WNT-pathway activation in IBD-associated colorectal carcinogenesis: Potential biomarkers for colonic surveillance *

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Abstract. Objectives: The Wnt-pathway dominates the sporadic carcinogenesis whereas p53 plays a pivotal role in the colitis-associated counterpart. The expression of Wnt-signaling proteins and p53 during colitis-associated carcinogenesis was determined.

Methods: A tissue microarray was constructed with colonic samples from 5 groups of patients: controls (C, n = 10), IBD without neoplasia (IBD, n = 12), non-dysplastic IBD with neoplasia elsewhere in the colon (IBD-NE, n = 12), dysplastic lesion in IBD (IBD-DYS, n = 12), and IBD-associated colorectal cancer (IBD-CRC, n = 10). Immunohistochemistry was performed for β-catenin, cyclin D1 and p53. p53 sequence analysis was performed in some cases.

Results: Nuclear β-catenin expression was found in 0%, 0%, 50%, 55% and 100% of the patients in the C-, IBD-, IBD-NE-, IBD-DYS- and IBD-CRC-groups, respectively. Non-dysplastic IBD mucosa with neoplasia detected elsewhere showed nuclear expression in 50% of the cases compared to 0% in IBD mucosa without neoplasia (p = 0.02). Cyclin D1 staining had similar expression patterns. Overexpression of p53 was only detected in the IBD-DYS (66.7%) and IBD-CRC groups (50%).

Conclusion: In contrast to previous findings, our results suggest activation of the Wnt-pathway in the early phase of colitis-associated carcinogenesis. Furthermore, as Wnt activation was observed in 50% of the IBD-NE cases, nuclear β-catenin may facilitate detection of neoplasia.

Keywords: Colorectal cancer, inflammatory bowel disease, Wnt-pathway, surveillance, p53

1. Introduction

It is well established that patients with extensive and longstanding inflammatory bowel disease (IBD) have a higher life-time risk of developing colorectal cancer (CRC) than subjects in the general population [10]. In order to prevent IBD-associated CRC, patients are offered surveillance colonoscopies. However, colonoscopic surveillance in IBD is hampered by the fact that IBD-associated carcinogenesis follows an ‘inflammation-dysplasia-carcinoma’ sequence instead of the ‘adenoma-carcinoma’ sequence as is seen in sporadic CRC, which makes endoscopic discerning of premalignant lesions far more difficult [13]. Furthermore, colonoscopic surveillance in IBD is invasive, time consuming and its effectiveness has still to be proven. Therefore, biomarker-assisted surveillance may potentially be a valuable additional diagnostic tool in the detection of premalignant lesions in IBD.

The molecular pathogenesis of colitis-associated carcinogenesis differs from its sporadic counterpart. Previous data suggested that Wnt-pathway activation dominates early sporadic carcinogenesis, whereas Wnt activation occurs less frequently and, if occurring at all, at a late stage of colitis-associated carcinogenesis [30]. In contrast, mutations in the tumor suppressor gene p53 occur frequently in the early stages of colitis-associated carcinogenesis, whereas in sporadic CRC p53 mutations are only observed in advanced colonic neoplasia [27,32]. An intriguing observation in this respect is that p53 mutations have been observed in non-

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dysplastic IBD colonic mucosa adjacent to dysplastic areas, which may be a feature of colonic field cancerization [4,5]. Comparable data on Wnt-pathway activation in non-dysplastic colonic mucosa in patients with IBD-associated neoplastic lesions are not available. Recognition of these field related biomarkers of neoplastic progression may facilitate the detection of early neoplasia in case of surveillance. The aims of the current study were therefore to determine the expression of Wnt-signaling proteins and p53 during colitis-associated carcinogenesis and to assess their use as biomarkers of colonic field cancerization in longstanding colitis.

2. Patients and methods

2.1. Patients and tissue samples

Paraffin embedded tissue specimens from endoscopic biopsies and surgical resection material were obtained from the Department of Pathology of the University Medical Center Utrecht, The Netherlands. The study was carried out in accordance with the ethical guidelines of this center concerning informed consent about the use of patient’s materials after surgical procedures. Tissue samples of five groups of patients were collected. The first group (C) consisted of controls with complaints of abdominal pain or diarrhoea (n = 10), in whom colonoscopy was performed to rule out pathology. Histological examination of these biopsies showed normal mucosa. In the second group (IBD) IBD-patients with chronically inflamed colonic mucosa without neoplasia were included (n = 12). The third group (IBD-NE) consisted of IBD-patients with neoplasia (eight CRC and four dysplasia) detected on colonoscopy. From these patients non-dysplastic mucosa was studied with a median distance between the biopsies and the neoplastic lesion of 11 cm (range: 2–78 cm). The fourth group (IBD-DYS) consisted of IBD-patients with dysplasia (six with low-grade dysplasia (LGD) and six with high-grade dysplasia (HGD)). The fifth group (IBD-CRC) consisted of patients with IBD-associated colorectal adenocarcinomas (n = 10). The histological criteria by Riddell et al. [26] for colitis-associated dysplasia and carcinoma were applied. Indefinite for dysplasia cases were excluded. Since there is no robust evidence of a difference in carcinogenesis between Crohn’s disease (CD) and ulcerative colitis (UC), biopsies from patients with either disease were included in this study [13].

2.2. Tissue microarray

A tissue microarray (TMA) was constructed according to a standard protocol described by Kononen et al. [17]. Specific parts of the samples were selected on corresponding haematoxylin and eosin (H&E) stained slides after which three core biopsies (diameter: 0.6 mm) were taken from each sample and punched into a recipient block using a manual tissue micro arrayer (Beecher instruments, Sun Prairie, WI, USA). The slides were cut from the TMA with a microtome (Leica Microsystems, Wetzlar, Germany). Staining with antibodies was performed in triplicate, resulting in a maximum of nine tissue cores per patient.

2.3. Immunohistochemistry

Immunohistochemistry (IHC) was performed using monoclonal antibodies for p53 (Biogenex, San Ramon, CA, USA, BP5312-1; 1:200, Citrate, automatically stained by Bond™, Biovision Systems/Leica Microsystems, Wetzlar, Germany), β-catenin (BD Transduction Laboratories, Lexington, KY, USA, clone 14; 1:5000, ARS, overnight incubation at 4°C), and Cyclin D1 (Neomarkers, Suffolk, UK, clone SP4 rabbitmonoclonal, 1:100, ARS, overnight incubation at 4°C). Briefly, 4 µm sections were deparaffinized, blocked for endogenous peroxidase activity by immersion with 0.3% H2O2 in methanol for 20 min. Antigen retrieval was performed in Tris/EDTA buffer (10 mM/1 mM; pH 9.0) for 10 min at 120°C (using a bench autoclave). After cooling for 10 min and washing in PBS, non-specific binding sites were blocked in PBS with 5% normal goat serum for 10 min, followed by antibody incubation as indicated previously. Antibody binding was visualized using Powervision + poly-HRP detection system (Immuno Vision Technologies Co., Brisbane, CA, USA). The chromogen used for β-catenin staining was 3,3-DiAmino Benzidine tetra-chloride, DAB (Sigma, MO, USA) and for cyclin D1 staining PowerDAB™ (Immuno Vision Technologies Co., Brisbane, CA, USA). Sections were counterstained with H&E.

2.4. Staining interpretation

The specimens were considered positive for p53 if the epithelial cells exhibited intense brown nuclear staining in the entire tissue sample or segments thereof (more than 20% positive cells) [15,31]. A faint scattered staining pattern of p53 should not be consid-
ered as a marker of gene mutation or inactivation [15, 18]. Interpretation of cyclin D1 staining was based on intensity and divided into three categories: negative or weakly positive (−), positive (+), and strongly positive (++) Both the positive and strongly positive samples were regarded as cyclin D1 overexpression [36]. β-catenin staining was evaluated in the cell membrane and nucleus. A membranous staining pattern, as can be observed in normal cells was considered negative, whereas a reduced membranous expression accompanied by increased cytoplasmic and nuclear staining was deemed positive [14]. The percentage of nuclear stained cells was semi-quantitatively analyzed and divided into three categories: low (<10% of cells), moderate (10–50% of cells) or high (>50% of cells). If more than 10% of cells showed positive nuclear staining, this was interpreted as indicative of Wnt activation [16,20]. All slides were evaluated by two observers conjointly (MS and MC). Discordant cases were reviewed by a second experienced gastrointestinal pathologist (JO).

2.5. p53 Sequence analysis

On rare occasions a stop codon (null) mutation of p53 can be associated with completely negative staining due to abrogation of translation of a stable protein fragment [1]. When this was suspected, sequence analysis was performed to verify the mutation.

Prior to DNA isolation, hematoxylin and eosin-stained histological slides were screened for the presence of at least 40% of tumor cells. DNA was extracted from formalin-fixed tissue sections of 15 μm thickness. These were deparaffinized with xylene, rehydrated in serial-graded water-ethanol solution (100% and 70%, respectively) and rinsed in deionized water. Subsequently, DNA was extracted by incubation at 55°C in 1 ml extraction buffer overnight (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 0.25 M EDTA pH 8.0, 0.5% SDS) to which 20 μl proteinase K (10 mg/ml) was added (Roche Diagnostics, Basel, Switzerland). Phenol-chloroform purification was performed and DNA precipitation was established by ethanol.

Exons 4–9 were amplified in seven different PCRs. For the amplification of DNA from paraffin sections, 1 U Ampli Taq Gold DNA polymerase (Perkin Elmer, Norwalk, CT, USA) was used with 2 mM MgCl2. The first PCR was performed with primers without the sequencing templates. In the second PCR with 1 μl of the first amplified product, primers were used with sequencing templates. The PCR products were sequenced and were analyzed subsequently as described previously [19]. The mutated codons were numbered from the first coding nucleotides.

2.6. Statistical analysis

The Fisher’s exact test was used to determine the statistical significance of differences in expression between groups. Due to the heterogeneity within a tumor sample, the three cores taken from a biopsy were considered independently. The tissue core with the highest score of the three slides was selected, resulting in three values per patient [6]. Statistical analysis was performed using SPSS Version 12.0 Statistical Software. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Clinicopathological characteristics

The clinicopathological characteristics of patients are listed in Table 1. No statistically significant differences in age, gender or IBD phenotype and disease duration were observed between the five groups.

3.2. Expression of β-catenin

Increased expression of nuclear β-catenin was found in 50% of patients in the IBD-NE group, in 55% of patients in the IBD-DYS group, and in 100% of the IBD-CRC cases, whereas in the C and IBD groups no nuclear β-catenin staining was observed. Remarkably, an increased nuclear β-catenin expression was detected in 50% of IBD-NE cases, as compared to a 0% expression in IBD patients without neoplasia (p = 0.02). The nuclear β-catenin positive and negative tissue samples were located at a median distance of 7 cm (range 2–20 cm) and 13 cm (range 2–78 cm), respectively, from the neoplastic lesion. All positively staining non-dysplastic IBD mucosal samples adjacent to a (pre)malignant lesion were found in patients with neoplasia in the rectum. Percentages of overexpression per core (not individual patients) are depicted in Fig. 1a, with an increased expression in 19%, 46% and 77% of all IBD-NE, IBD-DYS and IBD-CRC cores, respectively. Membranous β-catenin was highly expressed in the C and IBD groups, whereas nuclear staining was absent in these groups. As expected, membranous
staining declined with increasing nuclear accumulation. Taken all cores into account, a decreased membranous expression was observed in 5%, 15%, 21%, 40% and 70% of the C, IBD, IBD-NE, IBD-DYS and IBD-CRC cores, respectively (Fig. 1b). No difference in nuclear β-catenin expression between samples with LGD or HGD was found in the IBD-DYS group. Positive nuclear and membranous staining is shown in Fig. 2a and b, respectively.

3.3. Expression of cyclin D1

The results of cyclin D1 expression were in line with those of nuclear β-catenin staining. Increased cyclin D1 expression was found in 22%, 33%, 50%, 70% and 80% of patients in the C, IBD, IBD-NE, IBD-DYS and IBD-CRC groups, respectively (Fig. 1b). No difference in nuclear β-catenin expression between samples with LGD or HGD was found in the IBD-DYS group. Positive nuclear and membranous staining is shown in Fig. 2a and b, respectively.

3.4. Expression of p53

Expression of p53 was only found in the IBD-DYS and IBD-CRC groups, with positive expression in 67% and 50%, respectively. All high-grade dysplastic lesions (n = 6) and 2 out of 5 low-grade dysplastic lesions were p53 positive. Overexpression of p53 was found in 50% of the IBD-DYS cores and 45% of the IBD-CRC cores (Fig. 1d). In five of the IBD-CRC patients, immunohistochemical expression of p53 was completely negative. A sequence analysis of the p53 gene was performed in these cases to search for a stop codon (null) mutation. Sequence analysis could not be performed in one patient due to a shortage of tissue. Two out of the remaining four patients had a p53 mutation in the tumor, resulting in abrogation of protein production (insertion in exon 7 and mutation V197bE), adding up to an overall abnormal p53 function in 70% of the IBD-CRC patients instead of 50%. Positive and negative expression for p53 is shown in Fig. 2e and f, respectively.

4. Discussion

This study indicates that, in contrast to the general assumption, Wnt-pathway activation occurs early and frequently in IBD-associated colorectal carcinogenesis. Moreover, even in non-dysplastic mucosa adjacent to neoplastic lesions nuclear β-catenin and cyclin D1, markers of an activated Wnt-pathway, could be detected, whereas p53 overexpression was not observed in these tissue specimens. The canonical Wnt-pathway (β-catenin mediated Wnt-signaling) controls proliferation and differentiation in the colonic stem cell com-
Fig. 1. Expression level per core. (a) Nuclear \(\beta\)-catenin expression: determined by percentage of positively stained nuclei grouped in low (<10%), medium (10–50%), and high (>50%). (b) Membranous \(\beta\)-catenin expression: grouped in positive (reduced of absent membranous staining, indicating an activated Wnt pathway) or negative (presence of membranous staining, indicating an inactivated Wnt pathway). (c) Cyclin D1 expression: determined by the intensity of the expression grouped in negative (−), positive (+) or strongly positive (++). (d) p53 expression: grouped in a positive expression or negative (no or <20% cells with expression of p53).

department and is involved in colon carcinogenesis [9, 23]. Loss of adenomatous polyposis coli (APC) gene function results in a shift from membranous to nuclear \(\beta\)-catenin and activates transcription of target genes such as cyclin D1 and c-myc [3].

Previously published data on the role of the Wnt-pathway in colitis-associated colorectal carcinogenesis is conflicting. Van Dekken et al. [34] found increased nucleo-cytoplasmic \(\beta\)-catenin staining in 88% and 79% of dysplastic and cancerous lesions, respectively, whereas Mikami et al. [21] demonstrated very low nuclear \(\beta\)-catenin expression in high- and low-grade dysplasia and ulcerative colitis UC-associated CRC. In another study, increased nuclear \(\beta\)-catenin staining was observed in 48% of UC-related cancers and abnormal APC expression in 67% of cases [2]. However, other studies have shown that the frequency of APC gene mutations and \(\beta\)-catenin gene mutations in UC-related cancers is lower than in sporadic carcinomas [3,30,33]. Conflicting data about the expression of APC and \(\beta\)-catenin could be due to a wide variation of employed methods and selected patient populations.

A remarkable finding was the expression of nuclear \(\beta\)-catenin in non-dysplastic tissue adjacent to neoplastic lesions in half of our patients, which may be regarded as a feature of colonic “field cancerization”. This observation is in line with previously published data showing pancolonic chromosomal instability in UC patients with a neoplastic lesion, DNA aneuploidy in non-dysplastic mucosa of IBD patients with neoplasia, and telomere shortening in non-dysplastic tissue of patients with neoplasia elsewhere [22,24,25,28,29]. Our findings, however, are the first to suggest that the Wnt-pathway is already activated in the field surrounding a dysplastic or malignant lesion in IBD. Although these Wnt-proteins cannot be used as solitary markers of colorectal carcinogenesis, they may function as an additional diagnostic tool for a more biomarker-assisted colonic surveillance. Patients may be assigned to different risk groups, based on the expression level of Wnt-pathway proteins in the non-dysplastic biopsies obtained during surveillance colonoscopy. However, larger studies are needed to confirm our observations. Interestingly, all patients in the IBD-NE group with nuclear \(\beta\)-catenin staining had distal left-sided malignancies. Unfortunately the limited number of patients in this study impedes further speculation on a possible relation between tumor location and activation of the Wnt-pathway. The observation of nuclear \(\beta\)-catenin expression in left-sided CRCs, however, seems
Fig. 2. Images illustrating immunohistochemical expression profiles of \(\beta\)-catenin (a, b), cyclin D1 (c, d) and p53 (e, f). Magnification 100× and 400×. Sample (a) shows a nuclear expression of \(\beta\)-catenin and sample (b) a membranous staining pattern. Sample (c) demonstrates an increased expression of cyclin D1, whereas sample (d) shows a negative expression of cyclin D1. Sample (e) shows an increased expression of p53 and sample (f) a negative p53 staining pattern. The negative staining in (f) is caused by a stop codon mutation in dysplastic mucosa, see insert. The upper panel shows the dysplastic lesion with the arrow indicating the insertion causing the stop codon mutation. The lower panel shows the normal mucosa.

Positive p53 staining was found in two-third and half of the dysplastic and malignant lesions, respectively. This relatively low percentage of p53 positivity in the IBD-CRC group, compared to the IBD-DYS group, was at least in part explained by stop codon (null) mutations of p53 leading to a completely absent protein expression pattern (20%, \(n = 2\)). Immunohistochemical overexpression of p53 has been reported in the majority of UC-associated carcinomas [7, 11, 25]. Some studies have suggested that genetic mutations in p53, possibly due to oxidative injury, can already be detected in inflamed non-dysplastic mucosa of UC patients [12]. However, immunohistochemical studies found only weak to moderately intense p53 expression in non-dysplastic tissue of UC patients [35]. In our study, we also did not detect strong nuclear p53 staining in non-dysplastic IBD mucosa. Therefore, in contrast to nuclear \(\beta\)-catenin, p53 immunohistochemical staining of non-dysplastic mucosa does not serve as biomarker for malignant transformation elsewhere in the colon.

Some limitations of our study have to be considered. First, the degree of expression of markers may be heterogeneously distributed within a single lesion. For that reason, three tissue cores were selected from each sample in order to minimize the chance of sampling error [8]. This, however, was not always feasible, as the tissue core, which was punched out of the donor block, might not contain enough tissue. Second, the number of patients per group was relatively low, which
requires a cautious interpretation of the results. Nevertheless, significant differences between groups were found. Third, the patients included in this study were selected from tertiary centers; therefore the incidence of concurrent PSC was higher than in a normal IBD population.

In conclusion, our results suggest an early role for the Wnt-pathway in colitis-associated colorectal carcinogenesis. Furthermore, nuclear β-catenin may serve as a biomarker for colorectal field cancerization, facilitating early detection of neoplasia during colonic surveillance.

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