CHAPTER 3

Promoter methylation precedes chromosomal alterations in colorectal cancer development

Cellular Oncology 2006, 28: 247-257

Sarah Derks
Cindy Postma
Peter T.M. Moerkerk
Sandra M. van den Bosch
Beatriz Carvalho
Mario A.J.A. Hermsen
Walter Giaretti
James G. Herman
Matty P. Weijenberg
Adriaan P. de Bruïne
Gerrit A. Meijer
Manon van Engeland

Abstract

Background: Colorectal cancers are characterized by genetic and epigenetic alterations. This study aimed to explore the timing of promoter methylation and relationship with mutations and chromosomal alterations in colorectal carcinogenesis.

Methods: In a series of 47 nonprogressed adenomas, 41 progressed adenomas (malignant polyps), 38 colorectal carcinomas and 18 paired normal tissues, we evaluated promoter methylation status of *hMLH1, O^6MGMT, APC, $p14^{ARF}$, $p16^{INK4A}$, RASSF1A, GATA-4, GATA-5*, and *CHFR* using methylation-specific PCR. Mutation status of *TP53, APC* and *KRAS* were studied by p53 immunohistochemistry and sequencing of the *APC* and *KRAS* mutation cluster regions. Chromosomal alterations were evaluated by comparative genomic hybridization.

Results: Our data demonstrate that nonprogressed adenomas, progressed adenomas and carcinomas show similar frequencies of promoter methylation for the majority of the genes. Normal tissues showed significantly lower frequencies of promoter methylation of *APC, $p16^{INK4A,}$ GATA-4*, and *GATA-5* (P-values: 0.02, 0.02, 1.1×10^{-5} and 0.008 respectively). P53 immunopositivity and chromosomal abnormalities occur predominantly in carcinomas (P values: 1.1×10^{-5} and 4.1×10^{-10}).

Conclusions: Since promoter methylation was already present in nonprogressed adenomas without chromosomal alterations, we conclude that promoter methylation can be regarded as an early event preceding *TP53* mutation and chromosomal abnormalities in colorectal cancer development.

Introduction

Colorectal cancer development is characterized by the growth of a benign precursor lesion of which eventually a small percentage will progress into a carcinoma [28]. The genetic alterations underlying the adenoma to carcinoma transition have been extensively studied over the past two decades. Pioneering research of Vogelstein and co-workers has proposed a progression model in which genetic alterations as *APC* and *TP53* mutations and allelic loss of 5q and 18q play animportant role [2,16,17,24,43]. Previously, we introduced the concept that chromosomal instability does not merely constitute genetic noise but occurs in nonrandom patterns. Accumulation of losses in 8p21-pter, 15q11-q21, 17p12-13 and 18q12-21 and gains in 8q23-qter, 13q14-31, and 20q13 are strongly associated with advanced lesions and can be used as indicator of progression towards malignancy [22,32]. Recently, it has become clear that initiation and progression of cancer also involves epigenetic alterations such as DNA methylation and that genetic and epigenetic alterations interact in driving the development of cancer [34]. Colorectal cancer development is associated with epigenetic silencing of the DNA repair genes *hMLH1* [10] and *O^{6}MGMT* [9], the WNT signal transduction regulator *APC* [13], the Ras signalling molecule *RASSF1A* [44], the transcription factors *GATA-4* and *GATA-5* [1] and the cell cycle regulators *CHFR* [8,40], *p16^{INK4A}* and *p14^{ARF}* [21,33]. Although extensive knowledge exists on epigenetic and genetic changes in colorectal cancer, little is known about the exact relationship between these two [7,19]. In this cross-sectional study we address epigenetic and genetic (at the level of the single gene as well as at the level of whole chromosomes) alterations in colorectal cancers and its precursor lesions. Using a multi-gene approach we investigate the timing of promoter methylation and define how these epigenetic events are related to genetic events in colorectal cancer development.

Material and Methods

Patient material

This study was performed on a subset (n=139) of colorectal adenoma and carcinoma tissues which has been analyzed for structural chromosomal abnormalities by comparative genomic hybridization (CGH) previously [22]. Part of this subset has also been analyzed for mutation status of *APC* (n=96) and *KRAS* (n=78) [18,22]. We extended this series by adding 20 colorectal adenoma and carcinoma cases, bringing the overall total to 159 tissues. This series consists of 47 colorectal adenomas without signs of malignancy at time of resection (nonprogressed adenomas (nA)), 41 malignant polyps (colorectal adenomas containing a focus of carcinoma) and 38 additional

solitary colorectal carcinomas (Cs). Of the 41 malignant polyps, the adenoma part, referred to as progressed adenomas (pA) (n=41), and the carcinoma part (Cmp) (n=33) were microdissected and analyzed separately. If present we added morphologically normal mucosa within the resection specimen (n=18) of patients with solitary carcinomas (Cs) to these series. For each tissue sample, DNA was extracted from fifteen 10-µm paraffin sections, dissecting the most tumor-rich areas, allowing a maximum of 20% nontumor cell contamination.

Overall, the tissues were obtained from 95 patients, 46 males and 49 females (mean age of 67 years: range 40-89). Twenty-four patients exhibited multiple tumors; 4 patients presented with multiple adenomas, 1 patient presented with multiple carcinomas and 19 patients exhibited 1 or more adenomas adjacent to a carcinoma. The histological characteristics are listed in Table 1.

Table 1. Histological characteristics of 159 colorectal adenoma and carcinoma tissues

	Adenoma	**(n)**	**Carcinoma**	**(n)**
Tissue	non-progressed adenomas (nA)	47	carcinomas part of malignant polyp (Cmp)	33
	progressed adenoma (pA)	41	Solitary carcinoma	38
Histologic type	tubular	38		
	tubulovillous	42		
	villous	5		
	serrated	3		
Degree of dysplasia	mild	13		
	moderate	50		
	severe	25		
Differentiation grade			well	13
			moderate	52
			poor	6
TNM			I	23
			II	30
			III	17
			IV	1

Promoter methylation analysis

DNA methylation in the CpG islands of the *hMLH1, O^6MGMT, APC, $p14^{ARF}$, $p16^{INK4A}$, RASSF1A, GATA-4, GATA-5* and *CHFR* gene promoters was determined by chemical modification of genomic DNA with sodium bisulfite and subsequent methylation-specific PCR (MSP) as described in detail elsewhere [11,20,42]. Briefly, 1 μg of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega, Madison, USA) again treated with NaOH, precipitated with ethanol, and resuspended in H2O. To facilitate MSP analysis on DNA retrieved from formalin-fixed, paraffin embedded tissue, DNA was first amplified with flanking PCR primers that amplify bisulfite-modified DNA but do not preferentially amplify methylated or unmethylated DNA. The resulting fragment was used as a template for the MSP reaction. Primer sequences have been described before [1,5,42]. All PCRs were performed with controls for un methylated alleles (DNA from normal lymphocytes), methylated alleles [normal lymphocyte DNA treated in vitro with SssI methyltransferase (New England Biolabs)], and a control without DNA. Ten μl of each MSP reaction were directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. The methylation status was assessable in 96% of the total number of analyses. To asses reproducibility, 234 MSP reactions have been performed in duplicate starting from DNA amplification with flanking PCR primers, the reproducibility was 90%. To exclude false priming, sequencing of the methylated amplicon of *APC* was performed, revealing extensive methylation of all amplicons, including the primer binding region.

P53 immunohistochemistry

P53 immunohistochemistry (n=146) was performed using the mouse monoclonal antibody DO7 (DAKO, Glostrup, Denmark). Immunoperoxidase staining for p53 in formalin-fixed, paraffin-embedded tissue sections was performed by a horseradish peroxidase labeled streptavidin-biotin method. Four μm sections were mounted on 0.1% poly-L-lysine coatedglass slides, deparaffinized, and rehydrated through graded alcohols to water. Endogenous peroxidase activity was blocked by incubation with 0.3% H2O2 in methanol. Sections were immersed in 10 mM sodium citrate buffer, pH 6.0, and subjected to heatinduced antigen retrieval with microwave. To block non-specific protein binding, sections were pre-incubated with normal rabbit serum (1:50, DAKO) for 10min at room temperature. Mouse monoclonal antibody against p53 (1:500 DO7, DAKO) was applied, and tissue sections were incubated overnight at 4°C. Then sections were rinsed with PBS, and treated with biotinylated rabbit anti-mouse IgG (1:500, DAKO) for 30 min at room temperature, rinsed with PBS, and then incubated with streptavidine-biotin-HRP complex (1:200, DAKO) for 1 hour at room temperature. After washing with PBS the complex was visualized with diaminobenzidine

and H2O2 for 3 min. Sections were then counter stained with hematoxylin, dehydrated in graded alcohols, cleared in xylene and cover slipped. The area percentage of positive nuclei was scored with a point counting approach using a video overlay measuring system (Qprodit Leica, Cambridge, UK). An area percentage of 20 was used as threshold for positivity [31].

KRAS mutation analysis

KRAS mutation status of 78 colorectal adenoma and carcinoma tissues have been analyzed previously [22]. Fifty-two additional tissues were analyzed by PCR using an oligonucleotide 20-mer panel of codons 12 and 13 (TIB Molbiol, Advanced Biotechnology Center, Genova, Italy) as previously described [18]. Extracted DNA from peripheral blood lymphocytes from healthy donors was used as wild type *KRAS* codon 12 GGT-gly and codon 13 GGC-gly controls, and extracted DNA from 6 different colon cancer cell lines was used as control for known *KRAS* mutations.

Array-CGH analysis

One hundred-thirty-nine colorectal adenoma and carcinoma tissues have been analyzed by conventional CGH previously [22]. The 20 additionally collected neoplasms were analyzed by array CGH analysis using 5K BAC arrays [6,36]. In short, we used a fullgenome array printed in the house containing approximately 5000 clones with an average resolution of 1 Mb (http://www.vumc.nl/microarrays/index.html). After amplification of BAC clone DNA by ligation-mediated polymerase chain reaction (PCR) according to Snijders et al. (2001) all clones were printed in triplicate. Printing of clones was performed on codelinkTM slides (Amersham BioSciences, Roosendaal, NL) at a concentration of 1 μg/μl, in 150 mM sodium phosphate, pH 8.5, using a SpotArray72 printer (Perkin Elmer Life Sciences, Zaventum, BE). After printing slides were processed according to the manufacturer's protocol. Labeling and hybridization of tumor and reference DNAs were performed as described in detail by Snijders et al. (2001) with some modifications, namely, hybridizations took place in a hybridization station (HybArray12TM – Perkin Elmer Life Sciences, Zaventum, BE) and slides were scanned with Agilent DNA Microarray scanner (Agilent technologies, Palo Alto, USA), omitting the DAPI staining step. Segmentation and quantification of the spots was done using Imagene 5.6 software (Biodiscovery Ltd, Marina del Rey, California). Local background median intensity was subtracted from the signal median intensity for both test and reference channels and a ratio tumor/reference was calculated. The ratios were normalized against the mode of all ratios of the autosomes. As the clones were spotted in triplicate, the median value of the corresponding three intensities was taken into account for each clone in the array. Clones fromwhich the intensities of the three spots had a standard deviation >0.2 were excluded. Furthermore, clones with more than 20% missing values in all carcinomas were excluded for further analysis. All the analyses were

done excluding chromosome X, as in every hybridization a sex-mismatched reference DNA was used for quality control of the experiment. Clone positions were considered according to freeze May 2004. After using a smoothing algorithm [25], DNA copy number ratios obtained by array CGH were recoded as gains and losses at the resolution of whole chromosome arms compatible with the data obtained by chromosome CGH.

Data analysis

Differences in frequencies of gene methylation between the different stages of disease progression and associations between promoter methylation and mutations were evaluated by the Pearson's $\chi 2$ or Fisher exact test where appropriate. The total number of methylated genes, referred to as methylation index (MI), is defined as the number of genes methylated divided by the number of genes analyzed. The same accounts for the total number of gains and losses, referred to as chromosomal events, and the number of losses (8p21-pter, 15q11-q21, 17p12-13 and 18q12-21) and gains (8q23-qter, 13q14-31, and 20q13) associated with advanced lesions, referred to as cancer associated events [22]. The Mann Whitney U and Kruskal Wallis nonparametric test were used for comparing means of continuous variables. The McNemar test for paired cases was used to test the methylation differences between a subset of solitary carcinomas (Cs) and paired normal tissue. Simple linear regression analysis was performed to investigate the correlation between the number of methylated genes and the number of chromosomal abnormalities. For all analyses SPSS software version 11.0 was used. All reported *P* values are two-sided, and a *P* value <0.05 was considered statistically significant.

Results

Promoter methylation in relation to adenoma-carcinoma progression

In order to investigate the timing of promoter methylation in colorectal cancer development, we studied the frequency of promoter methylation of genes which function in regulating diverse cell functions in the colorectal adenoma and carcinoma tissues. Our data demonstrate that nonprogressed adenomas (nA), progressed adenomas (pA), carcinoma parts of malignant polyps (Cmp) and solitary carcinoma (Cs) showed similar frequencies of promoter methylation for the majority of genes (Table 2). No difference in mean methylation index (MI) (total number of methylated genes divided by the number of genes analyzed) between the different categories of neoplasm's was observed. However, $p14^{ARF}$ methylation was found in 71.1% and 73.5% of the nonprogressed adenomas (nA) and progressed adenomas (pA) respectively and decreases to

53.3% and 37.1% of the carcinoma parts of malignant polyps (Cmp) and solitary carcinoma (Cs) respectively (*P* value: 0.006).

Since we observed that promoter methylation of the studied genes is already present in nonprogressed adenomas (nA), we were interested in the presence of promoter methylation in matching normal mucosa. For 18 solitary carcinomas (Cs) morphological normal tissue from the resection specimen was available (Table 3a). We performed a nonparametric test for matched pairs and compared the methylation profile of 18 solitary carcinomas to their corresponding normal mucosa. In 158 of the 162 (9 genes x 18 pairs) possible combinations the gene methylation status was assessable in the carcinoma as well as in the normal tissue. In 48.7% of the pairs (77/158) no difference in gene methylation was observed within pairs (N=C) (Table 3b). In 66.2% (51/77) of these pair both components were unmethylated while in 33.8% (26/77) the carcinomas as well as the corresponding normal tissue showed promoter methylation. In 43% (68/158) of all pairs gene methylation was present in the carcinomas while absent in the corresponding normal tissue (C>N), and only in 8.2% (13/158) of the pairs promoter methylation was only observed in the normal tissue (N>C).

The McNemar test for paired cases showed that promoter methylation of *APC, p16^{INK4A,} GATA-4* and *GATA-5* occurred significantly more frequent in the carcinomas when compared to corresponding normal mucosa (*P*-values 0.02, 0.02, 1.1 x 10^{-5} and 0.008 respectively) (Table 3b). Although promoter methylation of *hMLH1, O^{6}MGMT* and *CHFR* was present in the normal tissues, this was predominantly when the carcinomas was also methylated. For example, promoter methylation of *hMLH1* was observed in 50.0% of the adenomas and 72.2% of the paired carcinomas (this high frequency could be explained by the fact that in 6 of the 18 pairs (33.3%) the carcinomas part showed microsatellite instability, data not shown. In 5 of these 6 pairs, the carcinoma as well as the paired normal tissue showed *hMLH1* methylation). In 8 of the 9 pairs in which normal tissue showed *hMLH1* promotermethylation, carcinoma tissue was methylated as well. In 6 cases a difference in methylation was present of which in 5 cases *hMLH1* was methylated in the carcinomas while unmethylated in the paired normal mucosa. Comparable patterns were observed for promoter methylation of *O^{6}MGMT, RASSF1A* and *CHFR*. More difference between paired carcinomas and normal tissues were observed for *p14^{ARF}* methylation. In 4 of the 9 cases in which a difference within pairs was observed, normal tissue displayed gene methylation while *p14^{ARF}* was not methylated in the corresponding carcinomas.

Table 2. Timing of promoter methylation, genetic and chromosomal abberations.

	nA	pA	Cmp	Cs	P value
Number of cases	47	41	33	38	
Promoter methylation (%)					
hMLH1	44.7	31.7	36.4	55.3	NS
O6-MGMT	66.7	72.5	54.5	60.5	NS
RASSF1a	19.1	24.4	24.2	29.7	NS
APC	61.7	51.2	33.3	44.7	NS
$p14^{ARF}$	71.1	73.5	53.3	37.1	0.006
$p16^{INK4A}$	28.9	36.6	36.4	31.6	NS
GATA-4	77.3	77.5	75.8	86.5	NS
GATA-5	95.7	87.8	84.4	82.9	NS
CHFR	48.6	56.7	50.0	55.9	NS
Mean methylation index	0.57	0.56	0.49	0.53	NS
Genetic alterations					
p53 immunopositivity (n=146)	14.3	34.2	55.2	59.5	1.1×10^{-5}
APC mutation (n=96)	50 (17/34)	63.2 (24/38)	58.3 (7/12)	66.7 (8/12)	NS
KRAS mutation (n=130)	34.3	40.0	28.6	28.1	NS
Chromosomal alterations					
Number of chromosomal events	6.2	12.4	12.2	10.6	4.1×10^{-6}
Number of CAE	0.9	3.0	3.3	3.0	3.6×10^{-10}

Note: Results of epigenetic and genetic analyses of nonprogressed adenomas (nA), progressed adenomas (pA), carcinoma parts of a malignant polyps (Cmp) and solitary carcinomas (Cs). Listed are the frequencies of promoter methylation of 9 genes, methylation index (total number of methylated genes divided by the number of genes analyzed), p53 immunopositivity, *APC* and *KRAS* mutation, number of chromosomal abnormalities (chromosomal events) and number of cancer associated events (losses at 8p21-pter, 15q11-q21, 17p12-13, 18q12-21, and gain at 8q23-qter, 13q14-31 and 20q13) per group. Data on p53 immunopositivity, APC and KRAS mutations were available for a subset of cases; MSP and CGH have been done on all cases.

Table 3a.Promoter methylation frequencies in 18 paired carcinoma and normal tissues.

Table 3b. Promoter methylation frequencies in 18 paired carcinoma and normal tissues.

	hMLH1	*O⁶MGMT*	*RASSF1A*	*p14ARF*	*p16INK4A*	*APC*	*GATA-4*	*GATA-5*	*CHFR*
N (%)	50	38.9	16.7	33.3	0	16.7	16.7	16.7	27.8
C (%)	72.2	61.1	44.4	38.9	38.9	61.1	94.4	72.2	55.6
N=C (%)	66.7 (12/18)	44.4 (8/18)	38.9 (7/18)	50.0 (9/18)	61.1 (11/18)	44.4 (8/18)	22.2 (4/18)	46.7 (7/15)	64.7 (12/17)
C>N (%)	27.8 (5/18)	38.9 (7/18)	44.4 (8/18)	27.8 (5/18)	38.9 (7/18)	50 (9/18)	77.8 (14/18)	53.3 (8/15)	29.4 (5/17)
N>C (%)	5.6 (1/18)	16.7 (3/18)	16.7 (3/18)	22.2 (4/18)	0 (0/18)	5.6 (1/18)	0 (0/18)	0 (0/15)	5.6 (1/18)
P value	NS	NS	NS	NS	0.02	0.02	4.1 x 10-6	0.008	NS

Note: Results of promoter methylation in 18 paired carcinoma (C) and normal tissue (N). N=C: no difference in gene methylation between carcinoma and normal tissue. C>N: gene methylated in carcinoma and unmethylated in normal tissue. N<C: gene methylated in normal tissue and unmethylated in carcinoma.

Promoter methylation in relation to genetic alterations

In order to study the relationship between promoter methylation and genetic alterations we analyzed mutations of three key genes involved in development of colorectal cancer, i.e. *TP53, APC* and *KRAS*.

Disruption of the p53 pathway, amongst others, can occur by loss of function of *TP53* itself and by *p14ARF* methylation [46]. The frequency of *p14ARF* methylation significantly decreased in tumor progression (Table 2). In contrast, aberrant p53 status, indicated by p53 immunopositivity, increased from 14.3% in the nonprogressed adenomas (nA) through 34.2% in the progressed adenomas (pA) to 55.2% and 59.5% in the carcinoma parts of malignant polyps (Cmp) and solitary carcinomas (Cs) respectively (*P* value: 0.001). Case by case, *p14ARF* methylation shows an inverse relation with p53 immunopositivity, approaching statistical significance (*P* value: 0.07).

For *APC* and *KRAS*, a similar pattern was found. *APC* was mutated in 50% and 63.2% of the nonprogressed and progressed adenomas (nA and pA), respectively, compared to 58.3% and 66.7% of the carcinomas parts of malignant polyps (Cmp) and solitary carcinomas (Cs) [22]. *KRAS* mutation was observed in 34.3% and 40.0% of the nonprogressed and progressed adenomas (nA and pA), respectively, and in 28.6% and28.1% of the carcinomas parts of malignant polyps (Cmp) and solitary carcinomas (Cs) (Table 2). While neither the frequencies of *APC* mutation nor *APC* promoter methylation differ between the different stages of tumor development, case by case analysis indicated an inverse relation (*P* value: 0.06). A similar pattern and inverse relation was observed for *KRAS* mutation and promoter methylation of *RASSF1A* and *hMLH1* (*P* values: 0.01 and 0.001 respectively).

Promoter methylation in relation to chromosomal alterations

The timing and interrelationship of promoter methylation and chromosomal alterations in tumor progression were analyzed by studying promoter methylation in cases without chromosomal abnormalities and by relating gene methylation status to the mean number of chromosomal and cancer associated events. Simple linear regression analyses revealed no correlation between the MI and the number of chromosomal abnormalities or number of cancer associated events. As described previously, the number of chromosomal abnormalities and especially the number of cancer associated events are associated with progressed lesions (*P* values: 4.1×10^{-6} and 3.6×10^{-10} respectively) (Table 2) [22]. Figure 1 shows that while the mean number of chromosomal events and cancer associated events increases during tumor progression the mean MI remains stable. In 13 cases (11 nonprogressed adenomas (nA) and 2 solitary carcinomas (Cs)) no chromosomal alterations were observed. The 12 adenomas without chromosomal abnormalities did not differ in MI from adenomas with chromosomal abnormalities. Interestingly, the 2 solitary carcinomas (Cs) without chromosomal abnormalities (both cases exhibit microsatellite instability; data not shown) were characterized by aMI of 0.96, while the MI of carcinomas harboring chromosomal alterations was 0.56 (*P* value: 0.006). No association between promoter methylation of a specific tested gene and the number of chromosomal abnormalities was observed.

One of the cancer associated chromosomal changes, deletion of 8p21, includes the *GATA-4* gene, which was also a frequent target of epigenetic changes in these tumors. Promoter methylation as well as loss of heterozygosity could combine leading to loss of function of *GATA-4*. *GATA-4* methylation was found in respectively 77.3% and 77.5% of the nonprogressed adenomas (nA) and progressed adenomas (pA) and in 75.8% and 86.5% of the carcinoma parts of malignant polyps (Cmp) and solitary carcinoma (Cs) respectively. In contrast, the frequency of loss of 8p21-pter increases in tumor development from 12.8% and 39.0% of the nonprogressed adenomas (nA) and progressed adenomas (pA) respectively to 45.5% and 42.1% of the carcinoma parts of malignant polyps (Cmp) and solitary carcinoma (Cs) respectively (*P* value: 0.005). The frequency in which loss of 8p21-pter is combined with *GATA-4* methylation (*GATA-4* M/8p-) increases during tumor development from 9% in nonprogressed adenomas (nA) to 25%, 30% and 32% in progressed adenomas (pA), carcinoma parts of malignant polyps (Cmp) and solitary carcinoma (Cs) respectively. The frequency in which only GATA-4 is methylated (*GATA-4*/8p) is stable and occurs in 68.2% of the nonprogressed adenomas (nA) and 52.2%, 45% and 54% of the progressed adenomas (pA), carcinoma parts of malignant polyps (Cmp) and solitary carcinoma (Cs) respectively. Loss of 8p21-pter without concomitant methylation of *GATA-4* (*GATA-4* U/8p-) is infrequent occurring in 4.5% and 15.2% of the nonprogressed adenomas (nA) and progressed adenomas (pA) and in 15% and 8.1% of the carcinoma parts of malignant polyps (Cmp) and solitary carcinoma (Cs).

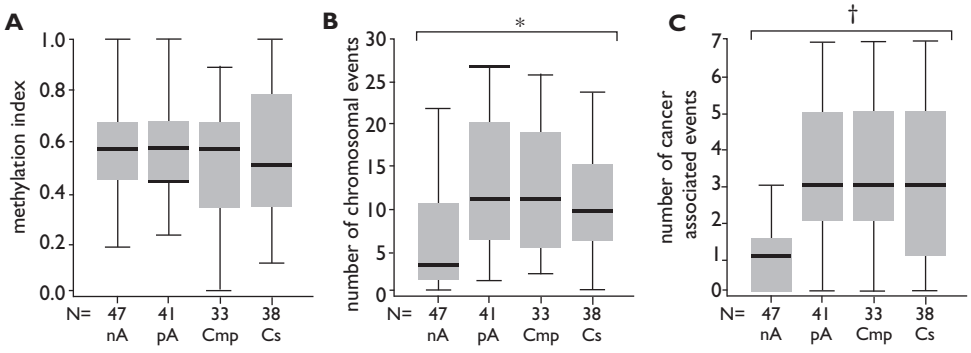

Figure 1. Promoter methylation and chromosomal alterations in colorectal cancer development.
nA= nonprogressed adenoma; pA= progressed adenoma; Cmp= carcinoma part of a malignant polyp; Cs= solitary carcinoma. (A) Promoter methylation has been analyzed for 9 DNA repair- and tumor suppressor genes. The methylation index (total number of methylated genes divided by the number of genes analyzed) stays stable during tumor development, while (B) the total number of chromosomal alterations (chromosomal events), analyzed on genomic level, and (C) the number of cancer associated events (losses at 8p21-pter, 15q11-q21, 17p12-13, 18q12-21, and gain at 8q23-qter, 13q14-31 and 20q13) increase.
* *P* value: 4.1×10^{-6}, † *P* value: 3.6×10^{-10}.

Discussion

In this study we attempt to elucidate the timing and interrelation of promoter methylation and genetic alterations in colorectal cancer development. Therefore we studied genetic and epigenetic events known to be associated with colorectal cancer development.

Considering the timing of epigenetic events in tumor progression, our results indicate that promoter methylation of the studied genes can be regarded as an early event in colorectal carcinogenesis. A high frequency of promoter methylation of multiple DNA repair- and tumor suppressor genes is already present in adenomas without any histological signs of progression, and malignant lesions showed similar frequencies of methylation. Even in morphologically normal mucosa from patients with solitary carcinomas (Cs) promoter methylation of *hMLH1, MGMT, RASSF1A, p14^{ARF}* and *CHFR* was observed, but, with exception of *p14^{ARF}* methylation, in lower frequencies compared to the carcinomas. *P16^{INK4A}, APC, GATA-4,* and *GATA-5* methylation occurred predominantly in the carcinomas. Promoter methylation in normal tissues was in most cases consistent with the methylation profile of the paired carcinoma. However, additional studies involving normal colonic mucosa from individuals without cancers are required to determine the exact timing of promoter methylation of the studied genes.

Interestingly, hypermethylation of *p14^{ARF}* was more frequently present in nonprogressed adenomas (nA) and progressed adenomas (pA) when compared to carcinoma parts of malignant polyps (Cmp) and solitary carcinomas (Cs). This observation can possibly be explained by the concept that the transition from an adenoma to a carcinoma can be considered as a transition from a heterogeneous cellular population to one that is more homogeneous [17]. Even though promoter methylation is a dynamic process, this indicates that *p14^{ARF}* methylation is not necessarily associated with a definitive growth advantage.

Furthermore, since the tumor suppressor functions of *p14^{ARF}* is dependent upon the presence of functional p53 [14], *p14^{ARF}* methylation is possibly of greater importance in early stages of disease progression where *TP53* mutations are not highly prevalent. This is supported by the observation that the frequency of p53 immunopositivity, as a marker of *TP53* mutations, increases during colorectal cancer development.

The concept that epigenetic alterations occur most frequently during the early stages of tumor development as well as the presence of promoter methylation of *hMLH1* and *MGMT* in normal colonic tissues of patients with colon cancers has also been shown by others [3,27,35]. Baylin and Ohm recently hypothesized that the early epigenetic alterations predispose cells to acquire the genetic abnormalities that proceed the neoplastic process [3]. In addition, Tlsty et al. showed that hypermethylation of the *p16^{INK4A}* promoter in mammary epithelial cells is associated with entrance into a state of unrestricted proliferation accompanied by chromosomal instability [37,38]. The actual mechanism involved is unknown but as epigenetical silencing of mismatch repair (MMR) genes causes a mutator phenotype [45] one would hypothesize that promoter methylation of "stability genes", such as *p16^{INK4A}* which retains proper cell cycle control, can initiate chromosomal instability. In this study however, promoter methylation of *p16^{INK4A}* shows no association with chromosomal instability. Also no association between promoter methylation of the mitotic checkpoint control gene *CHFR* and an increased number of chromosomal abnormalities was observed. These results are consistent with a study of Bertholon et al. [4] which showed that methylation of *CHFR* is not associated with chromosomal instability in cell lines. Apparently, in colorectal cancer, promoter methylation of other control genes needs to be evaluated to determine if epigenetic changes are indeed associated with the initiation of chromosomal instability.

Furthermore, we studied the relationship between epigenetic and genetic alterations in tumor development and inverse relations between promoter methylation and gene mutation within important regulatory pathways were observed. We confirmed previous reports that *p14^{ARF}* methylation shows an inverse correlation to *TP53* mutation in colorectal cancer [12,14,34], which has also been observed in bladder cancer [30] and non-small cell lung cancer [23]. In head and neck squamous cell carcinomas (HNSCC) an inverse correlation between *TP53* mutation status

and *cyclin A1* methylation, another downstream target of TP53 has been described [39]. Similar relations have been shown for *APC* promoter methylation and mutation [13] and *KRAS* mutation and *RASSF1A* methylation [41] indicating that gene mutation and promoter methylation do not frequently occur simultaneously in the same pathway, but rather may act in a mutual exclusive or complimentary fashion.

A different approach to study the relationship between epigenetic en genetic silencing of a gene was to examine the relationship between promoter methylation of a gene, *GATA-4*, and deletion of the chromosomal location of the gene, loss of 8p21-pter. Both events occur frequently in tumor development, but no association was observed. Hypermethylaton of *GATA-4* was not restricted to tissues with or without chromosomal loss of 8p21-pter. *GATA-4* methylation occurs prior to loss of 8p21-pter and the number of cases in which both events were present increased during tumor progression. In addition, no association between promoter methylation of a gene and the number of chromosomal alterations was observed. An observation which is in agreement with a recent study on hepatocellular carcinogenesis in which no correlation between the degree of chromosomal structural alterations and that of aberrant promoter methylation was present [26]. Furthermore, in nonprogressed adenomas without chromosomal abnormalities, high frequencies of promoter methylation were already present. Together, these observations suggest that promoter methylation of the selected DNA repair- and tumor suppressor genes precede chromosomal abnormalities in colorectal cancer development.

In summary, the data indicate that promoter methylation of the selected genes can be considered as an early event which occurs prior to *TP53* mutations and chromosomal instability. The association between gene methylation and pre-malignant lesions is highly relevant for methylation-marker based colorectal cancer screening. The observation that the presence of promoter methylation in normal tissues corresponds to the methylation profile of paired carcinomas suggests that methylation levels in normal colonic mucosa could serve as marker of risk of development of CRC. Given that aberrant DNA methylation can also be detected in stool DNA [15,29], studying methylation as common event in pre-malignant lesions is promising to provide novel specific biomarkers for risk assessment and secondary prevention [37].

Acknowledgements

Supported by the TransNational University Limburg and the Dutch Cancer Society (grant KWFVU02-2618).

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