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Epigenetic Regulation of WNT Signaling Pathway Genes in Inflammatory Bowel Disease (IBD) Associated Neoplasia

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Abstract

Introduction—WNT signaling pathway dysregulation is an important event in the pathogenesis of colorectal cancer (CRC) with *APC* mutations seen in more than 80% of sporadic CRC. However, such mutations in the WNT signaling pathway genes are rare in inflammatory bowel disease (IBD) associated neoplasia (dysplasia and cancer). This study examined the role of epigenetic silencing of WNT signaling pathway genes in the pathogenesis of IBD-associated neoplasia.

Methods—Paraffin-embedded tissue samples were obtained and methylation of ten WNT signaling pathway genes, including *APC1A*, *APC2*, *SFRP1*, *SFRP2*, *SFRP4*, *SFRP5*, *DKK1*, *DKK3*, *WIF1* and *LKB1*, was analyzed. Methylation analysis was performed on 41 IBD samples, 27 normal colon samples (NCs), and 24 sporadic CRC samples.

Results—Methylation of WNT signaling pathway genes is a frequent and early event in IBD and IBD-associated neoplasia. A progressive increase in the percentage of methylated genes in the WNT signaling pathway from NCs (4.2%) to IBD colitis (39.7%) to IBD-associated neoplasia (63.4%) was seen (NCs vs. IBD colitis, $p<0.01$; IBD colitis vs. IBD-associated neoplasia, $p=0.01$). In the univariate logistic regression model, methylation of *APC2* (OR 4.7, 95% CI: 1.1–20.63, $p=0.04$), *SFRP1* (OR 5.1, 95% CI: 1.1–31.9, $p=0.04$), and *SFRP2* (OR 5.1, 95% CI: 1.1–32.3, $p=0.04$) was associated with progression from IBD colitis to IBD-associated neoplasia, while *APC1A* methylation was borderline significant (OR 4.1, 95% CI: 0.95–17.5, $p=0.06$). In the multivariate logistic regression model, methylation of *APC1A* and *APC2* was more likely to be associated with IBD-associated neoplasia than IBD colitis. (OR *APC1A*: 6.4, 95% CI: 1.1–37.7 $p=0.04$; OR *APC2* 9.1, 95% CI: 1.3–61.7, $p=0.02$).

Summary—Methylation of the WNT signaling genes is an early event seen in patients with IBD colitis and there is a progressive increase in methylation of the WNT signaling genes during development of IBD-associated neoplasia. Moreover, methylation of *APC1A*, *APC2*, *SFRP1*, and *SFRP2* appears to mark progression from IBD colitis to IBD-associated neoplasia, and these genes may serve as biomarkers for IBD-associated neoplasia.

Keywords

IBD; Methylation; WNT signaling; Colorectal cancer

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the intestines afflicting about one million individuals in the US, with 30,000 new cases each year.¹ IBD includes two distinct disease categories, Crohn's disease (CD) and ulcerative colitis (UC), both of which are associated with an increased risk of CRC.^{2,3} Additionally, CRC is a major cause of increased mortality in IBD patients.⁴ The risk of CRC in IBD increases with prolonged disease duration and is greater in those with extensive colitis.⁵ This risk has been reported as ten- to 20-fold higher in UC patients with disease duration of 20 years or more, although current treatments may have modulated this risk.^{6–8} As a result of the recognition of this increased risk, regular surveillance colonoscopy at 1–3 years interval is recommended for patients with long-standing disease.^{9–12}

IBD-associated carcinomas arise in areas of dysplasia which are flat and difficult to recognize at colonoscopy.^{13,14} Consequently, one may have to take 33 or more colonic biopsies to have a 90% confidence of finding dysplasia. To increase the accuracy of colonoscopic surveillance to 95%, nearly twice the number of biopsy specimens are required.¹⁵ This makes surveillance labor intensive and expensive. Moreover, some of the recent studies have questioned the effectiveness of surveillance.^{16–18} In order to improve the effectiveness of surveillance, there is a great need for objective and reliable molecular markers for early detection of IBD-associated neoplastic lesions especially among patients with long-standing disease.

DNA methylation-dependent silencing of cancer related genes is an important and early event in CRC. Methylation of promoter-associated CpG islands leads to binding of various proteins having methyl binding domains like MeCP2, MBD2, and MBD3; these proteins may then initiate a cascade of events, which eventually lead to transcriptional silencing.¹⁹ Transcriptional silencing also involves changes in histone tails such as histone H3 lysine 9 methylation and histone H3 lysine 27 methylation as well as recruitment of histone deacetylases.^{20,21} All of these modifications result in changes to the local chromatin structure, which in turn result in a tightly compacted chromatin, which in turn restricts the access to transcription factors facilitating transcriptional silencing. Aberrant age-related as

well as cancer-specific methylation of genes like *p16*, *E-cadherin*, *hMLH1*, *p14*, *HPP1*, *ER*, etc., has already been reported in the setting of IBD-associated neoplasia (dysplasia and cancers).^{22–26} It is believed that chronic inflammatory states like IBD may predispose to accelerated aberrant methylation and inactivation of tumor suppressor genes, which in turn may accelerate the development of cancer.

The WNT signaling pathway is crucial to the development of sporadic CRC.^{27,28} Activating mutations in the WNT signaling pathway are seen in more than 80% of sporadic CRC.^{29,30} Signaling in the WNT signaling pathway begins when the WNT ligand, a secreted factor binds to the Frizzled (Fz) receptor and LRP 5/6 co-receptor to initiate the signaling cascade.³¹ Proteins of the disheveled (Dsh or dvl) family then interact with Fz receptors.³² These interactions subsequently lead to inhibition of complex formed by *APC*, *AXIN*, and Glycogen synthase kinase-3 β (*GSK-3 β*). The *APC/AXIN/GSK-3 β* complex normally plays a role in phosphorylation and degradation of β -catenin. As a result of the inhibition of the *APC/AXIN/GSK-3 β* complex, β -catenin accumulates and is translocated to the nucleus³³ where it upregulates the transcription of many cancer-related genes including *MYC*,³⁴ *cyclooxygenase-2 (COX-2)*,³⁵ and *cyclin D1* (Fig. 1).³⁶ Powerful developmental regulatory pathways, like the WNT signaling pathway, are controlled by stringent negative regulation, which, if disrupted, can lead to their aberrant activation and tumorigenesis. WNT signaling pathway is normally inhibited by soluble Fz-related proteins (SFRPs) and WNT inhibitory factor (WIF1), which bind to WNT ligands extracellularly³⁷ and by proteins of the dickkopf (DKK) family, which bind to the LRP surface molecule.³⁸ Hence, inactivation of *SFRPs*, *WIF1*, and *DKKs* by mechanisms such as promoter methylation can lead to activation of WNT signaling in cancer cells. Similarly, inactivation of *APC* by mutation or promoter methylation or both can lead to prevention of degradation of β -catenin as *APC* normally plays a role in β -catenin degradation by complexing with other proteins as described previously. Although mutations of WNT signaling pathway genes including *APC* have been described in sporadic CRC, mutations of these genes are rare (0% to 6%) in IBD-associated neoplasia.³⁹

Recent reports have explored the role of WNT signaling in IBD-associated neoplasia, but as yet little is known about the epigenetic regulation of this pathway in IBD-associated neoplasia.^{40–42} Increased nuclear β -catenin and reduced cytoplasmic APC1A expression has recently been reported in the setting of UC-associated cancers.^{43,44} Since mutation of WNT signaling genes is a rare event in IBD-associated neoplasia, we hypothesized that methylation of genes in the WNT pathway may be responsible for dysregulation of WNT signaling in these lesions. In the current study, we investigated if promoter methylation of WNT pathway genes occurs during the course of IBD-associated carcinogenesis and furthermore characterize the progression of the methylation events during the course of IBD associated neoplasia. This may help to find potential candidates for investigation as biomarkers for early detection of IBD-associated neoplasia.

We now show that methylation of WNT signaling pathway genes is an early event that can be seen in longstanding IBD colitis and there is a progressive increase in methylation of the WNT signaling pathway genes during the development of IBD-associated neoplasia.

Materials and Methods

Patient Samples

Paraffin-embedded tissue samples were obtained from Johns Hopkins Hospital (JHH) pathology archives in accordance with regulations of the Institutional Review Board and HIPAA compliance. Tissue samples were obtained from 18 IBD patients who underwent colectomy from 1997 to 2006. Eleven of out 18 patients had IBD-associated neoplasia

(either dysplasia or cancer) while seven were non-cancer IBD controls. A total of 41 IBD tissue samples were obtained including six IBD cancers, two high-grade dysplasias (HGDs), eight low-grade dysplasias (LGDs), and 25 noncancerous IBD colitis samples. Slides were first reviewed by an expert gastrointestinal pathologist at the JHH and then 5-um-thick sections of the desired paraffin-embedded tissue blocks were procured. In the case of IBD patients with dysplasia/cancer, we studied dysplasia/cancer samples as well as nondysplastic samples from other non-neoplastic areas of the colon. We also investigated methylation of the WNT signaling pathway genes in 27 NCs from 27 patients as well as 24 samples from 24 sporadic CRC patients. Sporadic CRC patients were stage matched to the IBD-associated cancer patients to determine if there were any differences between methylation of these genes between sporadic and IBD-associated cancers.

Methylation Analysis

Methylation analysis was performed using the methylation-specific polymerase chain reaction (MSP) strategy, as previously described.⁴⁵ DNA was extracted following a standard phenol-chloroform extraction protocol. Bisulfite modification of DNA was done using the EZ DNA methylation KitTM (Zymo Research) as per manufacturer's instructions. Methylation-specific PCR was carried out in a 25- μ l reaction containing 10 $\times$ MSP buffer, 10 mM dNTPs, 33 pmol of each of the methylated or unmethylated primers, 0.5 unit of JumpStartTM REDTaq[®] DNA polymerase and 4 μ l of bisulfite-treated DNA. Amplification cycles were as follows: one cycle of 95°C for 5 min followed by 35 cycles of 95°C for 30 s, annealing temp for 30 s, 72°C for 30 s, and a final extension step of 72°C for 5 min. In vitro methylated DNA (IVD) was used as a positive control for MSP. IVD was created by treating cell line DNA with Sassy methylated (New England Biolabs) as directed. DKO, which is a double knockout derivative of the CRC cell line Hct116 with knockout of the major DNA methyltransferases (DNMT1^{-/-} and DNMT3b^{-/-}) was used as an additional negative control. DKO lacks methylation at 95% of the known CpG sites.⁴⁶ Seven and a half microliters of each amplification reaction was loaded and run on 2% agarose gel containing GelStarTM Nucleic Acid Gel Stain (Cambrex Bio Science) and visualized by ultraviolet illumination.

We tested the promoter methylation of 10 WNT signaling pathway genes including *APC1A* (adenomatous polyposis coli1a), *APC2* (adenomatous polyposis coli2), *SFRP1* (secreted frizzled related protein1), *SFRP2* (secreted frizzled related protein2), *SFRP4* (secreted frizzled related protein4), *SFRP5* (secreted frizzled related protein5), *DKK1* (dickkopf1), *DKK3* (dickkopf3), *WIF1* (WNT inhibitory factor1), and *LKB1* (serine threonine kinase). All of these genes, except *APC2*, have been previously described to be hypermethylated and silenced in CRC by candidate gene approaches.^{47–50} Hypermethylation of *APC2* was recently described by Schuebel et al. in their transcriptome-wide approach to find genes hypermethylated in colon cancer.⁵¹ Table 1 summarizes the primer sequences used for the MSP reaction and the annealing temperatures used for the respective PCR reactions. Percentage of methylated genes for each tissue type sample was calculated using the following formula: (number of genes methylated)/(number of genes tested) $\times$ 100. The means of samples belonging to each tissue type were then compared.

Statistical Analysis

Categorical variables were analyzed using Chi-square tests, while continuous variables were analyzed using Mann–Whitney *U* test. *P* values of less than 0.05 were considered significant. Logistic regression was used to calculate the odds ratios (ORs) and 95% confidence interval. All statistical analysis was performed using the STATA 9.2 software package (College Station, TX).

Results

All IBD patients had long-standing disease with median disease duration of 12 years (12.5 years for those with IBD-associated neoplasia vs. 12 years for controls; $p=0.2$). The median age of IBD colitis samples was 52 years, while the median age of patients with IBD-associated neoplasia was 54 years ($p=0.4$). The cancers from IBD patients were Stages I and II and patients with sporadic CRC were stage-matched to IBD-associated cancers. Family history of cancer was present in 36% of the patients with IBD-associated dysplasia or cancer, while 42% of the patients with IBD colitis without neoplasia had a positive family history of cancer.

DNA was extracted and successful methylation analysis was performed in 99.2% of samples. Methylation of the WNT signaling pathway genes was seen for all genes in our samples, except for *LKB1*, which was uniformly unmethylated. *LKB1* was therefore excluded from further analyses. Table 2 summarizes the methylation frequencies of WNT signaling pathway genes according to the tissue type analyzed. Methylation of the WNT signaling pathway genes varied according to the tissue type analyzed. NCs were uniformly unmethylated for all the genes, except *WIF1*, which was methylated in 7%, and *SFRP2*, which was methylated in 29%. Methylation of some genes can occur in NCs as a process of aging, a process termed age-related methylation. There was no age-related methylation pattern seen for either *SFRP2* or *WIF1* among the NCs. Methylation of all the WNT signaling pathway genes (except *LKB1*) was seen frequently in samples with IBD colitis and IBD-associated neoplasia. The frequency of methylation of the WNT signaling pathway genes seen in samples with IBD colitis was significantly higher than those seen in the NCs (see p values in Table 2), except for *SFRP2* where the difference was not statistically significant ($p=0.10$). Furthermore, the frequency of methylation of the WNT signaling pathway genes (except *LKB1*, which was unmethylated in all IBD-associated neoplasias) increased in samples with IBD-associated neoplasia compared to samples with IBD colitis. Methylation of *APC1A*, *APC2*, *SFRP1*, and *SFRP2* was significantly higher in IBD-associated neoplasia compared to IBD colitis (*APC1A* 43.8% vs. 16.0%, $p=0.05$; *APC2* 81.3% vs. 48.0%, $p=0.03$; *SFRP1* 87.5% vs. 54.1%, $p=0.03$ and *SFRP2* 86.7% vs. 52.0%, $p=0.03$, respectively). Figure 2 shows the methylation profile of WNT signaling pathway genes in one of the IBD patients with cancer as a representative sample. Methylation analysis of the genes in WNT signaling pathway is shown from the cancer and areas of non-cancerous surrounding colitis.

Furthermore, the methylation of all the WNT signaling pathway genes was tested in 24 sporadic CRC who were matched in stage (stages 1 and 2) to the corresponding IBD-associated cancers. The methylation frequencies of the WNT signaling pathway genes seen in the sporadic CRC was similar to those seen in IBD-associated neoplasias (including dysplasias and cancers) except for *SFRP4* and *WIF1*. *SFRP4* was significantly more methylated in IBD-associated neoplasia (81.3%) compared to sporadic CRC (37.5%) ($p<0.01$). On the contrary, *WIF1* was significantly more methylated in sporadic CRC (100%) compared to IBD-associated neoplasia (68.8%), $p<0.01$. The remainder of the frequencies were comparable ($p = ns$; data not shown).

We then calculated the percentage of methylated genes in the WNT signaling pathway for each sample and compared the means for samples belonging to each tissue type. A progressive increase in the percentage of methylated genes was seen from NCs (mean=4.2%) to IBD colitis (mean=39.7%) to IBD-associated neoplasia (mean=63.4%) was seen (NC vs. IBD colitis, $p<0.01$, IBD colitis vs. IBD-associated neoplasia, $p=0.01$) (Fig. 3). There was no significant difference in the percentage of methylated genes between sporadic CRC and IBD-associated neoplasia (61.3% vs. 63.4% respectively; $p=0.42$). However,

methylation is an early event in IBD-associated neoplasia as relatively early lesions like LGDs show a high level of percentage of methylated genes (mean=75%).

We next used logistic regression model to find out whether methylation of these genes can be used to predict the presence of IBD-associated neoplasia, i.e., dysplasia or cancer. In the univariate analysis methylation of *APC2* (odds ratio [OR] 4.7 [95% CI 1.1–20.63], $p=0.04$), *SFRP1* (OR 5.1 [95% CI 1.1–31.9], $p=0.04$), and *SFRP2* (OR 5.1 [95% CI 1.1–32.3], $p=0.04$) was significantly associated with higher risk for IBD-associated neoplasia compared to IBD colitis. Methylation of *APC1A* was found to be borderline significant (OR 4.1 [95% CI 0.95–17.5], $p=0.06$). Moreover, in the multivariate model using these four genes, including *APC1A*, *APC2*, *SFRP1*, and *SFRP2*, methylation of *APC2* and *APC1A* was found to be significantly associated with higher risk for IBD-associated neoplasia [OR for methylated *APC2* 9.1 [95% CI 1.3–61.7], $p=0.02$ and OR for methylated *APC1A* 6.4 [96% CI 1.1–37.7], $p=0.04$] with an ROC value of 77.36%.

Discussion

Our study demonstrates for the first time epigenetic involvement of the WNT signaling pathway in IBD-associated neoplasia. We have shown that methylation of the WNT signaling pathway genes first begins in patients with long-standing IBD colitis and that the frequency of methylation of these genes increases progressively during the development of IBD-associated neoplasia. The percentage of methylated genes increases significantly from NCs to IBD colitis to IBD-associated neoplasia. A limitation of our current study is the relatively small sample size and the lack of expression and immunohistochemical data to fully confirm the silencing of the methylated genes.

However, the frequent methylation of the WNT signaling pathway genes in patients with IBD colitis suggests that chronic inflammation may play an important role in the methylation of WNT signaling genes. Further increase in the methylation of these genes in neoplastic lesions (dysplasia and cancer) suggests a progressive role of methylation of WNT signaling pathway genes in the pathogenesis of IBD-associated neoplasia. This is further supported by the fact that relatively early lesions like LGDs in IBD patients show high levels of methylation of the WNT signaling pathway genes. In the current study, we did not analyze non-inflamed normal colons from patients with IBD. Recent data suggest that some degree of methylation can occur in the non-inflamed terminal ileum, but it is still significantly lower than that seen in inflamed mucosa.⁵²

Chronic inflammation can lead to chronic injury, which may predispose tumor-related genes to methylation and silencing. This process has also been described in other chronic inflammatory states like Barrett's esophagus, which predisposes to esophageal cancer,⁵³ chronic gastritis, which predisposes to gastric cancer,⁵⁴ and cirrhosis, which predisposes to hepatocellular cancer.⁵⁵ WNT signaling pathway genes are some of the genes affected by methylation in patients with IBD. Methylation of *p16*, *E-cadherin*, *hMLH1*, *p14*, *HPPI1*, *ER*, etc. has already been reported by us and others in the setting of IBD-associated neoplasia (dysplasia and cancers).^{22–26} We studied the current panel of genes because of their importance to the biology of colorectal cancers.^{27,28}

Dysplasia is regarded not only as the precursor, but also a marker, of coexisting malignancy in IBD patients. However, there is a great deal of controversy in the diagnosis of dysplasia. Inflammatory epithelial changes can mimic dysplasia, and there is a significant degree of intra- and inter-observer variability in the pathological diagnosis.⁵⁶ This is not limited to less experienced pathologists, since even expert pathologists can differ in the opinion too.^{57,58} This has led to the recommendation that two pathologists, one of whom is an experienced

gastrointestinal pathologist, should independently review biopsy specimens from cases of IBD-associated dysplasia.^{59–61} This criterion was also used when we procured samples for the current study. Our findings of early methylation of *APC2*, *SFRP1*, *SFRP2*, and *APC1A* in IBD-associated dysplasia may provide a more objective marker for the diagnosis of these lesions. In fact, all patients with LGD and HGD showed 100% methylation of the *APC2*, which suggests that this could be used as a marker for dysplasia.

APC1A (classical *APC*) is a classical tumor suppressor gene. Its gene product forms a complex with GSK-3 β , axin/conductin, and β -catenin. Subsequently, β -catenin is phosphorylated and degraded. Mutation of *APC1A* prevents this degradation and causes accumulation of β -catenin in the cell. The β -catenin then translocates to the nucleus where it upregulates the transcription of many cancer-related genes. *APC1A* is mutated in 80% of sporadic CRC.²⁹ Additionally, *APC1A* mutations usually occur early in sporadic colorectal carcinogenesis since lesions like tubular adenomas have been found to harbor *APC1A* mutations. Earlier studies, which looked into the mutation of *APC1A* in IBD, found a 0–6% mutation rate, and that too in advanced lesion like HGD and cancers.^{39,62} In contrast to its low mutation frequency, *APC1A* protein expression was found to be abnormal in 67% of the UC-associated neoplasia, which cannot be explained by low mutation frequency.⁴⁴ Our results suggest that methylation of *APC1A* is an early and frequent event in IBD-associated neoplasia, and our results now suggest that methylation-associated silencing may account for the decreased protein expression of *APC1A* in these lesions. Since methylation of *APC1A* is more common than mutation, epigenetic inactivation may be a predominant mechanism for inactivation of *APC1A* and subsequent development of cancer in IBD patients.

APC2, identified more recently, has a high degree of homology with *APC1A*.⁶³ *APC2* can also modulate WNT signaling like *APC1A*.^{63,64} Apart from mutations in *APC1A*, IBD-associated neoplasias also differ in the timing and frequency of mutation of other tumor suppressor gene *P53*. *P53* mutations occur late in sporadic CRC, being more frequent in cancers compared to adenomas, whereas *P53* mutations occur early and are more frequent in IBD-associated neoplasia. Early lesions like LGDs and sometimes even non-dysplastic mucosa in ulcerative colitis can have *P53* mutations.^{65,66} Interestingly, *APC2* has been shown to interact with a protein, 53BP2, which in turn interacts with *P53* and an anti-apoptotic gene *Bcl2*. This suggests a mechanistic role of *APC2* in the *P53/Bcl2*-linked pathway of cell cycle progression and cell death.⁶⁷ Interaction of *APC2* with *P53* pathway is very interesting if viewed in the context of IBD-associated neoplasia. Early dysplastic lesions show a high level of *P53* mutation and *Bcl2* overexpression.^{65,68,69} Similarly, we have found *APC2* to be methylated to high levels in early dysplastic lesions. Therefore, *APC2* methylation, *P53* mutation, and *Bcl2* over-expression might play a synergistic role in the pathogenesis of these lesions. This will require further investigation.

The frequent inactivation of *SFRP1* and *SFRP2* in IBD-associated neoplasias further highlights the importance of canonical WNT pathway in the pathogenesis of IBD-associated neoplasia. *SFRPs* can block the WNT signaling pathway either by interacting with WNT proteins or by forming nonfunctional complexes with frizzled receptors Fz.⁷⁰ Methylation of *SFRPs* can thus lead to activation of WNT signaling pathway even in the absence of mutations in *APC* and β -catenin.⁴⁸

Recently, there has been increasing interest in developing stool DNA based biomarkers. In fact, the current ACS guidelines concluded that there is now sufficient data to include stool DNA as an acceptable option for CRC screening.⁷¹ DNA shed into stool theoretically provides a more comprehensive sampling of abnormal cells than random punch biopsies for cancer surveillance among IBD patients. Stool DNA testing for hypermethylation of the

SFRP-1 promoter has already been shown to be a sensitive and specific screening tool for sporadic CRC.⁷² Our findings of methylation of *APC1A*, *APC2*, *SFRP1*, and *SFRP2*, if validated in prospective studies, could be used to devise stool-based DNA methylation tools for early detection of dysplasia and cancer in IBD patients.

Conclusions

Methylation of the WNT signaling pathway genes is seen in patients with IBD colitis and the frequency of methylation of the WNT signaling pathway genes increases progressively during development of IBD-associated neoplasia. Moreover, the findings of early methylation of *APC1A*, *APC2*, *SFRP1*, and *SFRP2* in IBD-associated neoplasia may provide objective markers and a method for early detection of IBD-associated neoplasia. In IBD surveillance, strategies for CRC prevention would be strengthened by the addition of objective markers for dysplasia and cancer, which will be independent of the expertise of the pathologist. Further studies will be required to validate the findings of the current study.

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Figure 1.

Overview of the genes involved in the WNT signaling pathway. When the pathway is turned on by binding of WNT, a cascade of events leads to translocation of β -catenin to the nucleus, which in turn upregulates the expression of many cancer-related genes.

ID	Tissue type	APC1A	APC2	SFRP1	SFRP2	SFRP4	SFRP5	DKK1	DKK3	WIF1	LKB1
4a	Cancer										
4b	Colitis										
4c	Colitis										

Figure 2.

Methylation profile of individual WNT signaling pathway genes in paired samples from one of the IBD-associated cancers showing the increase in methylation from colitis to cancer. *Black bars* represent methylation, whereas *white bars* represent unmethylated sample. Note that the patient has acquired methylation of *APC1A*, *SFRP1*, *SFRP2*, and *WIF1* during progression to cancer.

Figure 3.

Percentage of methylated genes in different tissue types. Percentage of methylated genes increased from normal colons ($n=24$, mean=4.2%) to IBD colitis ($n=25$, mean=39.7%) to IBD-associated neoplasia ($n=16$, mean=63.4%) was seen (NC vs. IBD colitis, $p < 0.01$, IBD colitis vs. IBD-associated neoplasia, $p=0.01$). There was no significant difference between IBD neoplasia (mean=63.4%) and sporadic CRC (mean=61.3%) $p=0.42$.

Table 1

Primer Sequences, Annealing Temperatures, and Cycle Number for the Tested Genes

Genes	Forward	Reverse	Annealing Temp.	Cycles
APC1A-U	GTGTTTATTGTGGAGTGTGGGT	CCAATCAACAACTCCCAACAA	60	35
APC1A-M	TATTGCGGAGTGC GGTC	TCGACGAACTCCCGACGA	60	35
APC2-U	TGGTAGTGTGTTTGT TAGTTGGATTG	ACCAAAAATCCCAACCCAAAATAACCTCAAAACA	56	35
APC2-M	GTCGTTTGT TAGGTTCCGATC	GACCCGAAATAACCTCGAAACG	56	35
SFRP1-U	GTTTTGTAGTTTTTGGAGTTAGTGTGTGT	CTCAACCTACAATCAAAAACAACACAAACA	60	35
SFRP1-M	TGTAGTTTTCGGAGTTAGTGTGCGC	CCTACGATCGAAAACGACGCGAACG	60	35
SFRP2-U	TTTTGGGTGGAGTTTTTGGAGTTGTGT	AACCCACTCTCTCACTAAATACAACCTCA	60	35
SFRP2-M	GGGTCGGAGTTTTTCGGAGTTGCGC	CCGCTCTCTTCGCTAAATACGACTCG	60	35
SFRP4-U	GGGGGTGATGTTATTGTTTTGTATTGAT	CACCTCCCCTAACATAAACTCAAAACA	60	35
SFRP4-M	GGGTGATGTTATCGTTTTGTATCGAC	CCTCCCCTAACGTAAACTCGAAACG	60	35
SFRP5-U	GTAAGATTTGGTGTTGGGTGGGATGTTT	AAAACCTCAACCCAAACCTCACCATACA	60	35
SFRP5-M	AAGATTTGGCGTTGGGCGGGACGTTT	ACTCCAACCCGAACCTCGCCGTACG	60	35
DKK1-U	GGGGTTGGAATGTTTTGGGTTTGT	ACCTAAATCCCCACAAAACCATACCA	60	35
DKK1-M	GTCGGAATGTTTCGGTTCGC	CTAAATCCCCACGAAACCGTACCG	60	35
DKK3-U	GGGGTTTTGGTTTTTTTTTGT TTTTGGGT	AACCACCACCTATATATCCCAAAACACA	60	35
DKK3-M	CGGTTTTTTTCGTTTTTCGGGC	CGCCTATATATCCCGAAACGCG	60	35
WIF-1-U	GGTTTTTGAGTGTTTTTTTTTGGGTTT	AATACAATACACCCAATAAAACACCCA	60	35
WIF-1-M	GTTTTTGAGTGTTTTTTTTTCGGGTTC	AATACGATACGCCAATAAAACG	60	35
LKB1-U	GGATGAAGTTGATTTTGATTGGGTT	ACCCAATACAAAATCTACAAACCAACA	60	35
LKB1-M	ACGAAGTTGATTTTGATCGGGTC	CGATACAAAATCTACGAACCGACG	60	35

Table 2
Summary of Methylation of WNT Signaling Genes in Different Tissue Types

Genes	IBD-associated neoplasia (dysplasia and cancer) (N=16)			NC vs. IBD colitis	IBD colitis vs. IBD-associated neoplasia
	NC (N=27)	IBD colitis (N=25)	IBD-associated neoplasia (dysplasia and cancer) (N=16)		
Percentage Methylation					
<i>oo</i>	0	16.0	43.8	0.04 ^a	0.05 ^b
<i>APC2</i>	0	48.0	81.3	<0.01 ^a	0.03 ^a
<i>SFRP1</i>	0	54.1	87.5	<0.01 ^a	0.03 ^a
<i>SFRP2</i>	29.6	52.0	86.7	0.10	0.03 ^a
<i>SFRP4</i>	0	68.0	81.3	<0.01 ^a	0.35
<i>SFRP5</i>	0	44.0	62.5	<0.01 ^a	0.25
<i>DKK1</i>	0	28.0	50.0	<0.01 ^a	0.15
<i>DKK3</i>	0	8.3	12.5	0.13	0.67
<i>WIF1</i>	7.4	41.7	68.8	<0.01 ^a	0.09
Mean of percentage methylation	4.2	39.7	63.4	<0.01 ^a	0.01 ^a

p values were calculated using chi-square test for genes and Mann-Whitney U test for mean of percentage methylation

^a Significant p value

^b Borderline significant p value