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ORIGINAL ARTICLE – CANCER RESEARCH

Hypermethylation of *ITGA4*, *TFPI2* and *VIMENTIN* promoters is increased in inflamed colon tissue: putative risk markers for colitis-associated cancer

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Abstract
Purpose Epigenetic silencing of tumor suppressor genes is involved in early transforming events and has a high impact on colorectal carcinogenesis. Likewise, colon cancers that derive from chronically inflamed bowel diseases frequently exhibit epigenetic changes. But there is little data about epigenetic aberrations causing colorectal cancer in chronically inflamed tissue. The aim of the present study was to evaluate the aberrant gain of methylation in the gene promoters of *VIM*, *TFPI2* and *ITGA4* as putative early markers in the development from inflamed tissue via precancerous lesions toward colorectal cancer.
Methods Initial screening of different cancer cell lines by using methylation-specific PCR revealed a putative colon cancer-specific methylation pattern. Additionally, a demethylation assay was performed to investigate the methylation-dependent gene silencing of *ITGA4*. The candidate markers were analyzed in colonic tissue specimens from patients with colorectal cancer ($n = 15$), adenomas ($n = 76$), serrated lesions ($n = 13$), chronic inflammation ($n = 10$) and normal mucosal samples ($n = 9$).
Results A high methylation frequency of *VIM* (55.6 %) was observed in normal colon tissue, whereas *ITGA4* and *TFPI2* were completely unmethylated in controls. A significant gain of methylation frequency with progression of disease as well as an age-dependent effect was detectable for *TFPI2*. *ITGA4* methylation frequency was high in precancerous and cancerous tissues as well as in inflammatory bowel diseases (IBD).
Conclusion The already established methylation marker *VIM* does not permit a specific and sensitive discrimination of healthy and neoplastic tissue. The methylation markers *ITGA4* and *TFPI2* seem to be suitable risk markers for inflammation-associated colon cancer.

Keywords Epigenetic · DNA methylation · Colon cancer · Colitis · Gastrointestinal tract · Biomarker

Introduction

Colon cancer is one of the most frequent occurring cancers in the Western civilization. Furthermore, it is one of the leading causes of cancer-related deaths (Ferlay et al. 2010). One of the prevalent risk factors for developing colorectal cancer is inflammatory bowel diseases (IBD). Thus, patients with long-standing Crohn's disease have a significantly elevated lifetime risk of developing colorectal cancer. As shown in a screening and surveillance study during a 17-year period, patients with long-standing Crohn's disease developed cancer with increased risk depending on younger age at diagnosis, longer disease course and extent of the inflammation (Basseri et al. 2012). Similarly,

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the incidence rates of colorectal cancer are increased in patients with ulcerative colitis (Eaden et al. 2001). Particularly for risk groups like IBD patients, there is a need for defining risk markers for assessment of developing cancer. The mortality of colitis-associated and sporadic colorectal cancer (CRC) can be reduced significantly by frequent and regularly colorectal examination, which allows the early detection of premalignant adenomas and early-staged cancers (Center et al. 2009). The most effective screening method for colorectal cancer and the precancerous lesions is colonoscopy (Schoen et al. 2012). Yet the low compliance of patients has prompted the development of noninvasive screening tests. However, the vast majority of existing noninvasive screening tests, like the fecal occult blood test (FOBT), presents only low sensitivity and specificity, especially for precancerous lesions like adenoma (Hol et al. 2010).

In the past few years, it has become clear that abnormal hypermethylation of gene promoters and the associated loss of gene function is one of the early driving events of colonic carcinogenesis (Jones and Baylin 2007). Methylation of DNA occurs predominantly at the 5′-position of cytosines in the context of CG dinucleotides. In mammals, most of these dinucleotides are accumulated in so-called CpG Islands located in over 40 % of mammalian gene promoters. Methylation of the CpGs in gene promoters results in silencing of the respective gene. The finding of abnormal methylation of distinct genes like tumor suppressors due to carcinogenesis provides a new marker for early colon cancer detection (Lao and Grady 2011). Several abnormally methylated genes in colon cancer have been described recently. The aim of the present study was to identify and evaluate the benefit of a methylation marker panel for the early detection of colorectal cancer and its precancerous lesions considering that a large portion of colon cancers arise from chronic inflammation.

As putative risk markers for colitis-associated carcinogenesis, the methylation status of *tissue factor pathway inhibitor (TFPI2)*, *integrin alpha-4 (ITGA4)* and *vimentin (VIM)* gene promoters was examined. The *TFPI2* gene encodes a Kunitz-type serine proteinase inhibitor that protects the extracellular matrix of cancer cells from degradation and inhibits in vitro colony formation and proliferation. It is thought that loss of *TFPI2* function could predispose cells toward a pro-invasive program, consistent with an important role for this protein in later stages of carcinogenesis (Glockner et al. 2009; Hahn et al. 2008).

The *ITGA4* gene encodes the alpha-4 subunit of an integrin family member. Integrins are heterodimeric membranous receptor proteins consisting of two subunits, namely alpha and beta. They are receptors for VCAM1, whereas integrin alpha-4/beta-7 is a receptor for MADCAM1. Integrins have important functions in extracellular control of cell survival and differentiation via cell–cell interactions and cell–matrix communication. *ITGA4* is a putative tumor suppressor because of its ability to control cell adhesion (Ausch et al. 2009).

The *VIM* gene encodes a protein constituent of intermediate filaments. Its expression is considered a classic marker of mesenchymal cells, such as fibroblasts; hence, *VIM* should not be expressed by normal colonic epithelium. Anyhow apparently it is being hypermethylated during colon carcinogenesis and already being used as a promising methylation marker (Chen et al. 2005). In the present study, *VIM* was analyzed for comparison purposes of the evaluated marker panel.

This work emphasizes the significance of methylation changes during carcinogenesis and evaluates the potential of different methylation markers for the early detection of CRC.

Materials and methods

Cell lines

Initial screening of *ITGA4*, *TFPI2* and *VIM* promoter methylation was performed in cell lines LS174T, CaCo-2, Colo678, SW480, HT-29, HCT116, LS1034, HCEC, Colo357, HEK293, HeLa, HepG2, IGR-1, Jurkat, LNCaP, MCF7, MDA-MB231, Raji and SH-SY5Y. Cells were purchased from American Type Culture Collection and from DSMZ-German Collection of Microorganisms and Cell Cultures. LS174T, CaCo-2 and HCEC were obtained as generous gifts from A. M. Otto (Technical University of Munich, Germany), R. Brigelius-Flohé (German Institute of Human Nutrition (DIfE, Nuthetal, Germany), and the Nestlé Ltd. Research Centre (Lausanne, Switzerland), respectively. Cells were cultured according to protocols of the suppliers. Every cell line was tested negative for mycoplasma infection within 6 month before use.

Tissue samples

Colon tissue samples including 15 carcinomas, 76 adenomas, 13 serrated lesions and 9 normal colon tissue specimens from diverticulosis patients were obtained during colonoscopy and fresh-frozen. Additionally, ten chronically inflamed FFPE colon tissues from IBD patients were analyzed. Histologic findings of tissues are given in Table 1. The mean age of patients was 71.7 years, and 50.5 % of the subjects were male. The tissues were provided by the Division of Gastroenterology at the Maria Heimsuchung Caritas-Klinik Pankow (Berlin, Germany) and the Department of Medicine II, Division of Gastroenterology, University of Würzburg (Germany). The study was carried out

Table 1 Methylation state of *TFPI2*, *ITGA4* and *VIM* analyzed by MSP of human colon tissue

Sample no.	Histopathological feature	Age	Analyzed gene		
			TFPI2	*ITGA4*	*VIM*
1	Normal control	n.a.	U	U	M
2	Normal control	n.a.	U	U	U
3	Normal control	n.a.	U	U	M
4	Normal control	n.a.	U	U	U
5	Normal control	n.a.	U	U	M
6	Normal control	n.a.	U	U	M
7	Normal control	n.a.	U	U	M
9	Normal control	n.a.	U	U	U
10	Normal control	n.a.	U	U	U
11	Low grade chronic colitis	53	U	M	M
12	Low-mid grade chronic colitis	43	U	M	M
13	Heavy chronic colitis, ulcer	72	U	U	U
14	Inflammatory polyp	68	U	M	U
15	Chronic inflammation, polyp	93	U	M	U
16	Low grade chronic inflammation	69	U	M	U
17	Inflamed hyperplastic polyp	50	U	M	U
18	Inflamed hyperplastic polyp	74	M	M	U
19	Polypoid inflamed mucosa	50	M	M	M
20	Chronic colitis	n.a.	M	U	M
21	Serrated lesion	75	M	U	M
22	Serrated lesion	53	U	M	U
23	Serrated lesion	67	M	U	M
24	Serrated lesion	84	U	M	U
25	Serrated lesion	45	U	M	M
26	Serrated lesion	48	U	M	M
27	Serrated lesion	65	U	M	M
28	Serrated lesion	41	U	M	M
29	Serrated lesion	55	U	U	U
30	Serrated lesion	69	M	M	M
31	Serrated lesion	24	U	U	U
32	Serrated lesion	62	M	M	M
33	Serrated lesion	46	M	M	M
34	Adenoma	50	U	M	M
35	Adenoma	71	M	M	M
36	Adenoma	73	M	M	M
37	Adenoma	83	M	M	U
38	Adenoma	55	M	M	M
39	Adenoma	68	U	M	U
40	Adenoma	66	U	U	M
41	Adenoma	64	U	U	M
42	Adenoma	85	U	M	U
43	Adenoma	85	U	M	M
44	Adenoma	77	U	M	M
45	Adenoma	54	U	M	M
46	Adenoma	67	M	M	U
47	Adenoma	87	M	M	M
48	Adenoma	87	M	M	M
49	Adenoma	71	M	M	U
50	Adenoma	71	U	M	U
51	Adenoma	80	U	M	M
52	Adenoma	82	M	M	U
53	Adenoma	72	U	M	U
54	Adenoma	67	U	U	U
55	Adenoma	57	+/−	M	M
56	Adenoma	74	U	M	M
57	Adenoma	83	U	M	U
58	Adenoma	46	U	M	U
59	Adenoma	54	U	U	U
60	Adenoma	69	M	M	M
61	Adenoma	64	M	M	M
62	Adenoma	44	U	M	M
63	Adenoma	95	M	U	U
64	Adenoma	69	U	M	U
65	Adenoma	65	U	U	M
66	Adenoma	86	M	M	M
67	Adenoma	76	M	M	M
68	Adenoma	65	M	M	U
69	Adenoma	65	U	M	M
70	Adenoma	67	M	M	M
71	Adenoma	95	M	M	M
72	Adenoma	63	M	M	M
73	Adenoma	69	M	M	U
74	Adenoma	53	M	M	M
75	Adenoma	71	M	M	M
76	Adenoma	53	M	M	M
77	Adenoma	67	M	M	M
78	Adenoma	72	M	M	M
79	Adenoma	50	M	M	M
80	Adenoma	80	M	M	M
81	Adenoma	61	M	M	M
82	Adenoma	69	M	M	M
83	Adenoma	65	M	M	M
84	Adenoma	73	M	M	M
85	Adenoma	53	M	M	M
86	Adenoma	75	M	M	M
87	Adenoma	75	M	M	U
88	Adenoma	73	M	M	M
89	Adenoma	59	M	M	M
90	Adenoma	70	M	M	M
91	Adenoma	72	U	M	M
92	Adenoma	64	M	M	M

Table 1 continued

Sample no.	Histopathological feature	Age	Analyzed gene		
			TFPI2	*ITGA4*	*VIM*
93	Adenoma	46	M	M	U
94	Adenoma	74	M	M	M
95	Adenoma	79	M	M	M
96	Adenoma	60	M	M	M
97	Adenoma	79	M	M	M
98	Adenoma	59	U	M	U
99	Adenoma	84	M	M	M
100	Adenoma	70	M	M	M
101	Adenoma	77	M	M	M
102	Adenoma	66	M	M	M
103	Adenoma	72	M	M	M
104	Adenoma	85	M	M	M
105	Adenoma	76	M	M	M
106	Adenoma	68	M	M	U
107	Adenoma	74	M	M	M
108	Adenoma	75	M	M	M
109	Carcinoma	65	M	M	M
110	Carcinoma	51	M	M	M
111	Carcinoma	87	M	M	M
112	Carcinoma	84	U	M	M
113	Carcinoma	60	M	M	U
114	Carcinoma	58	M	M	M
115	Carcinoma	78	M	M	M
116	Carcinoma	70	M	M	M
117	Carcinoma	76	M	U	U
118	Carcinoma	44	M	M	M
119	Carcinoma	90	M	M	M
120	Carcinoma	61	M	M	M
121	Carcinoma	73	M	M	U
122	Carcinoma	78	M	M	U
123	Carcinoma	67	M	M	M

n.a. not available, *U* unmethylated, *M* methylated

in compliance with the Declaration of Helsinki. Written informed consent was received from participants prior to inclusion in the study. Permission for the study was given by the Ethics Commission of the University of Potsdam, Germany (Decision 14/2011).

DNA extraction and bisulfite conversion

Genomic DNA was extracted from cell pellets and from human tissue samples by use of the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. For FFPE tissues, the QIAamp DNA FFPE tissue kit (Qiagen) was used. The DNA obtained from 2×10^6 cells was eluted in 100 µl ddH_2O. Two different methods were used to convert the genomic DNA by bisulfite treatment. As described previously (12), 250 ng–2 µg DNA was denatured by NaOH (final concentration 0.3 mol/L) for 15 min at 37 °C. After that, 12 µl of 0.1 mol/L hydroquinone and 208 µl of 3.6 mol/L NaHSO3 (both freshly prepared) were added and incubated for 16 h at 55 °C. The bisulfite-converted DNA was purified using the Wizard DNA Clean-Up System (Promega) and eluted in 50 µl H_2O. Desulfonation was carried out by addition of 5 µl 3 mol/L NaOH and incubation for 15 min at 37 °C. The DNA was precipitated by ethanol and resuspended in 50 µl H_2O. In addition to this method, the EZ DNA Methylation Kit (Zymo Research) was used to modify the genomic DNA according to the manufacturer`s protocol.

Nested methylation-specific PCR (MSP)

Initially, MSP was performed in a 25-µl reaction volume containing 5 µl of bisulfite DNA as template, 1 U Dream Taq DNA polymerase (Thermo Scientific), 400 nmol/L forward and reverse primers, 0.1 mmol/L dNTPs, 1× Dream Taq Buffer, 4 mmol/L $MgCl_2$ and H_2O. As a positive control, 10 ng of EpiTect Control DNA (Qiagen, Hilden) was used. In a second nested PCR, 2 µl of the first amplicon was added to 23 µl reaction mix as above but with 400 nmol/L nested forward and reverse primers. Subsequently, the nested MSP products were analyzed on an agarose gel. Primer sequences and PCR conditions are listed in Supplementary Table ESM 1.

Demethylation assay with 5-aza-2′-deoxycytidine

In order to determine the inhibition of DNA methylation of the *ITGA4*-promoter, we treated the colon cancer cell line HCT116 with 5-aza-2′-deoxycytidine (5-aza-dC; Sigma-Aldrich). The cells were treated with final concentrations from 5, 10 and 15 µM 5-aza-dC, whereas PBS was used as solution control. The untreated cells were the negative control. After 96 h, the cells were harvested and frozen for further protein analysis.

Protein extraction and Western blot analysis

Briefly, the cell pellets were lysed in 200 µl RIPA buffer (50 mM Tris/HCl, 150 mM NaCl, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS in PBS) and 30-min incubation at 4 °C. Subsequently, the lysed cells were centrifugated at 10,000 rpm for 30 min and the supernatant was used for protein analysis.

For the Western blot analysis, 100-µg protein was used for each lane. The protein aliquots were separated on SDS-PAGE and subsequently transferred to PVDF membrane filters, followed by blocking with 5 % milk powder in TBST

buffer. The membranes were probed with the primary antibody anti-ITGA4 (Epitomics, Burlingame) and anti-β-actin (Abcam, Cambridgeshire), reacted with the corresponding horseradish peroxidase-conjugated secondary antibody (Cell Signaling, Boston) and detected using a chemiluminescence reaction. The membranes were exposed to X-ray film to visualize the detected proteins of interest.

Statistics

The levels of significance between the disease-dependent groups were calculated by the software *Prism 6* (Graphpad Software, La Jolla, USA) using *Fisher's exact test*. Differences between the groups were considered significantly in case of $p < 0.05$.

Results

Methylation pattern of candidate markers in cell lines

Initially, the organ specificity of the candidate methylation markers was studied by analyzing the promoter methylation status of *ITGA4*, *TFPI2* and *VIM* by MSP in a total of 19 cell lines. These cells included seven colon cancer cell lines and one normal colon epithelial cell line as well as eleven cell lines from different organs. Whereas the target genes were unmethylated in the normal colon cell line HCEC, they were consistently methylated in the analyzed colon cancer cells except for SW480 where *VIM* was unmethylated (Table 2). However, cell lines originating from different tissues exhibited a vastly heterogenic methylation profile. Accordingly, all of the examined gene promoters were unmethylated in cell lines HEK293 derived from embryonic kidney, HeLa from cervical carcinoma and SH-SY5Y from neuroblastoma. The other non-colon cell lines exhibited inconsistently methylated gene promoters of *ITGA4*, *TFPI2* and *VIM* (Table 2). A representative MSP analysis of the *VIM* promoter is exemplarily shown (Fig. 1).

Table 2 Promoter methylation status of *ITGA4*, *TFPI2* and *VIM* genes in cell lines

Cell line	Origin of tissue	*ITGA4*	*TFPI2*	*VIM*
CaCo-2	Colon adenocarcinoma	M	M	M
Colo678	Colon carcinoma	M	M	M
HCT116	Colon carcinoma	M	M	M
HT-29	Colon adenocarcinoma	M	M	M
LS1034	Cecum carcinoma	M	M	M
LS174T	Colon adenocarcinoma	M	M	M
SW480	Colon adenocarcinoma	M	M	U
HCEC	Colon normal	U	U	U
Colo357	Pancreas carcinoma	U	M	U
HEK293	Embryonic kidney cells	U	U	U
HeLa	Cervical carcinoma	U	U	U
HepG2	Hepatocellular carcinoma	U/M	U	M
IGR-1	Melanoma	U/M	U/M	U
Jurkat	AL-leukemia	U	U/M	U
LNCaP	Prostate carcinoma	U	U/M	U/M
MCF-7	Breast adenocarcinoma	M	U	U/M
MDA-MB231	Breast adenocarcinoma	M	U	U/M
Raji	B lymphocytes, Burkitt's Lymphoma	U	M	M
SH-SY5Y	Neuroblastoma	U	U	U

U unmethylated, *M* methylated, *U/M* unmethylated and methylated DNA detectable

Demethylation assay and reexpression of *ITGA4*

The methylation-dependent inhibition of *TFPI2* gene expression has been shown already (Heller et al. 2008). In contrast, *VIM* is not expressed in colon tissue independently from the methylation status (Chen et al. 2005), but gains hypermethylation during carcinogenesis. In order to determine the methylation-dependent inhibition of the gene expression of *ITGA4,* we performed a demethylation assay using 5-aza-2′-deoxycytidine, a common demethylating agent. The methylation status of the treated cells was analyzed by MSP, and ITGA4 reexpression was investigated by Western blotting.

The *ITGA4* promoter was fully methylated in the colon cancer cell line HCT116. After cell treatment with 0, 5, 10 and 15 µM 5-aza-2′-deoxycytidine, genomic DNA was isolated and prepared by bisulfite treatment. Subsequently, the methylation status of the *ITGA4* promoter was analyzed by MSP. As expected, the treatment with 5-aza-2′-deoxycytidine (5-aza-dC) revealed demethylation of the *ITGA4* promoter, which was shown by a PCR signal in the unmethylated MSP reaction (Fig. 2).

The Western blot analysis of the protein lysate of the treated cells revealed a transcriptional and translational reactivation of *ITGA4*. We observed that the protein level of ITGA4 was increased with the 5-aza-dC treatment. The non-treated and solvent controls did not show any detectable ITGA4 protein appearance (Fig. 3). These results indicate that the methylation of the *ITGA4* promoter directly mediates the transcriptional and translational silencing of *ITGA4* in colon cancer cells.

Methylation status of *ITGA4*, *TFPI2* and *VIM* in normal, precancerous and carcinoma tissue

Aware of the uniform methylation status of *ITGA4*, *TFPI2* and *VIM* in colon cancer cell lines, we analyzed a total of 123 colon biopsies from patients with different disease

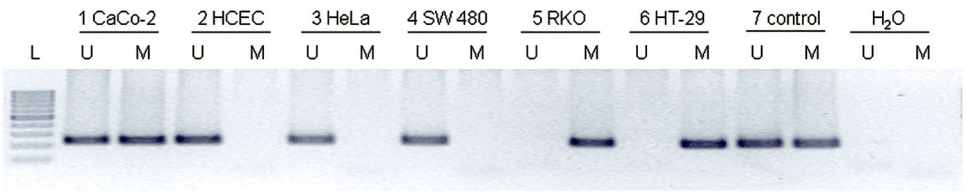

Fig. 1 Verification of *VIM* gene promoter methylation in cell lines by MSP. PCR products of unmethylated (U) and methylated (M) *VIM* from sodium bisulfite-treated genomic DNA from cell lines are visualized by ethidium bromide staining. *1–6*—analyzed cell lines as indicated; *7*—positive methylated and unmethylated control DNA; *8*—negative control; *L*—base length standard

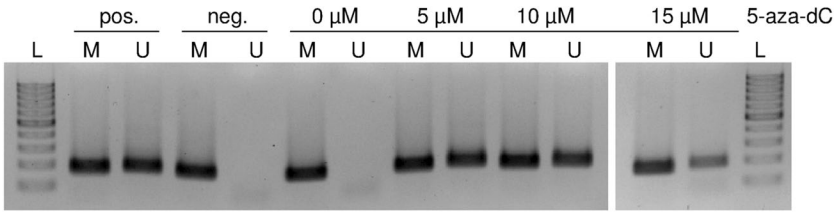

Fig. 2 DNA methylation changes in the *ITGA4*-promoter after 5-aza-dC treatment. The methylation status of *ITGA4* in HCT116 cells treated with 5, 10 and 15 µM 5-aza-dC for 96 h was analyzed by MSP. The non-treated control (neg.) and the solvent control with PBS (0 µM) displayed no detectable unmethylated signal. In vitro methylated and unmethylated control DNA (pos.) was used as positive control

states. In the subset of normal controls, neither *ITGA4* nor *TFPI2* promoters were methylated (Table 1). Surprisingly, a methylated *VIM* promoter was already found in five out of nine (55.6 %) normal colon mucosa specimens.

In inflamed tissue, methylation of the analyzed gene promoter regions was a common feature as well. The non-neoplastic mucosa samples of IBD patients showed relatively higher methylation levels in *ITGA4* and *TFPI2* than in normal colon mucosa samples (80 and 30 %, respectively) (Fig. 4a, b). *ITGA4* methylation was significantly higher in inflamed than in normal tissue ($p = 0.0007$). The *VIM* promoter was methylated in 40 % of IBD-derived samples as well (Fig. 4c).

Colon tissue samples from patients with serrated lesions and adenomas represented the precancerous subset. A significantly more frequent methylation of the *ITGA4* promoter was observed in serrated lesions (69.2 %; $p < 0.0001$) as well as in adenomas (93.4 %; $p < 0.0001$) compared to the normal subset (Fig. 4a). Overall, a hypermethylated *TFPI2* promoter has been detected less frequently than *ITGA4* methylation in the precancerous subset (Table 1). A methylation of the *TFPI2* promoter was found in 38.5 % of serrated biopsies and in 67.1 % of adenoma samples which was significantly more frequent compared to controls ($p < 0.0001$) (Fig. 4b). No significant increase in *VIM* methylation rates was found in the precancerous states as opposed to normal colon. The *VIM* gene exhibited promoter methylation in 30.8 and 75 % of serrated lesions and adenomas, respectively (Fig. 4c).

Additionally, a subset of colon carcinoma specimens consisting of UICC stages from I to IV revealed a

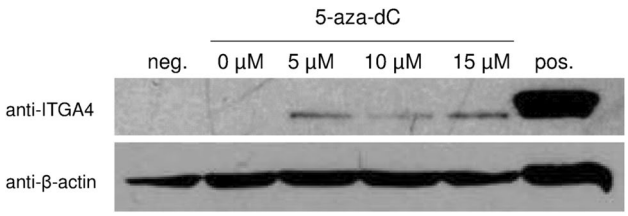

Fig. 3 Upregulation of *ITGA4*-gene expression after 5-aza-dC treatment. HCT116 cells are treated with 5 µM (3), 10 µM (4) or 15 µM (5) 5-aza-dC for 96 h resulting in reexpression of the ITGA4 protein. The untreated negative control (1) and the solvent control PBS (2) showed no detectable signal. The positive control (Raji cell lysate—6) displayed a strong signal. The protein β-actin is used as loading control

highly frequent methylation of all observed gene promoters. Methylation rates of *ITGA4* and *TFPI2* were 93 and 93.3 %, respectively, in CRC biopsies (Fig. 4a, b). This represented a significant increase compared to the normal subset ($p < 0.0001$). Analysis of the *VIM* promoter revealed a methylation rate of 73.3 % (11/15) in CRC specimens.

An overview of the complete data set of tissue analysis is given in Table 1.

Influence of gender and age on candidate marker promoter methylation

A possible gender-specific change in methylation was analyzed in the three genes. The samples were equally

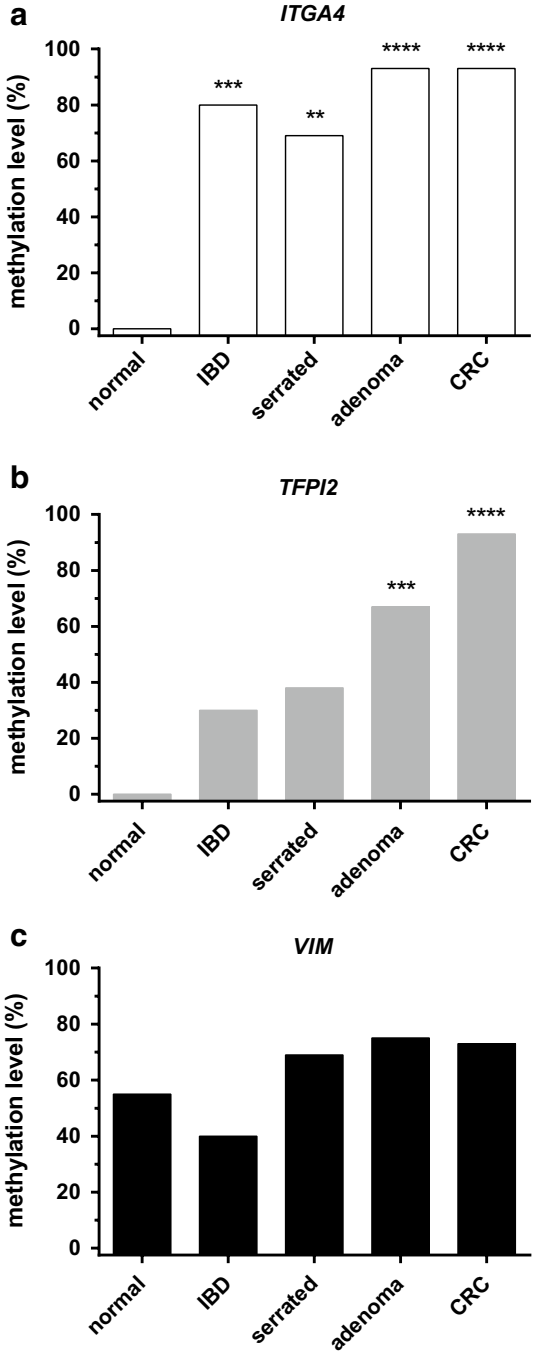

Fig. 4 Fraction of samples with promoter methylation of the analyzed genes dependent of the histopathological features. The biomarkers have been analyzed in control and tumor samples by methylation-specific PCR (MSP). *Bars* represent the biopsies being methylated in **a** *ITGA4*; $^{**}p = 0.0017$; $^{***}p = 0.0007$; $^{****}p < 0.0001$, **b** *TFPI2*; $^{***}p = 0.0001$; $^{****}p < 0.0001$ and *VIMENTIN* (**c**). *IBD* Inflammatory bowel disease, *CRC* colorectal carcinoma

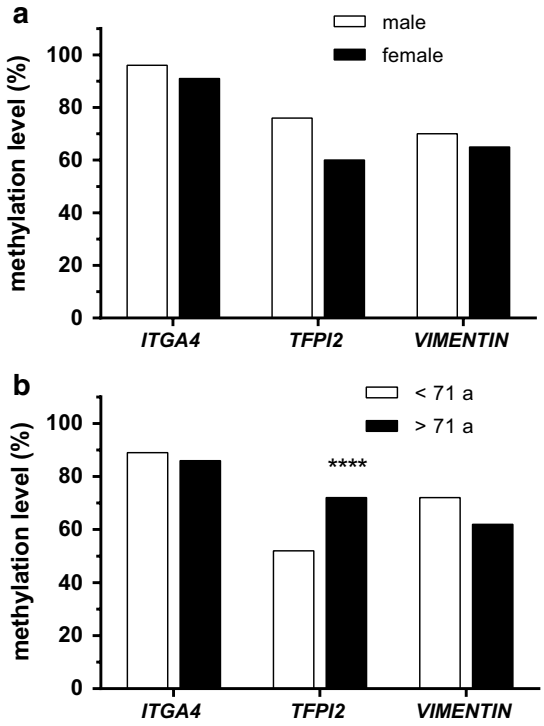

Fig. 5 Fraction of biopsies methylated in *ITGA4*, *TFPI2* and *VIM* in dependence of gender (**a**) and age (**b**). *Bars* represent the biopsies being methylated in *ITGA4*, *TFPI2* and *VIM,* respectively ($^{****}p < 0.0001$)

distributed in male (49.5 %) and female (50.5 %). Regardless of the histopathological features, the gender-specific analysis of the MSP displayed a methylated *ITGA4* promoter in 96.3 % of the male and in 90.9 % of female samples (Fig. 5a). The *TFPI2* promoter was methylated in 75.9 % of male and 60 % of female specimens. Furthermore, the *VIM* promoter was methylated in 70.4 % of male and in 65.4 % of female samples. Overall, a less frequent methylation rate of all three genes was found in female samples. However, no significant difference was calculated.

Age is an important risk factor for the development of CRC, and aging colonic mucosa exhibits beside a genome-wide hypomethylation a locus-specific hypermethylation as well. In order to test a possible link between age- and site-specific gain of methylation, the obtained results from the different tissue samples were analyzed in an age-dependent manner. Therefore, the patients were divided into two populations. The group "<71 a" ($n = 64$) was defined as patients younger than the average age at sampling point. Accordingly, the group ">71 a" ($n = 50$) was defined as patients older than the average age at sampling point. The methylation level of *ITGA4* was almost similar in the group "<71 a" with 89.1 % and in the older group ">71 a" with 86 % (Fig. 5b). The methylation level of *VIM* decreased slightly in the older subset where 62 % of the biopsies exhibited *VIM* promoter methylation compared with

71.9 % of the younger group. These differences were not statistically significant. Thus, changes in methylation frequency of the gene promoters of *ITGA4* and *VIM* were not age-dependent. Interestingly, the methylation levels of *TFPI2* varied significantly between the two populations ($p < 0.0001$). A methylated *TFPI2* promoter was found in 72 % of the older subset versus 51.6 % in the younger group (Fig. 5b).

Combination of methylation markers *ITGA4* and *TFPI2* in tissue samples

In order to define the minimal yet best-informative methylation marker panel, we combined the results of the three genes *ITGA4*, *TFPI2* and *VIM*. As expected, an increased hypermethylation of all three gene markers was observed in sporadic tumors, adenomas and serrated lesions, as well as in IBD samples. However, we decided to use only *ITGA4* and *TFPI2* for a putative methylation marker panel. Based on the findings of methylated *VIM* promoters in normal controls, the methylation analysis of this gene appeared to be more prone for false-positive results.

Accordingly, the analyzed MSP results revealed in none of the normal control samples neither a methylated *ITGA4* nor a *TFPI2* promoter (Fig. 6). In contrast, tissue samples derived from patients with IBD were in 9/10 cases methylated at least in one of the analyzed genes (90 %), whereas in eight out of the ten cases, one of the analyzed genes was unmethylated as well (Table 1). Both, *ITGA4* and *TFPI2,* were methylated in only two IBD samples ($p = 0.0455$). Analysis of the serrated lesions revealed at least two genes methylated in three serrated lesions (23 %; $p = 0.0275$). In adenomas, the methylated proportion of at least one gene was 93.4 % of the adenomas ($p < 0.0001$). All of the two genes were methylated in 90.8 % of the adenoma samples. Most importantly, all of the CRC samples (100 %) were methylated in at least one gene ($p < 0.0001$). Even 86.7 % of CRC tissues revealed a methylated promoter in both analyzed genes ($p < 0.0001$).

Discussion

Colon cancer is characterized by the accumulation of genetic and epigenetic alterations during carcinogenesis. By detecting these molecular changes in precancerous and early cancerous stages, the mortality rates of colon cancer can be reduced significantly (Bretthauer 2010; Jemal et al. 2010; Rabeneck et al. 2010; Zauber et al. 2012). In order to assess the suitability of frequently methylated putative tumor suppressors as markers for the early detection of colon cancer and its precursors, we evaluated the aberrantly methylated genes *ITGA4*, *TFPI2* and *VIM* in colon tissues of different disease stages. Previously, several studies have demonstrated the high potential of the selected genes for the early detection of colon carcinogenesis (Ausch et al. 2009; Chen et al. 2005; Glockner et al. 2009).

In this study, the initial finding of colon cancer-specific hypermethylation of *ITGA4*, *TFPI2* and *VIM* in cell lines was indicative for the suitability of all three genes as possible methylation markers in colon carcinogenesis. Correspondingly, the actively transcribed genes *ITGA4* and *TFPI2* were unmethylated in the normal colon cell line HCEC as expected. However, no *VIM* promoter methylation was detected in HCEC as well, although the *VIM* gene is transcriptionally silent in colonic epithelial cells.

In contrast, we were able to show a methylation-dependent silencing of the *ITGA4* promoter in colon cancer cells. After treatment with the DNMT inhibitor 5-aza-2′-deoxycytidine, a demethylation of the *ITGA4* promoter and a reexpression of the ITGA4 protein could be observed. This data prove DNA methylation to be the responsible mechanism for epigenetic gene silencing of *ITGA4*, which is frequently seen in colorectal carcinogenesis. These

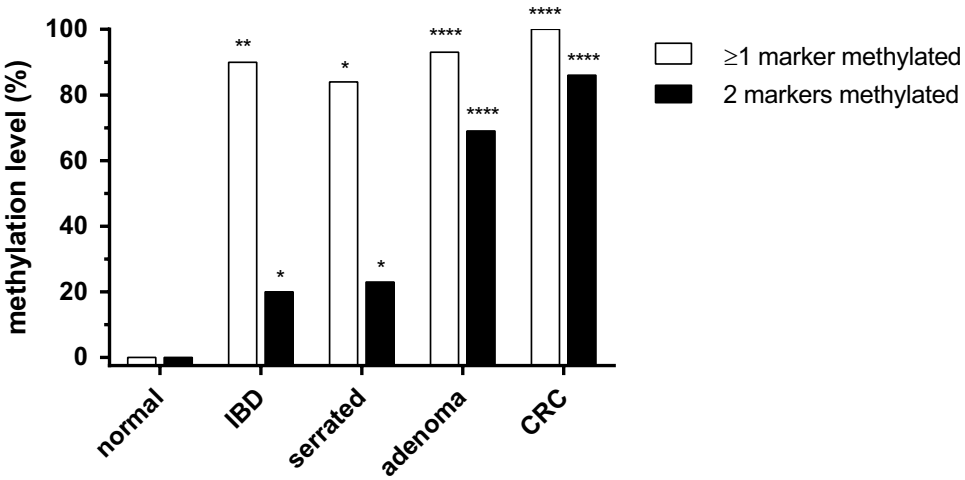

Fig. 6 Fraction of samples with promoter methylation of the analyzed genes *ITGA4* and *TFPI2* dependent of the histopathological features. The biomarker panel has been analyzed in control and tumor samples by methylation-specific PCR (MSP). *Bars* represent the biopsies being methylated in *ITGA4*, *TFPI2* and *VIM*. *IBD* Inflammatory bowel disease (*$p < 0.05$; **$p < 0.01$); serrated lesions (*$p < 0.05$), adenoma (****$p < 0.0001$), *CRC* colorectal carcinoma (****$p < 0.0001$)

findings support our assumption that *ITGA4* promoter hypermethylation appears to be a suitable marker for colon cancer detection.

Based on these findings, we analyzed human colon biopsies of different histological stages. All three candidate markers revealed high methylation rates in colon cancer and adenoma tissue. The already established methylation marker *VIM* appeared to be relatively common methylated in CRC and adenoma tissue. Several studies have been published reporting quite diverse sensitivities of CRC detection by *VIM* methylation analysis, ranging from 38 to 81 % (Ahlquist 2010; Ahlquist et al. 2008; Carmona et al. 2013). In the present work, *VIM* showed an intermediate value in relation to this range. Thereby, it represented the lowest sensitivity for CRC detection obtained among the three markers when evaluated individually (73.3 %). However, *VIM* appears to be minor suitable for colon cancer detection since we found high methylation rates throughout all histologic states including normal colon mucosa. The consequence is low specificity for the detection of neoplastic and pre-neoplastic lesions. Nevertheless, several studies have reported lower *VIM* methylation rates in normal controls ranging from 0 to 12 % (Baek et al. 2009; Itzkowitz et al. 2007). This discrepancy may be explained by the use of mucosal samples from diverticulosis patients as normal controls in our study. Although these tissue samples had a normal appearing histology, we cannot exclude possible inflamed regions within the biopsies causing a hypermethylated *VIM* promoter.

However, the additionally analyzed gene promoters of *ITGA4* and *TFPI2* did not show any methylation in the control samples. Levels of *ITGA4* methylation in CRC samples were prominently high and could be used to significantly discriminate advanced adenoma and carcinoma from normal mucosa at the tissue level. Interestingly, serrated lesions and adenoma tissue samples showed methylation levels as high as carcinoma tissue, making *ITGA4* methylation a robust biomarker. Unexpectedly, the non-neoplastic inflamed tissue samples exhibited a high *ITGA4* methylation rate as well.

Additionally, the *TFPI2* methylation levels were significantly elevated in CRC and adenoma tissue. Although the analysis of the methylation data revealed an age-dependent increase in the proportion of methylated biopsies in *TFPI2,* it became clear that the putative methylation markers *ITGA4* and *TFPI2* appeared to be methylated early during colorectal carcinogenesis and therefore are valuable markers for early detection of neoplastic lesions.

Importantly in inflamed tissue-derived biopsies, the hypermethylation of the analyzed gene promoter regions was a common feature as well. Consistent findings have been made in many other chronic inflammation conditions like Barrett's esophagus or *H. pylori* infection of the stomach (Hahn et al. 2008; Nakajima et al. 2006). Patients with chronic gastrointestinal inflammations have an elevated risk of developing colorectal cancer (Bernstein et al. 2001). A possible explanation for the high methylation rates is provided by the high level of oxidative stress in inflamed regions leading to recruitment of DNA methyltransferase 1 (DNMT1) to the damaged chromatin regions and subsequent methylation of the DNA (Foran et al. 2010). Furthermore, it has been shown that proinflammatory factors like interferon gamma are able to induce the expression of DNMT3b leading to higher levels of 5-methylcytidine (Kominsky et al. 2011). Additionally, TNF-alpha induces cyclooxygenase-2 (*COX-2*) expression subsequently leading to higher prostaglandin E2 (PGE2) levels. The eicosanoid PGE2 is known to activate *DNMT3b* expression as well and induces hypermethylation of gene promoters (Xia et al. 2012). It has also been proposed that unrepaired inflammation-mediated halogenated DNA damage products can mimic 5-methylcytosine due to their high affinity for methyl-binding proteins and ability to induce methylation of the daughter strand after DNA replication, leading to the establishment and propagation of aberrant methylation (Valinluck and Sowers 2007).

Generally, altered DNA methylation seems to be a result of chronic inflammation and not of acute inflammation (Niwa et al. 2010). For that reason, the duration of inflammation is an important risk factor for the development of CRC (Bernstein et al. 2001). In IBD tissue, it seems that hypermethylation of certain genes precedes dysplasia and neoplastic changes. Malignancy in IBD patients originates in precursor cells localized in or nearby the dysplastic mucosa (Grivennikov et al. 2010; Hartnett and Egan 2012; Itzkowitz and Yio 2004). However, the molecular events contributing to sporadic CRC, as mutations in *WNT* or *EGFR* signaling and methylation of CIMP genes, are considered to be different in inflammation-associated CRC (Samowitz et al. 2007). In detail, the timing and the frequency of epigenetic aberrations are altered by the underlying inflammatory process (Feagins et al. 2009). Thus, the methylation frequency of genes like O6-methylguanine DNA methyltransferase (*MGMT*), human mutL homolog 1 (*hMLH1*) and *P16* was relatively low in IBD-associated cancers compared to sporadic neoplasia (Mikami et al. 2007). However, the methylation of other genes like estrogen receptor (*ER*), runt-related transcript factor-3 (*RUNX3*) and methylated-in-tumor-1 (*MINT1*) seems to occur more frequent in IBD patients with neoplasia (Fujii et al. 2005; Garrity-Park et al. 2010). These findings might explain the inconsistent methylation frequencies in IBD tissue and precancerous tissues like serrated lesions in our study.

A major drawback in this study is the lack of information about the duration of inflammation in the colitis

patients. Therefore, it is difficult to correlate the disease duration and the methylation frequency of *ITGA4*, *TFPI2* and *VIM*. However, we assume that ulcerative colitis as a chronic disease with heavy impairments existed for several years at the time of surgery. Further analysis regarding the correlation of methylation frequencies in IBD-associated carcinogenesis is needed.

Nonetheless, biomarkers like the three genes examined in this study may be helpful as risk markers for the prognosis of IBD patients to develop CRC; especially, the high sensitivity of *ITGA4* for precancerous lesions makes it attractive as potential risk marker. Furthermore, the combination with *TFPI2* methylation analysis, which showed elevated methylation rates with progression of disease, seemed to lead to an increase in specificity with minor decrease in sensitivity for detection of CRC and its precursors. Apparently, both methylation markers were methylated in a small subset of IBD samples, which was not depending on the severity and extent of the inflammation. Although this study was limited to a small group of IBD samples, we propose that *TFPI2* and *ITGA4* methylation could serve as possible risk markers for the development of IBD-related colorectal cancer. For this purpose, it will be necessary to test these biomarkers in studies extended to larger cohorts of IBD patients to confirm the robustness of the findings. Consequently, this could improve the early diagnosis of high-risk patients with IBD when assessed in non-neoplastic tissues obtained by screening colonoscopy. A noninvasive stool-based test might improve compliance with surveillance, which is currently poor, even among high-risk patients. Algorithms incorporating stool DNA as a complement to colonoscopy could potentially lengthen the interval between surveillance examinations in marker-negative patients, which could also reduce the high cost of surveillance endoscopy. Conversely, a patient with a positive stool DNA test may benefit from colonoscopy at shorter surveillance intervals. The tissue study based on well-matched cases and controls showed that methylation markers are highly discriminant for IBD.

Besides already established methylation markers like *mSEPT9* (Payne 2010), there is a growing interest in new biomarkers for the early colon cancer detection (Nibbe and Chance 2009). Nevertheless, a sensitivity of 100 % will be practically impossible to reach by using methylation markers solely, because a subset of CRCs has low level or no detectable hypermethylated genes. Therefore, it will be necessary to combine methylation markers with alternative markers, like mutational DNA markers, as already proposed for other cancers (Cheon and Orsulic 2014). Additionally, RNA expression studies in colorectal cancer tissue revealed useful diagnostic markers like *ARNTL2* and *SERPINE1* (Mazzoccoli et al. 2012). Furthermore, it should be noticed that the detection of markers in precancerous lesions requires highly sensitive techniques. We recently introduced an ultrasensitive platform for the detection of known and unknown gene mutations in stool samples (Gerecke et al. 2013). An adaptation of this technique for detecting methylation could be used for sensitive methylation detection. For the purpose of a noninvasive screening in the future, the detection limits of methylation events should be evaluated equally in body fluids. A joint analysis of mutation and methylation markers for early detection of neoplastic diseases in body fluids like feces is desirable.

Conclusion

In conclusion, the gene methylation of *ITGA4* and *TFPI2* is an early and frequent event in precancerous and cancerous lesions of the colon and rectum. Furthermore, this methylation occurs in colon tissue from patients with chronic inflammation and was not detected in any normal colon samples. Finally, the methylation of *ITGA4* and *TFPI2* promoters is associated with a phenotypic down-regulation of these gene products. These factors make the *ITGA4* and *TFPI2* methylation a feasible epigenetic marker for early detection of CRC- and IBD-related cancer and may be useful for CRC screening in the future.

We have reported for the first time that *VIM* methylation was detected in normal colon tissue and therefore is not acceptable as specific early detection marker for colorectal neoplasia.

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Conflict of interest None.

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