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Mol Carcinog. Author manuscript; available in PMC 2017 August 03.

Published in final edited form as:

Mol Carcinog. 2017 February ; 56(2): 761–773. doi:10.1002/mc.22533.

Comparison of Biomarker Expression between Proximal and Distal Colorectal Adenomas: The Tennessee-Indiana Adenoma Recurrence Study

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Abstract

It is unclear if proximal and distal traditional adenomas present with differences in molecular events which contribute to cancer heterogeneity by tumor anatomical subsite. Participants from a colonoscopy-based study (n=380) were divided into subgroups based on the location of their most advanced adenoma: proximal, distal, or “equivalent both sides”. Eight biomarkers in the most advanced adenomas were evaluated by immunohistochemistry (Ki-67, COX-2, TGFβRII, EGFR, β-catenin, cyclin D1, c-Myc) or TUNEL (apoptosis). After an adjustment for pathological features, there were no significant differences between proximal and distal adenomas for any biomarker. Conversely, expression levels did vary by other features, such as their size, villous component, and synchronousness. Large adenomas had higher expression levels of Ki-67(P<0.001), TGFβRII (P<0.0001), c-Myc (P<0.001), and cyclin D1 (P<0.001) in comparison to small adenomas, and tubulovillous/villous adenomas also were more likely to have similar higher expression levels in comparison to tubular adenomas. Adenoma location is not a major determinant of the expression of these biomarkers outside of other pathological features. This study suggests similarly important roles of Wnt/β-catenin and TGF-β pathways in carcinogenesis in both the proximal and distal colorectum.

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Conflicts of Interest

We declare that there is no actual or potential conflict of interest.

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Keywords

colorectal adenoma; biomarkers; β-catenin; proximal; distal; immunohistochemistry

INTRODUCTION

The colorectum is dichotomized as proximal and distal based on its embryonic origin and varying physiological functions and gene expression [1–3]. Data suggest that colorectal cancer (CRC), one of most common cancers [4], is highly heterogeneous by tumor subsite. For example, in comparison to distal CRCs, proximal CRCs exhibit a higher incidence in women and the elderly, a higher mortality [5,6], a different histopathological appearance [7], and a higher prevalence of some distinct molecular alterations such as microsatellite instability (MSI) [8,9]. Some of these differences may be attributed to sessile serrated adenomas (SSA), the presumed precursor for most MSI-high CRCs, which is more prevalent on the right colon than on the left colorectum [10,11]. However, it remains unclear whether there are molecular events that also affect the more common traditional or conventional adenoma and contribute to subsite heterogeneity in CRC.

Proximal adenoma prevalence is greater than distal adenomas among some subgroups, such as older adults, African-Americans and women [12–17]. Although small adenomas (< 10 mm) are evenly distributed [18,19], advanced adenomas are more predominant in the distal colorectum [20–24]. A recent analysis of more than 1.2 million CRC cases suggested that adenoma initiation rates were higher for proximal tumors, whereas growth rates were higher for distal tumors [25]. However, despite substantial data regarding the correlation of adenoma localization with age, race, gender, and stage, little is known about possible underlying molecular events which may differentially contribute to the tumorigenic process. In this study, we compared a panel of biomarkers between proximal and distal adenomas. These biomarkers were selected because they have been reported to be important factors or pathways in colorectal tumorigenesis [26], including cell proliferation (Ki-67), apoptosis (terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)), cyclooxygenase-2 (COX-2), transforming growth factor beta receptor II (TGFβRII), epidermal growth factor receptor (EGFR), and Wnt/β-catenin pathway (β-catenin, cyclin D1 and c-Myc).

MATERIALS AND METHODS

Participants

The subset of participants is from a retrospective cohort study, the Tennessee-Indiana Adenoma Recurrence Study [27]. Out of the 1643 eligible individuals, 1020 (62.1%) participated. Eligibility criteria were: their first adenoma diagnosis during index colonoscopy had to have taken place between January 1996 to December 2002; they had an index diagnosis of synchronous (>1) or advanced (1.0 cm in diameter, villous/tubulovillious subtype, or high-grade dysplasia) adenoma; they were between the ages of 40 and 75; there was an absence of familial adenomatous polyposis or previous history of cancer, except for non-melanoma skin cancer; they were not already participating in an

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intervention trial to prevent adenoma recurrence; they had the ability to speak English and provide informed consent; they could not be a current resident in a correctional facility. Included in this analysis were the first 380 participants. In comparison to the entire cohort, individuals selected for this study were more likely to be female (39.2% vs 31.1%), white (89.5% vs. 80.6%), and less likely to have a large adenoma (28.7% vs. 34.8%). There were no statistically significant differences for age, the presence of synchronous adenomas, the location of the adenomas, the presence of the worst subtype, or the presence of high-grade dysplasia. Committees for the use of human subjects in research at each institution approved the study protocol.

Diagnosis data were abstracted from medical records. Formalin-fixed, paraffin-embedded, adenoma tissue blocks were obtained. 5 μm sections were made and placed on charged slides. Serial tissue sections were coated with a thin layer of paraffin and placed in a vacuum desiccator cabinet (Terra Universal Company, CA) at 4°C. The diagnosis was reviewed and confirmed by an experienced research pathologist (T.S.).

An analytic index adenoma was selected for each participant. If the participant had synchronous adenomas, the most advanced adenoma tissue was used, and was defined in the following order: 1) the presence of high-grade dysplasia, 2) the most villous component, or 3) the largest size. According to the index adenoma location, the participant was defined as "predominant proximal" (in which the index adenoma was proximal to the splenic flexure) or "predominant distal" (in which the index adenoma was in the splenic flexure or distal to it). Fifty-eight participants with histopathologically equivalent adenomas on both sides were categorized as "equivalent both sides", and both adenomas were used in the analysis.

Immunohistochemistry

The optimized staining protocols for Ki-67, cyclin D1, COX-2, EGFR, c-Myc, and TGFβRII are summarized in Supplementary Table 1. For negative controls, phosphate buffered saline (PBS) replaced primary antibodies. Both positive and negative control slides were processed with each batch of staining. The semi-quantitative scoring criteria for Ki-67, cyclin D1, COX-2 and EGFR were previously described [28]. The cut-off value for each biomarker was determined based on the protein expression level in adenoma tissue, as used in previous reports [29–33]. Briefly, Ki-67 was 0 (0–20% cells positive) and 1 (>20%) [28,29]. Cyclin D1 was recorded as 0 (<5% cells positive), 1 (5–30%), and 2 (>30%) [30]. COX-2 and TGFβRII were scored using a modified All red scoring system, which combines the estimated proportion of positive staining tumor cells with the average estimated intensity of staining [28,34]. EGFR was recorded as 0 (<10%), 1 (10–50%), and 2 (>50%)[31,32]. C-Myc was scored 0 (0%), 1 (<10%); 2 (10–50%), and 3 (>50%) [35]. The superficial, middle and basal thirds of polyps were also scored separately for zonal scores, using the same criteria as for the entire polyp.

Immunohistofluorescence

Our previous validation study showed a complex subcellular localization of β-catenin, which may exist in the nucleus, in the cytoplasm, or on the cell membrane [28]. It is generally accepted that β-catenin can function as an oncogene when it is translocated from membrane

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to nucleus, when it binds to T cell factor (TCF) or lymphocyte enhance family members, and when it transactivates its target genes [33]. In this study, fluorescence immunohistochemistry was performed to determine β-catenin nuclear accumulation and co-expression of transcription factor 4 (TCF4), so that the nuclear localization of β-catenin could be more clearly visualized than is possible with the conventional immunohistochemistry method. An optimized double immunofluorescent labeling protocol was performed. The deparaffinized slides were placed in R-buffer A and heated with a pressure cooker for antigen retrieval. The sections were incubated with 5% normal donkey serum for 10 min at 37°C, then by the primary antibody mixture of goat anti-TCF4 (Santa Cruz, Cat# sc-8631, 1:80) and mouse anti-β-catenin (BD Transduction Lab, Clone 14,1:200) for 1 hour at room temperature. After a rinse in PBS, sections were incubated with biotin conjugated anti-mouse (R&D, Cat# CTS002) for 30 minutes to amplify the β-catenin signal. After a thorough rinse in PBS, sections were incubated with a mixture of streptavidin-FITC (Zymed, Cat# 43-8311, 1:80) and Cy3 conjugated donkey anti-goat (Chemicon, Cat# AP180C, 1:100) for 30 minutes. After another thorough rinse, the sections were coverslipped with ProLong Gold Antifade Reagent with DAPI (Invitrogen, Cat# P36935) and stored in the dark at 4 °C. Known positive adenoma tissue was used as a positive control. Negative controls were made by replacing primary antibodies with PBS. Nuclear or cytoplasmic immunostaining were considered equally as positive signals. Nuclear β-catenin accumulation in tumor cells was scored according to a four-category (0–3) method (Supplementary Figure 1). Nuclear TCF4 was not recorded because of its widespread expression in all nuclei of adenoma epithelial cells.

Apoptosis Assay

Apoptosis was detected with the Promega DeadEnd™ Colorimetric Apoptosis Detection System (Promega BioScience, CA), which is based on a modified TUNEL assay. The positive control was human liver, and the negative control was obtained by omitting the TdT enzyme after peroxidase inactivation. To accurately record the apoptotic index (the percentage of TUNEL-positive cells versus the total number of cells) [36], computer-aided quantitative analysis was performed with an Olympus BX40 microscope, a Retiga FAST 1394 color digital camera and BioQuant NOVA Prime imaging software (BioQuant, TN). Positive cells in the adjacent stroma were excluded, or if they were into the luminal space, if they were blank, folding, hemorrhaging, experiencing necrosis, poorly stained, and if they were in stromal areas. The quantitative imaging analysis method is summarized in Supplementary Figure 2.

Statistical Analysis

The chi-square test and the student’s t-test were used to analyze the characteristics of participants and the expression of biomarkers between predominant proximal and predominant distal adenomas. To compare the expression between proximal and distal adenomas in the “equivalent both sides” subgroup, Wilcoxon-signed rank tests or paired students’ t-tests (TUNEL only) were used. Analysis of variance was used for the comparisons involving more than three groups. The intraclass correlation coefficient was used to evaluate intra-observer variations of TUNEL. The correlations of the eight markers were also analyzed using Spearman correlation coefficients. In analyses including all

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participants, the mean scores of distal and proximal adenomas were calculated for the "equivalent both sides" subgroup. All analyses were two-sided, with a significance level of 5% and performed using SAS statistical software (v9.3).

RESULTS

Most participants had traditional and synchronous adenomas (Table 1). The predominant distal group was more likely than the predominant proximal group to have a large or advanced adenoma ($P<0.001$) or villous histology ($P=0.03$), and was less likely to have synchronous adenomas ($P<0.001$).

The typical distribution of positive cells and subcellular localization patterns had apparent differences by zonal location (Figure 1 and Supplementary Table 2). Except for c-Myc, EGFR and TUNEL, biomarker expression varied by adenoma characteristics (Table 2). When there were differences between adenoma types, in general, the more severe phenotype had a higher expression level than the less severe phenotype. For example, compared to small adenomas, large adenomas had higher expression levels of Ki-67, TGFβRII, c-Myc, and cyclin D1 (all $P<0.001$), as well as a higher Wnt pathway score ($P=0.008$). Adjustment for their location in the colon did not alter results (data not shown). To evaluate the individual contributions of synchronousness and histology to the observed differences, we compared the expression between individuals with synchronous non-advanced adenomas with a single advanced adenoma, and with at least one advanced adenoma in addition to synchronous adenomas. Ki-67, COX-2, TGFβRII β-catenin, c-Myc, Cyclin D1, and the Wnt pathway score were significantly different between the groups. Expression levels of these markers were higher among those with advanced adenomas regardless of the number of adenomas, with the exception of TGFβRII.

Compared to the predominant proximal group, the predominant distal group had significantly stronger expression of cyclin D1 ($P=0.01$) and EGFR ($P=0.05$) (Table 3). There was no significant difference between the groups for the other markers. After an adjustment for pathological features, there were no significant differences in expression for all markers between predominant proximal and predominant distal adenomas. This was also confirmed, with one exception, in an exploratory analysis comparing colon versus rectum differences. This was done by evaluating the proximal colon versus the distal colon (i.e. excluding rectal adenoma, data not shown) or by comparing the proximal colon, distal colon, and rectum. C-Myc levels were higher in rectum adenomas versus distal and proximal adenomas ($P=0.02$; data not shown). In a model in which all five biomarkers with a $P<0.20$ from the unadjusted models (c-Myc, cyclin D1, EGFR, β-catenin, and TGFβRII) were mutually adjusted for each other, there were no significant differences in expression between predominant proximal and predominant distal adenomas for all five of the markers.

Within the "equivalent both sides" group, the expression levels of all markers were similar between distal and proximal adenomas(Table 4), except for cyclin D1, which showed a higher expression level in the predominant distal adenoma in unadjusted analysis ($P=0.03$) and its Wnt pathway score ($P=0.045$). However, after an adjustment for adenoma size, subtype, degree of dysplasia, and synchronousness, there were no differences between

proximal and distal adenomas (data not shown in table). Most biomarkers were significantly correlated with each other, except for nuclear β-catenin, which only correlated with c-Myc expression (*P*=0.03) (Table 5).

DISCUSSION

We found no differences between proximal and distal adenomas in a panel of eight biomarkers involved in colorectal tumorigenesis after adjusting for other adenoma features. This held true whether comparing adenomas from different individuals or different adenomas within the same person, indicating a similar tumorigenesis process within traditional adenoma subtypes regardless of tumor location. The tumorigenesis process might be similar throughout the colorectum for sporadic traditional adenomas, at least as it relates to this panel of biomarkers. This study suggests that the TGF-β and Wnt/β-catenin pathways play important roles in both proximal and distal colorectal tumorigenesis, and that a synergistic role in the TGF-β, Wnt/β-catenin, COX-2 and EGFR pathways may exist to promote tumor progression throughout the human colorectum. This may have important implications for CRC prevention.

The proximal and distal segments develop from two embryonic areas with different physiological functions, and have different gene expression levels [1,2]. A large body of CRC and adenoma studies also support differences between segments in epidemiology, clinicopathology and molecular biology [5–9,37–40]. These findings suggest that there might be location-related molecular features in normal colorectum and neoplastic lesions. In recent years, some of these differences have been identified to be related to the serrated neoplasia pathway [11,41]. Thus far, little is known about underlying molecular events, other than the serrated neoplasia pathway, which may differentially contribute to the tumorigenic progress in traditional adenomas arising in the proximal versus distal colorectum. The current study, limited to comparisons of traditional adenomas, did not find differential expression for the selected biomarkers involved in cell proliferation/apoptosis and the signaling pathways of COX-2, TGFβ, EGFR, and Wnt/β-catenin. Our results are consistent with reports that non-steroidal, anti-inflammatory drug use is associated with a reduced risk of adenoma recurrence by a similar magnitude in both the distal and proximal colon [41], and that risk factors for traditional adenomas are largely similar between the proximal and distal colorectum [42]. In addition, nuclear β-catenin overexpression had no correlation with the CRC tumor site in a recent meta-analysis [43]. However, due to the substantial variability of molecular events in CRCs and the hundreds of potential alterations, we cannot definitively conclude that there is no heterogeneity for any biomarker between the proximal and distal colorectum based on our limited analysis of the eight biomarkers in this study. For example, a recent study reported distinct patterns of DNA methylation between traditional adenomas of the proximal and distal colon [44]. Nevertheless, this study has opened the way for further studies involving more (or other) pathways, other methods, or other types of markers (e.g. DNA mutations, DNA methylation, non-coding RNAs, etc) which may provide additional molecular evidence to clarify the potential tumorigenic differences between proximal and distal adenomas.

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Although polyp location was not associated with the expression of biomarkers evaluated in this study, we did observe that other important clinicopathological features of adenomas, such as tumor size, villous component, dysplasia, and synchronousness were significantly associated with the biomarker expression. This is consistent with previous studies. For example, in the Wnt/β-catenin pathway, nuclear β-catenin was significantly associated with high grade dysplasia or advanced adenomas, consistent with other reports regarding its key role in promoting an adenoma's progression to carcinoma [45–48]. The expression of nuclear cyclin D1, a responsive gene of the Wnt/β-catenin pathway, was significantly higher in advanced adenomas. These adenomas have higher cellular proliferation, larger size, and a higher villous component than in non-advanced adenomas. This is consistent with its potential role in adenoma progression [49–51]. The protein expression of c-Myc, another important target gene of the Wnt/β-catenin pathway, has been reported to be closely correlated with the size of a colorectal adenoma [45]. In general, we also found that expression levels were higher in more advanced adenomas, although this was not statistically significant for the villous component. C-Myc is not necessarily correlated with cellular proliferative activity in previous studies [45,52], but we observed a positive correlation between the c-Myc and Ki-67 levels in this study. We also found that, of all the markers, c-Myc might be the only one with location-specific expression (with higher levels in the rectum). Given the important role of *Myc*-directed transcriptional activation in CRCs [53] and the prevalence of expression in our study, our findings suggests that c-Myc overexpression might be a later-stage event than cyclin D1 in colorectal carcinogenesis [54].

In this study, TGFβRII expression was positively associated with more advanced adenomas, including the phenotypes of large, villous, and high-grade dysplasia. The epithelial cells in the adenomas were heterogeneously expressed with patchy strong staining and with zonal specificity at the surface, unlike the normal colonic mucosa, which shows a uniform distribution of TGFβRII expression in a moderate level in normal colon epithelial cells and stromal cells [55]. This finding suggests that the dysregulation of the TGF-β pathway in colorectal adenomas includes an abnormal TGFβRII expression, exhibiting a decreased expression in some tumor cells and an increased expression in other cells. In general, the increased expression of TGFβRII protein is associated with more advanced pathologic features of adenomas. Interestingly, out of eight selected biomarkers, only decreased TGFβRII was significantly associated with adenoma synchronousness. This suggests that reduction in TGFβRII may contribute to the initiation of colorectal adenomas. Our observations provide data to support the importance of the dysregulation of the TGFβ pathway in colorectal carcinogenesis in humans.

In addition to the Wnt/β-catenin and TGFβ pathways, the COX-2 pathway also has a key role in colorectal carcinogenesis, based on evidence from both animal and human studies [56,57]. Specifically, the administration of the COX-2-selective inhibitor celecoxib significantly decreases the occurrence of sporadic colorectal adenomas, not only by suppressing the growth of existing adenomas, but also by preventing the formation of new adenomas [58]. A previous study reported that large (> 1 cm) adenomas exhibited a significantly stronger expression of COX-2 than the very small (< 5 cm) adenomas, and an expression was absent in normal colonic epithelium, suggesting that epithelial COX-2 activity is important for the growth and/or survival of adenomatous epithelial cells [59].

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Although we did not observe a statistically significant difference according to the size of the adenomas in this study, we did find levels were higher in adenomas with a villous component or high-grade dysplasia. This is consistent with this statement. However, there may be important tumor-stromal interactions at the adenoma stage of neoplasia. Individuals with a high deep stromal expression of COX-2 developed significantly more recurrent adenomas (65%) than those with a low deep stromal expression (47%, p=0.04) [60]. Further, although much of the data suggest an important role for COX-2/PGE2 signaling in the promotion of tumorigenesis, many conflicting results have also been reported and further research is still required [61]. Our data supports the role of COX-2 in tumor progression in the human colorectum, but also indicates it may not be as important as the TGF-β and Wnt/β-catenin pathways. Future large-scale studies with detailed zonal quantitative analysis of epithelial/stromal expression of COX-2 may contribute to understanding the precise role of COX-2 in human colorectal carcinogenesis.

In addition to evaluating the individual markers within a pathway, it is also important to consider their relationship to each other because cancer development is a long-term, complex process requiring an accumulation of alterations in multiple genes and signaling pathways to form a complex cross-talk network. In the present study, most biomarkers were significantly correlated with each other. For example, cyclin D1 was also significantly correlated with other biomarkers such as Ki-67, EGFR, TGFβRII and c-Myc, suggesting a complexity of potential interactions of cyclin D1 with other genes, regardless of tumor location [62]. An illustration summarizing the relationships between the pathways examined in this study is provided in Figure 2. Previous animal studies using mouse models of Smad4/APC and Smad2/APC compound heterozygotes provided direct evidence of the cooperative involvement of TGF-β and Wnt signaling in carcinogenesis, including colon neoplasia [63,64]. The exact nature of the signaling cross-talk among the pathways is extremely complex and highly context-dependent. Nonetheless, it is well-described that the Wnt/β-catenin signaling plays a key role in both early and late colorectal tumorigenesis [65] [53]. Furthermore, this pathway interacts with other pathways to form complex signaling networks (see reviews [66–71]). The β-catenin downstream targets (cyclin D1 and c-Myc) are not only regulated by the Wnt/β-catenin pathway, but also directly (EGFR) and indirectly (EGFR, TGF-β, and COX-2) by other pathways. For example, EGFR-Erk directly increases the expression of cyclin D1 and c-Myc and indirectly enhances β-catenin-TCF transcription activity by down-regulating caveolin-1. The nuclear pSmad2/3 can directly associate with the lymphoid enhancer binding factor 1/T cell-specific factor (LEF1/TCF) to synergistically activate β-catenin target genes. This may partially explain our finding that, in comparison to β-catenin, its downstream targets (cyclin D1 and c-Myc) and cell proliferation activity (Ki-67) are more closely correlated with the other pathway biomarkers EGFR, COX2 and TGFβRII. However, this study showed that nuclear accumulation of β-catenin is associated with its downstream target gene c-Myc, but not cyclin D1. The lack of a correlation between nuclear cyclin D1 and nuclear β-catenin might be explained by a previous study. It found that the nuclear accumulation of cyclin D1 is mainly due to the loss of cyclin D1 nuclear export, altered nuclear trafficking, and proteolysis. This can result from direct mutations within cyclin D1, cancer-specific alternative splicing or mutations that target the upstream signaling pathway that regulate the phosphorylation-dependent nuclear

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export of cyclin D1 complexes [72]. Although larger studies are needed, our observational data provide support that, for traditional sporadic adenomas, a synergistic role among Wnt/beta-catenin, TGF-β, COX-2 and EGFR pathways may exist to promote tumor progression in the colorectum. Thus, multiple and key components in the associated pathways likely need to be simultaneously targeted for use as early detection biomarkers or for the effective prevention and treatment of CRC.

To the best of our knowledge, few studies focus on the comparison of the expression of carcinogenesis biomarkers in traditional colorectal adenomas between the proximal and distal colorectum. For this study, there are several factors that need to be considered in the interpretation. This study only included individuals with either synchronous or advanced adenomas, so this study may represent a more severe, or later, phenotype. This study was also limited to an analysis of combined categories of colorectum segments (i.e. proximal and distal or distal without rectum). If there are important location differences in early carcinogenesis or if there are important segmental differences, this study may not have been able to detect the differences. In this study, we used non-probability sampling within a larger cohort. Although we found few differences between the sample and the larger cohort, we cannot exclude the possibility of unmeasured selection biases or confounding, which may affect the generalization to the larger cohort in an unknown manner. The study is also based on a cross-sectional analysis and so cannot evaluate relationship or relevance of the selected markers to the prospective risk of colorectal neoplasia. The panel of markers was carefully selected for this study based on known genes and pathways important in the initiation and progression of CRC [26]. Although we did not observe statistically significant differences between proximal and distal adenomas in this study for any of the eight selected biomarkers, we did observe differences according to other adenoma features, such as advanced adenoma. Future studies are warranted to evaluate whether these markers may be used as predictors of advanced adenoma. Future studies in probability-based samples, which can also prospectively evaluate risk of metachronous adenoma or colorectal cancer and more extensive biomarkers, are also needed to confirm or refute our findings.

There are several strengths. Participants were recruited from a well-established retrospective cohort study of patients with a first time diagnosis. We had detailed patient information, and the diagnoses were standardized by a single pathologist. Our results with adenoma features, other than polyp location, were consistent with most previous studies, suggesting the data obtained in the current study are appropriate for the analyses. Finally, in general, the sample size was adequate for most comparisons, although the number of adenomas with high grade dysplasia was small.

In conclusion, our study indicated that the expression of selected biomarkers were largely similar between distal and proximal adenomas, after accounting for histological differences, indicating a similar carcinogenesis process in traditional adenoma formation for our study participants. The altered expression of β-catenin, cyclin D1 and TGFβRII was closely correlated with advanced features of adenoma, suggesting the important roles of Wnt/β-catenin and TGF-β pathways in the carcinogenesis in both the proximal and distal colorectum. Further studies are warranted to confirm our findings.

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Supplementary Material

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Acknowledgments

Grant sponsor: National Cancer Institute; Grant numbers: R01CA97386; P50CA95103; K07CA122451; P30CA068485.

This study is supported by the research grants R01CA97386 and P50CA95103 through the National Cancer Institute. Dr. Shrubsole was supported by K07CA122451 from the National Cancer Institute. The immunostaining of the biomarkers and participant recruitment were conducted by the Survey and Biospecimen Shared Resource and the Translational Pathology Shared Resource, both of which are supported in part by the Vanderbilt-Ingram Cancer Center (P30CA068485). The content of this paper is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health. A portion of this material is the result of work supported with resources and the use of facilities at the VA Tennessee Valley Healthcare System.

We thank Mr. Anthony L. Frazier for collecting tissue blocks and for his technical assistance in histology, Ms. Pamela S. Wirth for assistance with immunohistochemical staining, Ms. Sara Hollis and Ms. Hongmei Wu for laboratory assistance and tissue sample collection, and Dr. Zhi Chen for her statistical analysis assistance.

Abbreviations

CRC	colorectal cancer
MSI	microsatellite instability
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
COX-2	cyclooxygenase-2
TGFβRII	transforming growth factor beta receptor II
EGFR	epidermal growth factor receptor
PBS	phosphate buffered saline
TCF4	transcription factor 4
DAPI	4′,6-Diamidino-2-phenylindole
SSA	sessile serrated adenomas
TSA	traditional serrated adenoma
LEF1/TCF	lymphoid enhancer binding factor 1/T cell-specific factor
ERK	extracellular signal-regulated kinase
PI3K	phosphatidylinositol 3-kinase
$G_{\alpha s}$	G protein α subunits
$G_{\beta\gamma}$	G protein β^{γ} subunits
TGFα	transforming growth factor-α

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AC	adenylyl cyclase
PKA	protein kinase A
cAMP	cyclic AMP
AR	amphiregulin

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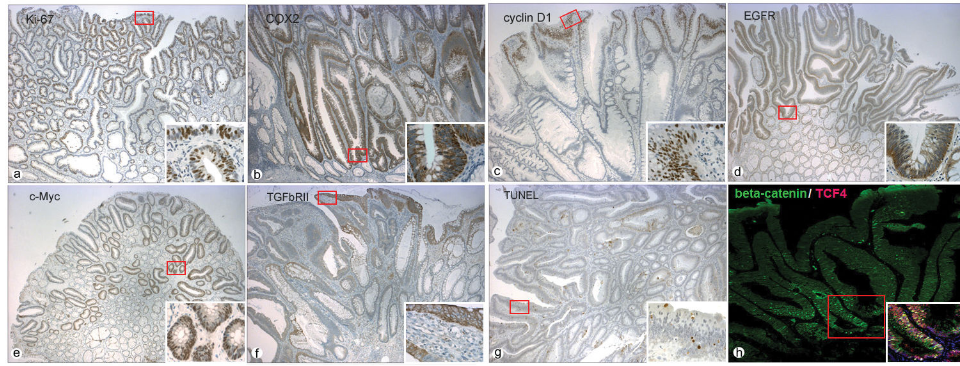

Figure 1.

The expression patterns of eight markers in colorectal adenomas. The positive cells of most markers (Ki-67, TUNEL, COX-2, cyclin D1, EGFR, c-Myc and TGFβRII) show surface predominant distribution, except nuclear β-catenin (h), which shows basal predominant distribution (in which the positive cells are mainly distributed in the basal zone of adenomatous polyp) (see Supplementary Table 2). The subcellular localization of each marker is shown with high-power magnification in the lower right corner of each figure. The positively stained markers may present in the nuclei (Ki-67, TUNEL, cyclin D1 and c-Myc), cytomembrane (TGFβRII and EGFR), or cytoplasm (COX-2) of tumor cells. The positive signal of β-catenin shows the complexity of subcellular localization in the cell membrane, cytoplasm and nuclei (h). The positive nuclear β-catenin (green) overlaid the positive TCF4 (red) shows in yellow color, and the nuclei are counterstained with DAPI.

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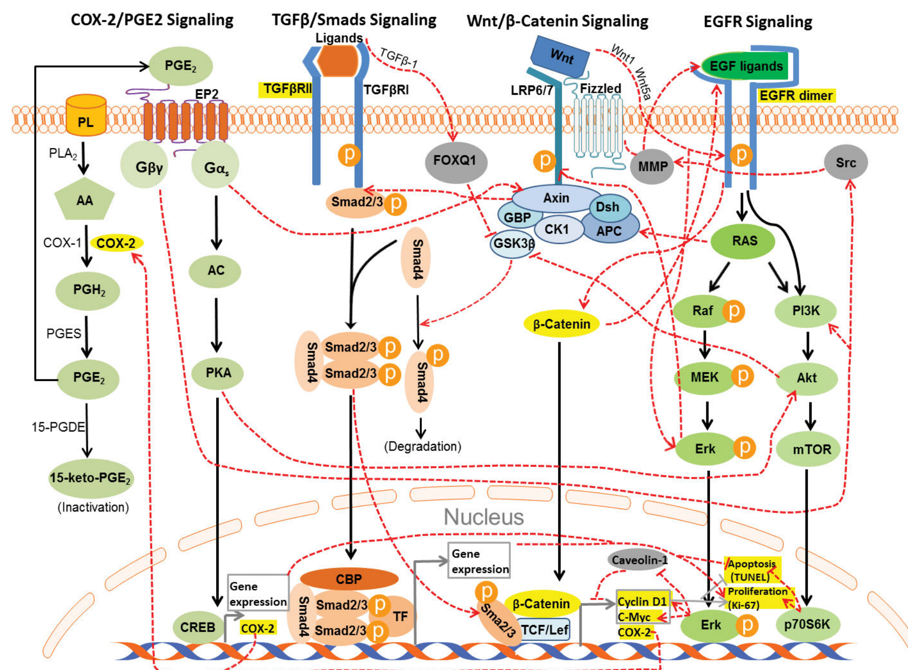

Figure 2. The cross-talk of the canonical Wnt/β-catenin pathway with the TGF-β, EGFR and COX-2 pathways

For the interaction between the Wnt/β-catenin and TGF-β pathways, the responsive Smads (Smads 2 and 3) can directly associate with lymphoid enhancer binding factor 1/T cell-specific factor (LEF1/TCF) to synergistically activate target genes [73,74]; the tumor suppressor Smad4 is negatively regulated by Wnt/GSK3 phosphorylations [75]; Axin, a core component of the β-catenin destruction complex, may function as an adapter, facilitating Smad3 association with the receptor complex thereby promoting TGF-β signaling [76]; FOXQ1, a member of the forkhead transcription factor family, can be induced by TGF-β1 and enhance the nuclear translocation of β-catenin [77]. For interaction between Wnt/β-catenin and EGFR pathways, a synergistic interaction has been found through various mechanisms at different levels. For example, the direct association between β-catenin and EGFR/c-Neu (ErbB1/ErbB2) heterodimers is identified in mammary gland tumors [78]. In intestinal tumor cells, APC and KRAS act synergistically in enhancing Wnt signaling, tumor formation and progression [79]. Overexpression of Wnt-1 and Wnt-5a activated EGFR signaling by stimulated EGFR tyrosine phosphorylation, activation of extracellular signal-regulated kinase (ERK)1/2, and matrix metalloproteinase-mediated release of soluble EGFR ligands through Frizzled receptors [80]. A potent activation of Wnt/β-catenin by EGFR is dependent on ERK MAP kinase-mediated phosphorylation of Wnt co-receptor LRP6 which dramatically increases the cellular response to Wnt. Moreover, EGFR directly phosphorylate β-catenin at Tyr142, which is known to increase cytoplasmic β-catenin concentration via release of β-catenin from membranous cadherin complexes [81]. The phosphatidylinositol 3-kinase (PI3K)/AKT pathway inactivates GSK3, via direct AKT-mediated phosphorylation to facilitate Wnt signaling pathway [82]. EGF treatment of human breast cancer cell lines MDA-MB-468 can induce a strong tyrosine phosphorylation of β-catenin, that blocks the interaction between β-catenin and E-cadherin and increases the invasiveness and metastatic

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potential of cancer cells [83]. In addition, EGFR/Erk signaling causes upregulation of cyclin D1 and c-Myc, and downregulation of caveolin-1 which in turn enhances β-catenin-TCF/LEF-1 transcriptional activity [84–86]. For interaction between Wnt/β-catenin and COX-2 pathways, a direct link between two pathways has been found through prostaglandin E2, one of the bioactive products of COX-2, which activates components of the canonical Wnt signaling system via G protein-coupled receptor EP2 and ravious signaling. The G protein α subunits ($G_{\alpha s}$) interact with Axin resulting displacement of APC and increase of nuclear β-catenin; $G_{\alpha s}$ can also activate Akt through PKA; G protein β^{γ} subunits ($G_{\beta^{\gamma}}$) interact with PI3K resulting phosphorylation and inactivation of GSK-3β via Akt. In addition, EP2-$G\beta^{\gamma}$ can also promote the transactivation of EGFR pathway in colon cancer cells through Src, which activates the proteolytic release of the EGFR ligands amphiregulin (AR) and transforming growth factor-α (TGFα), thereby stimulating the EGFR-signalling network. EP2-$G_{\alpha s}$ also stimulates expression of COX-2 by activating adenylyl cyclase (AC), resulting in increased cyclic AMP (cAMP) production, protein kinase A (PKA) activation and the phosphorylation of CREB. [69,71].

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Table 1

Clinicopathological characteristics of participants, Tennessee-Indiana Adenoma Recurrence Study

Participant Characteristics	All Cases	Subgroup of Tumor Type			*P*-value
		Predominant Proximal*	Predominant Distal†	Equivalent Both Sides‡	
	N=380	N=122	N=200	N=58	
Age, mean ± std (y)	60.4 ± 8.7	60.3 ± 8.4	60.1 ± 8.8	61.9 ± 8.8	0.37
Female, n (%)	149 (39.2)	52 (42.6)	81 (40.5)	16 (27.6)	0.13
White, n (%)	325 (89.5)	108 (92.3)	167 (86.5)	50 (94.3)	0.13
Synchronous adenomas, n (%)	317 (83.4)	104 (85.3)	155 (77.5)	58 (100.0)	0.0002
Location of adenoma(s), n (%)					<0.001
Proximal only	87 (23.6)	87 (74.4)	0	0	
Distal only	131 (35.6)	0	131 (67.9)	0	
Both proximal and distal	150 (40.8)	30 (25.6)	62 (32.1)	58 (100.0)	
Size of largest adenoma, n (%)					<0.001
< 1 cm	209 (55.0)	72 (59.0)	88 (44.0)	49 (84.5)	
1 cm	109 (28.7)	32 (26.2)	70 (35.0)	7 (12.1)	
Unknown	62 (16.3)	18 (14.8)	42 (21.0)	2 (3.5)	
Worst subtype (%)					0.03
Tubular	305 (80.3)	97 (79.5)	153 (76.5)	55 (94.8)	
Tubulovillous/villous	70 (18.4)	23 (18.9)	45 (22.5)	2 (3.5)	
SSA	3 (0.8)	1 (0.8)	2 (1.0)	0	
TSA	2 (0.5)	1 (0.8)	0	1 (1.7)	
High-grade dysplasia, n (%)	31 (8.2)	7 (5.7)	22 (11.0)	2 (3.5)	0.09
Advanced adenoma, n (%)	186 (49.0)	56 (45.9)	121 (60.5)	9 (15.8)	<0.001
Synchronous hyperplastic polyp, n (%)	39 (10.3)	12 (9.8)	20 (10.0)	7 (12.1)	0.88

SSA, sessile serrated adenomas; TSA, traditional serrated adenoma

*Most advanced adenoma (largest size, tubulovillous subtype, or high grade dysplasia) at right side

†Most advanced adenoma at left side

‡Multiple adenomas with same size and same level of advanced on both sides

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Table 2

Correlation between biomarker expression and clinicopathological characteristics among participants with distal or proximal predominant adenomas*

Biomarker Level†	Size of largest adenoma		Subtype		Dysplasia Level		Advanced Adenoma		Synchronous Adenomas		Combined Synchronous and Advanced		
	< 1 cm	1 cm	Tubular	Tubulovillous or Villous	No highgrade	Highgrade	No	Yes	No	Yes	Synchronous non-advanced adenomas	Single adenoma advanced	Synchronous adenomas 1 advanced
TUNEL, mean (std)	0.59 (1.21)	0.55 (1.68)	0.57 (1.60)	0.27 (0.49)	0.53 (1.50)	0.28 (0.35)	0.62 (1.58)	0.41 (1.30)	0.23 (0.45)	0.57 (1.58)	0.62 (1.58)	0.23 (0.45)	0.50 (1.58)
P-value	0.84		0.13		0.38		0.18		0.09		0.19		
Ki-67													
0	66 (42.8)	22 (21.6)	89 (36.0)	12 (17.7)	97 (33.2)	5 (17.2)	65 (45.1)	37 (20.9)	12 (19.1)	90 (34.9)	65 (45.1)	12 (19.1)	25 (21.9)
1	91 (57.2)	80 (78.4)	158 (64.0)	56 (82.4)	195 (66.8)	24 (82.8)	79 (54.9)	140 (79.1)	51 (81.0)	168 (65.1)	79 (54.9)	51 (81.0)	89 (78.1)
P-value	<0.001		0.004		0.08		<0.001		0.02		<0.001		
COX-2													
0	25 (15.6)	19 (18.6)	44 (17.7)	8 (11.8)	49 (16.7)	5 (17.2)	25 (17.2)	29 (16.4)	13 (20.6)	41 (15.8)	25 (17.2)	13 (20.6)	16 (14.0)
1	80 (50.0)	44 (43.1)	118 (47.6)	23 (33.8)	131 (44.7)	13 (44.8)	73 (50.3)	71 (40.1)	25 (39.7)	119 (46.0)	73 (50.3)	25 (39.7)	46 (40.4)
2	40 (25.0)	22 (21.6)	63 (25.4)	19 (27.9)	79 (27.0)	3 (10.3)	37 (25.5)	45 (25.4)	17 (27.0)	65 (25.1)	37 (25.5)	17 (27.0)	28 (24.6)
3	15 (9.4)	17 (16.7)	23 (9.3)	18 (26.5)	34 (11.6)	8 (27.6)	10 (6.9)	32 (18.1)	8 (12.7)	34 (13.1)	10 (6.9)	8 (12.7)	21 (21.1)
P-value	0.26		0.001		0.045		0.02		0.75		0.045		
EGFR													
0	74 (46.8)	46 (45.1)	112 (45.5)	25 (36.8)	133 (45.7)	8 (27.6)	66 (46.2)	75 (42.4)	24 (38.1)	117 (45.5)	66 (46.2)	24 (38.1)	51 (44.7)
1	52 (32.9)	30 (29.4)	80 (32.5)	20 (29.4)	92 (31.6)	10 (34.5)	49 (34.3)	53 (29.9)	17 (27.0)	85 (33.1)	49 (34.3)	17 (27.0)	36 (31.6)
2	32 (20.3)	26 (25.5)	54 (22.0)	23 (33.8)	66 (22.7)	11 (37.9)	28 (19.6)	49 (27.7)	22 (34.9)	55 (21.4)	28 (19.6)	22 (34.9)	27 (23.7)
P-value	0.59		0.12		0.10		0.24		0.08		0.22		
TGFβRII													
0–1	113 (71.1)	48 (47.1)	157 (63.6)	15 (22.1)	165 (56.5)	10 (34.5)	107 (74.3)	68 (38.4)	16 (25.4)	159 (61.6)	107 (74.3)	16 (25.4)	52 (45.6)
2	33 (20.8)	36 (35.3)	62 (25.1)	36 (52.9)	84 (28.8)	15 (51.7)	25 (17.4)	74 (41.8)	36 (57.1)	63 (24.4)	25 (17.4)	36 (57.1)	38 (33.3)
3	13 (8.2)	18 (17.7)	28 (11.3)	17 (25.0)	43 (14.7)	4 (13.8)	12 (8.3)	35 (19.8)	11 (17.5)	36 (14.0)	12 (8.3)	11 (17.5)	24 (21.1)
P-value	0.004		<0.001		0.03		<0.001		<0.001		<0.001		
β-catenin													
0	65 (41.1)	48 (47.1)	101 (41.1)	33 (48.5)	126 (43.3)	12 (41.4)	57 (39.9)	81 (45.8)	32 (50.8)	106 (41.3)	57 (39.9)	32 (50.8)	49 (43.0)
1	71 (44.9)	33 (32.4)	99 (40.2)	18 (26.5)	115 (39.5)	4 (13.8)	67 (46.9)	52 (29.4)	20 (31.8)	99 (38.5)	67 (46.9)	20 (31.8)	32 (28.1)

Biomarker Level[†]	Size of largest adenoma		Subtype		Dysplasia Level		Advanced Adenoma		Synchronous Adenomas		Combined Synchronous and Advanced		
	< 1 cm	1 cm	Tubular	Tubulovillous or Villous	No highgrade	Highgrade	No	Yes	No	Yes	Synchronous non-advanced adenomas	Single adenoma advanced	Synchronous adenomas 1 advanced
2–3	22 (13.9)	21 (20.6)	46 (18.7)	17 (25.0)	50 (17.2)	13 (44.8)	19 (13.3)	44 (24.9)	11 (17.5)	52 (20.2)	19 (13.3)	11 (17.5)	33 (29.0)
P-value	0.10		0.11		<0.001		0.002		0.39			0.003	
c-Myc													
0–1	54 (33.8)	26 (25.5)	79 (31.9)	16 (23.5)	91 (31.1)	6 (20.7)	49 (33.8)	48 (27.1)	20 (31.8)	77 (29.7)	49 (33.8)	20 (31.8)	28 (24.6)
2	89 (55.6)	45 (44.1)	121 (48.8)	30 (44.1)	144 (49.2)	10 (34.5)	81 (55.9)	73 (41.2)	26 (41.3)	128 (49.4)	81 (55.9)	26 (41.3)	47 (41.2)
3	17 (10.6)	31 (30.4)	48 (19.4)	22 (32.4)	58 (19.8)	13 (44.8)	15 (10.3)	56 (31.6)	17 (27.0)	54 (20.9)	15 (10.3)	17 (27.0)	39 (34.2)
P-value	<0.001		0.06		0.008		<0.001		0.44			<0.001	
Cyclin D1													
0	79 (49.7)	25 (24.8)	100 (40.7)	13 (19.1)	110 (37.8)	7 (24.1)	75 (52.1)	42 (23.9)	14 (22.2)	103 (40.1)	75 (52.1)	14 (22.2)	28 (24.8)
1	70 (44.0)	52 (51.5)	115 (46.8)	30 (44.1)	132 (45.4)	14 (48.3)	61 (42.4)	85 (48.3)	30 (47.6)	116 (45.1)	61 (42.4)	30 (47.6)	55 (48.7)
2	10 (6.3)	24 (23.8)	31 (12.6)	25 (36.8)	49 (16.8)	8 (27.6)	8 (5.6)	49 (27.8)	19 (30.2)	38 (14.8)	8 (5.6)	19 (30.2)	30 (26.6)
P-value	<0.001		<0.001		0.21		<0.001		0.004			<0.001	
Wnt pathway score[‡]													
0–1	51 (32.5)	21 (20.8)	70 (28.7)	11 (16.2)	79 (27.3)	5 (17.2)	47 (33.1)	37 (31.0)	15 (23.8)	69 (37.1)	47 (33.1)	15 (23.8)	22 (19.5)
2–3	87 (55.4)	54 (53.5)	123 (50.4)	32 (47.1)	147 (50.9)	11 (37.9)	79 (55.6)	79 (44.9)	30 (47.6)	128 (50.2)	79 (55.6)	30 (47.6)	49 (43.4)
4	19 (12.1)	26 (25.7)	51 (20.9)	25 (36.8)	63 (21.8)	13 (44.8)	16 (11.3)	60 (34.1)	18 (38.6)	58 (22.8)	16 (11.3)	18 (28.6)	42 (37.2)
P-value	0.008		0.01		0.02		<0.001		0.61			<0.001	

[*] Unless otherwise indicated, the frequency (percentage) is presented in each table cell.

[†] The scoring criteria for the % positive cells were as follows: c-Myc: 0=0%, 1=<10%, 2=10–50%, 3=>50%; Ki-67: 0=0–20%, 1=>20%; cyclin D1:0=<5%, 1=5–30%, 2=>30%; EGFR: 0=<10%, 1=10–50%, 2=>50%; COX-2 & TGFβRII: 0=negative, 1=<2/3 cells with slight staining or <1/3 cells with moderate staining including focal cells with intense staining, 2=>2/3 slight staining or 1/3–2/3 moderate staining, 3=>2/3 moderate or strong staining.

[‡] Wnt pathway score is the sum of the scores for β-catenin, c-Myc, and Cyclin D1.

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Table 3

Comparison of biomarker expression levels between left- and right-sided predominant adenomas

Biomarker Level	Subgroup of Tumor Type		*P*-value		
	Predominant Proximal N=122* n (%)	Predominant Distal N=200* n (%)	Unadjusted	Adjusted for Adenoma Features†	Adjusted for Other Biomarkers‡
TUNEL, mean(std)	0.52 (1.24)	0.49 (1.54)	0.88	0.93	
Ki-67			0.53	0.99	
0	41 (33.9)	61 (30.5)			
1	80 (66.1)	139 (69.5)			
COX-2			0.98	0.92	
0	21 (17.2)	33 (16.5)			
1	54 (44.3)	90 (45.0)			
2	30 (24.6)	52 (26.0)			
3	17 (13.9)	25 (12.5)			
EGFR			0.05	0.12	0.15
0	62 (51.2)	79 (39.7)			
1	38 (31.4)	64 (32.2)			
2	21 (17.4)	56 (28.1)			
TGFβRII			0.11	0.33	0.72
0–1	75 (61.5)	100 (50.3)			
2	34 (27.9)	65 (32.7)			
3	13 (10.7)	34 (17.1)			
β-catenin			0.10	0.10	0.06
0	43 (35.5)	95 (47.7)			
1	51 (42.2)	68 (34.1)			
2–3	27 (22.3)	36 (18.1)			
c-Myc			0.11	0.22	0.40
0–1	44 (36.1)	53 (26.5)			
2	57 (46.7)	97 (48.5)			
3	21 (17.2)	50 (25.0)			
cyclin D1			0.01	0.09	0.12

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Biomarker Level	Subgroup of Tumor Type		*P*-value		
	Predominant Proximal N=122* n (%)	**Predominant Distal N=200* n (%)**	**Unadjusted**	**Adjusted for Adenoma Features†**	**Adjusted for Other Biomarkers‡**
0	55 (45.1)	62 (31.3)			
1	53 (43.4)	93 (47.0)			
2	14 (11.5)	43 (21.7)			
Wnt pathway score			0.56	0.54	
0–1	36 (29.8)	48 (24.4)			
2–3	57 (47.1)	101 (51.3)			
ge;4	28 (23.1)	48 (24.4)			

TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; COX-2, cyclooxygenase-2; EGFR, epidermal growth factor receptor; TGFβRII, transforming growth factor beta receptor II

* Unless otherwise indicated, the frequency (percentage) is presented in each table cell.

† Adjusted for adenoma size, villous component, high-grade dysplasia, and synchronousness.

‡ Mutually adjusted for all biomarkers with a *P*-value < 0.20 in unadjusted models (c-Myc, cyclin D1, EGFR, β-catenin, and TGFβRII).

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Table 4

Comparison of biomarker expression levels between both sides equivalent adenomas*

Biomarker	Total		Basal		Middle		Surface	
	Difference	*P*	Difference	*P*	Difference	*P*	Difference	*P*
TUNEL†	−0.24	0.43	−0.16	0.53	−0.43	0.29	−0.12	0.74
Ki-67	0	0.61	0	0.13	0	0.29	0	0.79
COX-2	0	0.80	0	0.77	0	0.84	0	0.61
EGFR	0	0.21	0	0.39	0	0.06	0	0.72
TGFβRII	0	0.70	0	0.31	0	0.82	0	0.86
β-catenin	0	0.52	0	0.28	0	0.37	0	0.96
c-Myc	0	0.32	0	0.48	0	0.17	0	0.86
Cyclin D1	0	0.03	0	0.63	0	0.73	0	0.07
Wnt pathway score	0	0.045	0	0.28	0	0.15	0	0.52

TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; COX-2, cyclooxygenase-2; EGFR, epidermal growth factor receptor; TGFβRII, transforming growth factor beta receptor II

*Median difference and p-value from Wilcoxon signed rank test are presented except TUNEL.

†Mean difference and p-value from paired t-test are presented.

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Table 5

Correlation of biomarker expressions in colorectal adenomas*

	Ki-67		COX-2		EGFR		TGFβRII		β-catenin		c-Myc		Cyclin D1		Wnt PathwayScore	
	r	***P***	**r**	***P***	**r**	***P***	**r**	***P***	**r**	***P***	**r**	***P***	**r**	***P***	**r**	***P***
TUNEL	0.11	0.02	0.12	0.02	0.16	0.001	0.14	0.009	−0.08	0.12	0.20	<0.001	0.10	0.06	0.10	0.06
Ki-67			0.23	<0.001	0.14	0.008	0.30	<0.001	0.05	0.33	0.42	<0.001	0.37	<0.001	0.39	<0.001
COX-2					0.06	0.23	0.24	<0.001	−0.002	0.97	0.22	<0.001	0.17	<0.001	0.19	<0.001
EGFR							0.15	0.004	0.05	0.38	0.21	<0.001	0.17	0.001	0.19	<0.001
TGFβRII									−0.02	0.75	0.37	<0.001	0.35	<0.001	0.34	<0.001
β-catenin											0.11	0.03	0.07	0.17		
c-Myc													0.35	<0.001		

*Spearman correlation coefficients

TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; COX-2, cyclooxygenase-2; EGFR, epidermal growth factor receptor; TGFβRII, transforming growth factor beta receptor II