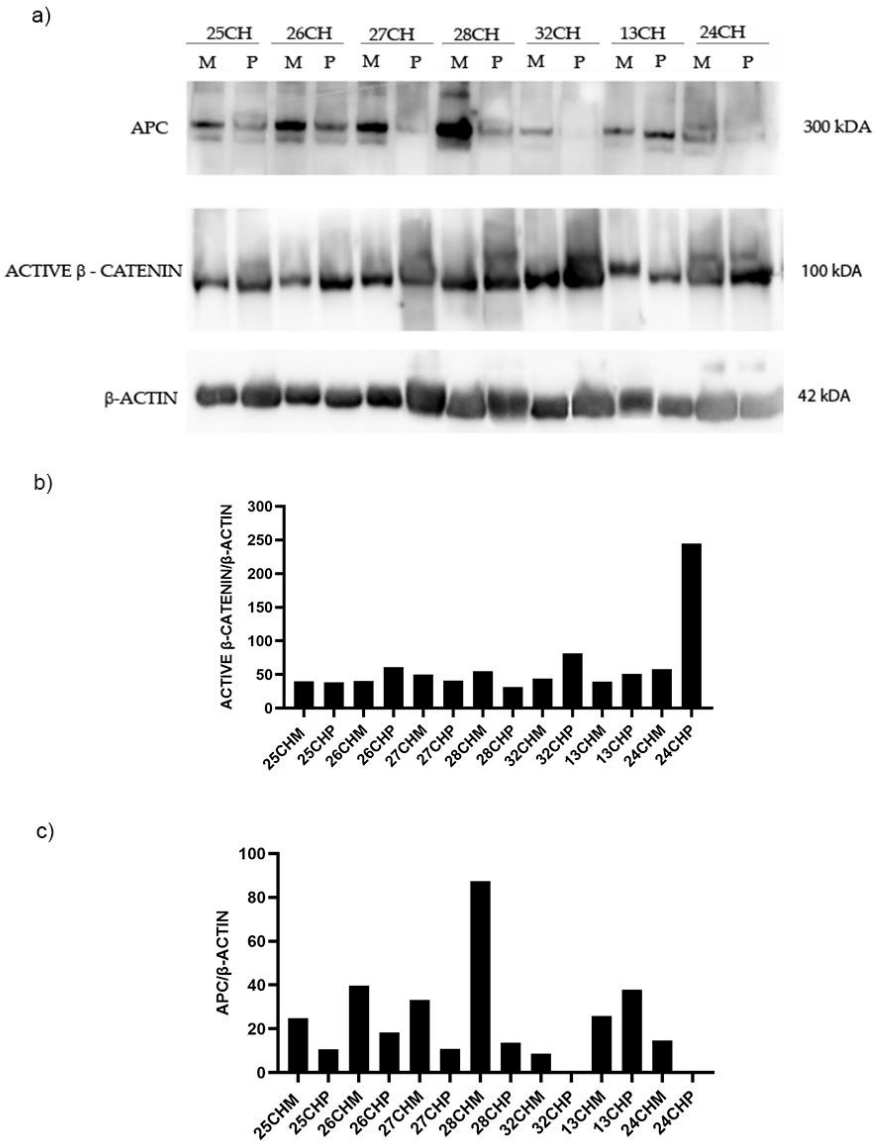

Figure 4. Western blotting analysis in sporadic adenomas determining the protein expression levels of APC and β-Catenin in polyp (P) vs. adjacent mucosa (M). Data shows are representative of three independent experiments. The average expression levels of panel "a" were determined by densitometric analysis (panel b and c) and calculated in relation to the β-Actin level. kD: Kilodalton as protein molecular weight unit.

4. Discussion

The current literature lacks significant Wnt/β-catenin/APC gene expression studies on adenomas. Our study is the first to analyze the modulation of Wnt/β-catenin pathway in early carcinogenesis. Understanding the significance of benign pre-cancerous lesions is widely recognized, yet studying them is complicated in terms of sampling, mainly due to the difficulty of obtaining biopsies and their small size. The long period required for polyps and cancer to develop, as well as the tendency of early-stage cancers to be asymptomatic in many individuals, allows a window of opportunity for polypectomy and cancer prevention, as well as for early diagnosis and highly effective drug administration for early-stage cancers. Blood-based tests could detect genetic changes associated with polyps released into circulation. Promoting the uptake and completion of follow-up testing and treatment holds significant potential to save lives. Therefore, understanding the

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progression from colorectal adenomas to colorectal carcinomas is essential for the development of new therapeutic strategies and improving clinical outcomes.

Tumor and adjacent mucosa biopsies are the definitive diagnostic procedures for histological evaluation. The adjacent mucosa, also known as "*apparently healthy"* mucosa, being closely associated with polyps in the tumor microenvironment (TME), may have already molecularly been impacted. Our data supports this hypothesis.

It is already known that *APC* is inactivated in FAP polyps. This study focused on evaluating APC gene and protein expression not only on pathological FAP tissue and tubular-villous adenomas, but also on apparently normal endoscopic mucosal tissue sampled at the 5-cm proximal. Very interestingly, lower APC expression was detected in the adjacent mucosa compared to the normal one.

APC has a functional role in the canonical (β-catenin-dependent) Wnt signaling pathways [48]. Most cases of CRCs are initiated by *APC* inactivating mutations, leading to constitutive activation of the Wnt/β-catenin signaling pathway. Most of the literature has extensively assessed the role of APC somatic and germline mutations in familial as well as sporadic forms of CRC.

It is known that *APC* is inactivated in FAP polyps. Our results showed a reduction in APC expression both in FAP and sporadic polyps. Additionally, a lower APC expression was detected in adjacent mucosa.

Moreover, APC can inhibit the initiation and development of colorectal tumor, independently of canonical Wnt signaling. APC assists in chromosome segregation, establishes cellular polarity and migration, and represses DNA replication [49]. *APC* mutations contribute in early adenoma creation leading to chromosomal instability by triggering spindle abnormalities and deregulation of microtubules/actin cytoskeleton. Moreover, *APC* mutations increase cell migration by reducing cell adhesion via deregulation of β-catenin and E-cadherin distributions among the cytoplasm and the cell membrane [50]. Xu et al. demonstrated that over-expression of erythropoietin-producing hepatocyte (EphB6), a member of the tyrosine kinase family, along with *APC* gene mutations, increases proliferation, migration, and invasion in colon epithelial cell line, IMCE, supporting the role of *APC* mutations in promoting tumorigenesis in CRC [51]. In our study APC gene was less expressed in colon tumor compared to the adjacent colonic mucosa and to the mucosa of healthy controls. Also APC protein resulted less expressed in colon tumor compared to the adjacent colonic mucosa. All results confirmed literature data in carcinomas.

LEF1 is a downstream mediator and nuclear effector of the Wnt/β-catenin signaling pathway [52]. In normal cells two LEF1 isoforms are in regulation of Wnt-dependent pathways as apoptosis, motility and gene transcription. The role of LEF1 is controversial, usually is detected and upregulated in most colonic carcinomas enhancing the progression [53]. Nevertheless, little is known about the expression of LEF1 in early carcinogenesis. LEF1 is generally excessively expressed in malignant tumors and may play a role in tumor growth and metastasis [54]. LEF1 knockdown in glioblastoma multiforme cells inhibits invasion, migration, proliferation, and the self-renewal potential of stem-like cells [55]. Myc induces the expression of LEF1 to activate the Wnt pathway in colon cancer [56]. LSD1 (lysine-specific demethylase 1) promotes bladder cancer progression by upregulating LEF1 and enhancing EMT [57]. LEF1 expression may contribute to cancer development [58–60], but there is a lack of evidence to support malignant phenotype changes. The underlying molecular mechanisms of LEF1 regulation for colonic adenocarcinoma progression remain unknown. Xiao et al. 2021 study findings, support LEF1 as a potentiator and potential therapeutic target for colonic adenocarcinoma. LEF1 enhances the motility of colonic adenocarcinoma cells via remodeling of lamellipodia/filopodia and the polymerization of F-actin/β-tubulin. LEF1 maintains the viability and growth of colonic adenocarcinoma cells through increasing proliferation, Lamin B1 expression, and decreasing apoptosis. In addition, LEF1 is closely related to EMT. However, an *in vivo* study published on Science [61] showed that LEF1 restricts ectopic crypt formation and tumor cell growth in colon adenomas from APC-deficient mice. Loss of *Lef1* markedly increased tumor initiation and tumor cell proliferation, reduced the expression of several Wnt antagonists, and increased *Myc* proto-oncogene expression and formation of ectopic crypts in *Apc*-mutant adenomas. These results uncover a

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previously unknown negative feedback mechanism in CRC, in which ectopic *Lef1* expression suppresses intestinal tumorigenesis by restricting adenoma cell dedifferentiation to a crypt-progenitor phenotype and by reducing the formation of cancer stem cell niches. Recent literature data therefore demonstrate how the controversy on the function of LEF1 in colorectal tumorigenesis is still open.

In our study, the expression of LEF1 in healthy tissues decreased in the adjacent mucosa and the polyp, supporting the recent hypothesis of LEF1's role as a tumour suppressor, already in adenomas.

BCL9 is considered a key developmental regulator and a well-established oncogenic driver in multiple cancer types, mainly through potentiating the Wnt/β-catenin signaling. BCL9 has been identified as an adapter and essential core component of nuclear β-catenin complexes [62] and functions as an adapter protein providing binding sites for the transcriptional machinery of Wnt signaling [63]. As an oncoprotein, BCL9 promotes cancer development mainly through sustaining cancer cell division [64], promoting proliferation and migration, inhibiting apoptosis [65], remodeling TME and immune system, and regulating chromosomal instability and karyotype for tumor evolution [66].

Wnt3a is a ligand that activates the canonical Wnt pathway. Wnt3a stimulates tumor progression in glioblastoma [67], breast and prostate cancers [68,69], and malignant mesothelioma [70]. Other studies have shown that Wnt3a serves as a tumor suppressor based on two main findings. One is that bones engrafted with Wnt3a-expressing multiple myeloma H929 cells are preserved; the other is that treatment of myelomatous SCID mice carrying the primary disease with recombinant Wnt3a stimulates bone formation and attenuates multiple myeloma growth [71]. Marit et al. reported that Wnt3a inhibits the proliferation of several B-acute lymphoblastic leukemia cell lines [72]. However, Qi L. et al., analyzed Wnt3a expression in a large array of colon cancer tissue samples to determine its role in colon-cancer progression and observed a significant correlation between Wnt3a expression and histological differentiation, clinical stages, metastasis, and recurrence. The resulting data indicates that the upper stream factor of the Wnt signaling pathway may play an important role in colon cancer progression and agree with a recent study on colorectal cancer, in which results reveal that Wnt3a is highly expressed in the primary and metastatic sites and is significantly associated with expression of the metastasis-related protein matrix metalloproteinase (MMP)-9 [73]. In our study the correlation between BCL9 and Wnt3a both in polyps and in the adjacent mucosa confirms two aspects: the first is that the canonical Wnt pathway is already activated in the adjacent mucosa; the second that it is also activated in early carcinogenesis. Finally our results confirm Wnt3a as tumor suppressor.

There is no evidence to prove the exact function of Wnt5a. Some studies suggest that Wnt5a is a tumor suppressor, while others suggest the opposite. During the development of colorectal cancer, Wnt5a showed different functions in different signal transduction pathways [74]

Wnt5a mRNA is expressed in most normal tissues including the colon, but is highly up-regulated in the progression from normal tissue to carcinomas [75]. The expression of Wnt5a protein, however, seems to be downregulated as it is frequently inactivated in CRC by tumour-specific methylation and, thus, could be a potential biomarker [76]. Wnt5a is suggested to act as a tumor suppressor for CRC by antagonizing the Wnt/β-catenin signaling [76].

Traditionally, Wnt5a is believed to be the non-canonical Wnt ligand and activates Ca^{2+}-dependent effectors and other non-canonical pathways through small Rho-GTPases and c-Jun-NH2-kinase [77]. However, its role in the progression of CRC is complicated and seems to be contradictory. Several studies proved that Wnt5a was silenced in most CRC cell lines and specimens due to frequent methylation in its promoter region [78,79]. The expression of Wnt5a was negatively correlated with the degree of tumor differentiation and the aggressive behavior [78,80]. Meanwhile, promoter methylation of Wnt5a was strongly associated with the microsatellite instability status of patients with CRC, and multiple histone modifications of Wnt5a were involved in its silencing and might promote colon cancer metastasis, providing evidence that epigenetic events may promote Wnt5a-mediated signaling in CRC [81,82]. On the contrary, other studies demonstrated that Wnt5a was upregulated consistently in intestinal polyps and tumor samples, and increased Wnt5a expression

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predicted the early recurrence or metastasis in colon cancer patients [83,84]. Wnt5a could also promote the migration of CRC cells by activating Fzd7-driven non-canonical Wnt signaling and enhance the cell stemness of CRC through activating the canonical Wnt signaling [85,86]

In our study the correlation between Wnt5a and LEF1 detected in healthy tissues could indicate that the Wnt non-canonical pathway is active in normal colonic tissue. Furthermore, these results also could denote a possible inflammatory state of donors undergoing screening colonoscopy, as both Wnt5a and LEF-1 are linked to the inflammatory state. Aberrant Wnt signaling is linked to defects in the chronic inflammatory response. Indeed, in the still normal colonic mucosa, various inflammatory mediators can actively contribute to the creation of a TME favorable to cell transformation, survival and proliferation [87]. The mutual interaction of epithelial cells within the TME influences the stages of tumorigenesis driven to a large extent by inflammation This may be an attractive therapeutic target to control inflammation in the colonic mucosa [88] Furthermore, IEC can drive the plasticity of stromal cells in the TME under the influence of extrinsic factors, such as diet, and microbiota composition contributing to inflammation and tumorigenesis in CRC [87,89] However, understanding the interactions between Wnt signaling and inflammatory/immune responses in tumor onset remains a necessary goal for both prevention and therapy, given that the majority of CRCs appear to be immunologically "cold" tumors unresponsive to therapies with immune checkpoint inhibitors [90].

5. Conclusions

This is the first study analyzing the gene expression of *APC, Wnt3A, Wnt5A, BCL9* and *LEF1* in colon polyps vs. adjacent mucosa and vs. normal mucosa from control individuals. These findings of altered expression levels of *Wnt* genes in apparently normal adjacent mucosa from patients with familial and sporadic colon polyps underline an interplay between tumor and surrounding colonic epithelium. This may aid in identifying patients at risk of developing cancer.

In conclusion, our study will improve the understanding of the pathogenesis in CRA. Finding essential genes, exploring their potential pathogenesis of colorectal adenoma, and developing gene-targeted drugs are urgent clinical and scientific problems to be solved.

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