BRIEF ARTICLE

Promoter methylation status of *hMLH1*, *MGMT*, and *CDKN2A/p16* in colorectal adenomas

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Abstract

AIM: To investigate aberrant DNA methylation of CpG islands and subsequent low- or high-level DNA microsatellite instability (MSI) which is assumed to drive colon carcinogenesis.

METHODS: DNA of healthy individuals, adenoma (tubular or villous/tubulovillous) patients, and colorectal carcinoma patients who underwent colonoscopy was used for assessing the prevalence of aberrant DNA methylation of human DNA mismatch repair gene mutator L homologue 1 (*hMLH1*), Cyclin-dependent kinase inhibitor 2A (*CDKN2A/p16*), and O-6-methylguanine DNA methyltransferase (*MGMT*), as well as their relation to MSI.

RESULTS: The frequency of promoter methylation for each locus increased in the sequence healthy tissue/adenoma/carcinoma. *MGMT* showed the highest frequency in each group. *MGMT* and *CDKN2A/p16* presented a statistically significant increase in promoter methylation between the less and more tumorigenic forms of colorectal adenomas (tubular *vs* tubullovillous and villous adenomas). All patients with tubulovillous/villous adenomas, as well as all colorectal cancer patients, showed promoter methylation in at least one of the examined loci. These findings suggest a potentially crucial role for methylation in the polyp/adenoma to cancer progression in colorectal carcinogenesis. MSI and methylation seem to be interdependent, as simultaneous *hMLH1*, *CDKN2A/p16*, and *MGMT* promoter methylation was present in 8/9 colorectal cancer patients showing the MSI phenotype.

CONCLUSION: Methylation analysis of *hMLH1*, *CDKN2A/p16*, and *MGMT* revealed specific methylation profiles for tubular adenomas, tubulovillous/villous adenomas, and colorectal cancers, supporting the use of these alterations in assessment of colorectal tumorigenesis.

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INTRODUCTION

Chromosomal instability and microsatellite instability (MSI) are two well-known pathways of colorectal carcinogenesis[1,2]. Recently, the CpG island methylation phenotype has been added to these two pathways. This novel pathway is characterized by the concordant methylation of the promoter regions of multiple genes that play a role in carcinogenesis[3], an alteration that has also been associated with the process of aging. CpG island DNA methylation, the most extensively studied epigenetic alteration in neoplasms, represses gene transcription by excessive and aberrant methylation of CpG-rich regions, called "CpG islands", in the 5' region of genes, leading to transcriptional silencing of the promoter and therefore inactivation of the gene[4,5].

Aberrant DNA methylation of CpG islands (CpG island methylator phenotype, CIMP) is also linked with subsequent low- or high-level DNA MSI[6-8]. Methylation of the human DNA mismatch repair gene mutator L homologue 1 (*hMLH1*) is the principal mechanism underlying the pathogenesis of sporadic high-level MSI (MSI-H) colorectal cancer (CRC)[6] and methylation of another DNA repair gene, O-6-methylguanine DNA methyltransferase (*MGMT*), is linked with low-level MSI (MSI-L)[7,8]. Moreover, epigenetic inactivation of the cyclin-dependent kinase inhibitor 2A (*CDKN2A/p16*) by methylation has been observed in both adenomas and CRC[9,10]. These hypermethylated genes are not only probable pathogenic events in the polyp to cancer progression sequence, but are also neoplasm-specific molecular events that have the potential to be used as molecular markers for pre-malignant tumors in the colon.

In this study, we used high-sensitivity methylation-specific polymerase chain reaction (PCR) (MSP) assays for *hMLH1*, *CDKN2A/p16*, and *MGMT*, three genes previously shown to be aberrantly methylated in pre-malignant neoplasms in the colon[6-10]. We have applied these assays to DNA extracted from blood, normal tissue, adenomas (tubular, villous, or tubulovillous), and colon cancer of patients who underwent first time colonoscopy at the University Hospital of Ioannina, Greece. We assessed the prevalence of aberrant DNA methylation and its correla-

tion to MSI status, as well as the temporal order and the time of their appearance during the different steps of adenoma to carcinoma progression, in an attempt to identify their possible use as molecular markers of colon carcinogenesis and furthermore their pathogenic role in the transformation of colon neoplastic adenomas to carcinoma.

MATERIALS AND METHODS

Study design
The study was designed to include patients undergoing first time colonoscopy for routine clinical indications, organized in four different groups, on account of their colonoscopic and histopathological data. Patients without colonoscopic findings and histologically normal mucosa consisted group Ⅰ (normal individuals - GⅠ). Patients with tubular adenomatous polyps comprised group Ⅱ (GⅡ). Patients with polyps of increased tumorigenic potential (tubulovillous or villous adenomatous polyps) formed group Ⅲ (GⅢ), and, finally, patients with colonoscopic findings and histological evidence of CRC comprised group Ⅳ (GⅣ).

Patients
Seventy nine patients (44 males and 35 females, mean age: 62.5 ± 13.9 years) who underwent colonoscopy for routine clinical indications or for colon cancer screening at the Hepatogastroenterology Unit of the University Hospital of Ioannina, were included in this study, after approval from the Review Board of the University Hospital of Ioannina. Each group consisted of approximately 20 consecutive patients defined by histopathological analysis. Thus, we obtained 18 patients with tubular adenomas (10 M/8 F), 21 patients with tubulovillous or villous adenomas (12 M/9 F), and 20 colon cancer cases (11 M/9 F). Finally, 20 adenoma-free patients matched by sex and age (11 M/9 F) formed the control group.

The study was performed on freshly obtained lesions from adenomas or cancerous tissue that were resected at the time of colonoscopy. Matched normal colorectal mucosa was obtained from the resection margin that was furthest from any malignant lesion. Blood samples withdrawn at the time of colonoscopy were also included in this study. Patients with a prior history of inflammatory bowel disease, genetic CRC syndromes, or any other cancers, were excluded from our study.

Collection of tissue and blood DNA samples
DNA from the blood, fresh normal, and abnormal tissue of patients who underwent colonoscopy was extracted using the QiaAmp DNA Mini Kit (Qiagen GmbH, Hilden, Germany).

Methylation-specific PCR
Methylation of the CpG promoter region of the three genes *hMLH1*, *CDKN2A/p16*, and *MGMT* was determined by MSP after bisulfite-modification of DNA samples (blood, cancerous, and non-cancerous tissue) as previously described[11,12]. These three genes have been

Table 1 Primer sequences, annealing temperature, and product sizes for methylation-specific polymerase chain reaction assays

CpG status	Genes	Forward primer (5'→3')	Reverse primer (5'→3')	Genomic position[2]	Annealing temperature (℃)	Product size (bp)
M	*hMLH1*	ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG	-716 to -602	55	115
U		TTTTGATGTAGATGTTTTATTAGGG TTGT	ACCACCTCATCATAACTACCCACA	-721 to -598	55	124
M	*MGMT*[1]	TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	+142 to +223	62	121
U		TTTGTGTTTTGATGTTTGTAGGTTTT TGT	AACTCCACACTCTTCCAAAAACAA AACA	+137 to +230	62	133
M	*CDKN2A/p16*	TTATTAGAGGGTGGGGCGGATCGC	CCACCTAAATCGACCTCCGACCG	+167 to +401	65	234
U		TTATTAGAGGGTGGGGTGGATTGT	CCACCTAAATCAACCTCCAACCA	+167 to +401	55	234

[1]All of the primers for the *MGMT* gene were modified with a 20 bp GC-rich tail (5'-GCGGTCCCAAAAGGGTCAGT-3') at their 5' end; [2]GenBank (PubMed). M: Methylated; U: Unmethylated; *hMLH1*: AB017806; *MGMT*: DD183709; *CDKN2A/p16*: DQ406745.

employed in several earlier methylation studies of CIMP and CRC. In brief, the procedure was as follows: The extracted DNA underwent bisulfite modification (2 μg DNA is necessary for each experiment) using the EZ DNA methylation Gold kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. The modified DNA was used immediately for MSP or stored at -20℃ for further analysis. The bisulfite-treated DNA was subject to MSP in a blinded manner using primer pairs designed to specifically amplify the methylated or unmethylated alleles for the respective genes (Table 1). Each PCR reaction mix consisted of a total volume of 50 μL containing: 3.5 mmol/L of MgCl$_2$ (2 mmol/L for the methylated reaction); 1 × PCR Gold Buffer (Applied Biosystems, Weiterstadt, Germany); 250 μmol/L deoxynucleotide triphosphates mixture (Promega, Madison, USA); 0.1 μmol/L of forward and reverse primers (Invitrogen GmbH, Karlsruhe, Germany); 2.5 Units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Weiterstadt, Germany), and the appropriate amount of bisulfite-treated DNA. The thermocycler conditions were in general as follows: 95℃ for 10 min; 55 cycles of 30 s each at 95℃, specific annealing temperature for 30 s, and 1 min at 72℃; and a final extension of 10 min at 72℃. The PCR products were then subjected to horizontal gel electrophoresis on a 25 g/L agarose gel, stained with ethidium bromide, and visualized under UV transillumination. All MSP assays were repeated at least twice to validate the results. A set of known methylated and unmethylated control DNA samples was included in each round of bisulfite treatment.

The specificities of the MSP assays were confirmed by sequence analysis of aberrant methylation bands using the Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, USA), in a LI-COR 4200 DNA sequencer (LI-COR Inc., USA), in parallel with the corresponding normal DNA samples, as described previously[12].

MSI testing
The MSI status of the 79 patients was assessed using the reference panel of five pairs of microsatellite primers: BAT25, BAT26, D2S123, D5S346, and D17S250, known as Bethesda markers[13]. PCR reactions and primer sequences have been described previously[14]. The 5'-la-

belled PCR products are loaded onto a 66 cm denaturing 6 mol/L urea-acrylamide gel and analyzed in a LI-COR 4200 DNA sequencer. Each PCR was run twice to ensure reproducibility of results in case the band shifts were not clearly informative in the first attempt. The MSI phenotype was identified by the presence of abnormal bands in the polyp/adenoma or carcinoma tissue DNA that were not present in blood or normal tissue DNA. MSI high (MSI-H) polyps/adenomas or carcinomas were defined as those where two of the five Bethesda markers were unstable. MSI low (MSI-L) samples were defined as a shift in only one of the five markers. Samples showing no allelic shifts were termed as MSI stable (MSS).

RESULTS

MSP for hMLH1, CDKN2A/p16 and MGMT
Table 2 shows the methylation patterns of the four distinct patient groups for the promoters of the genes examined. Promoter methylation was detected even in the G Ⅰ group (healthy individuals). In this case, only patients aged over sixty showed methylation of some promoters (with one exception for MGMT concerning a 28-year-old woman). This indicates that methylation in normal patients is correlated to the age. By contrast,, in the patients of the G Ⅱ, G Ⅲ, and G Ⅳ groups, the frequency of methylation is similar in older and younger patients (> 60 years and ≤ 60 years respectively, statistical data not shown), indicating that factors other than age are probably responsible for promoter methylation in these groups (Figure 1).

Among all samples investigated, promoter methylation of *hMLH1* or *CDKN2A/p16* was detected only in tissue samples and not in blood (indicating that methylation of these promoters is somewhat tissue/site specific). *MGMT* methylation was detected in tissue as well as in blood samples. The frequency of *MGMT* methylation was similar in blood and tissue samples only for the G Ⅰ patient group (46% in tissues *vs* 40% in blood, *P* = 0.704), as has been reported before and is also age related. However, in patient groups G Ⅱ, G Ⅲ, and G Ⅳ a significantly increased ratio in tissue samples *vs* blood samples (39% in tissues *vs* 6% in blood, *P* = 0.022; 76% *vs* 41%, *P* = 0.035 and 90% *vs* 64%, *P* = 0.043 for G Ⅱ, G Ⅲ and G Ⅳ, respectively) was observed. This implies that methylation of *MGMT* is

Table 2 Promoter methylation profiles for *hMLH1*, *CDKN2A/p16*, and *MGMT* in the four patient groups *n* (%)

Patient group	*hMLH1* (tissue)		*CDKN2A/p16* (tissue)		*MGMT* (tissue)		*MGMT* (blood)	
	M	U	M	U	M	U	M	U
G I (*n* = 20)	6/20 (30)	14/20 (70)	3/20 (15)	17/20 (85)	9/20 (46)	11/20 (54)	8/20 (40)	12/20 (60)
G II (*n* = 18)	7/18 (39)	11/18 (61)	4/18 (22)	14/18 (78)	7/18 (39)	11/18 (61)	1/18 (6)	17/18 (94)
	P = 0.563		*P* = 0.581		*P* = 0.666		*P* = 0.019[1]	
G III (*n* = 21)	10/21 (48)	11/21 (52)	14/21 (67)	7/21 (33)	16/21 (76)	5/21 (24)	7/17 (41)	10/17 (59)
	P = 0.576		*P* = 0.008[1]		*P* = 0.025[1]		*P* = 0.019[1]	
G IV (*n* = 20)	13/20 (65)	7/20 (35)	14/20 (70)	6/20 (30)	18/20 (90)	2/20 (10)	11/18 (64)	7/18 (36)
	P = 0.279		*P* = 0.837		*P* = 0.242		*P* = 0.245	

[1]Statistically significant in comparison to the previous group. M: Methylated; U: Unmethylated.
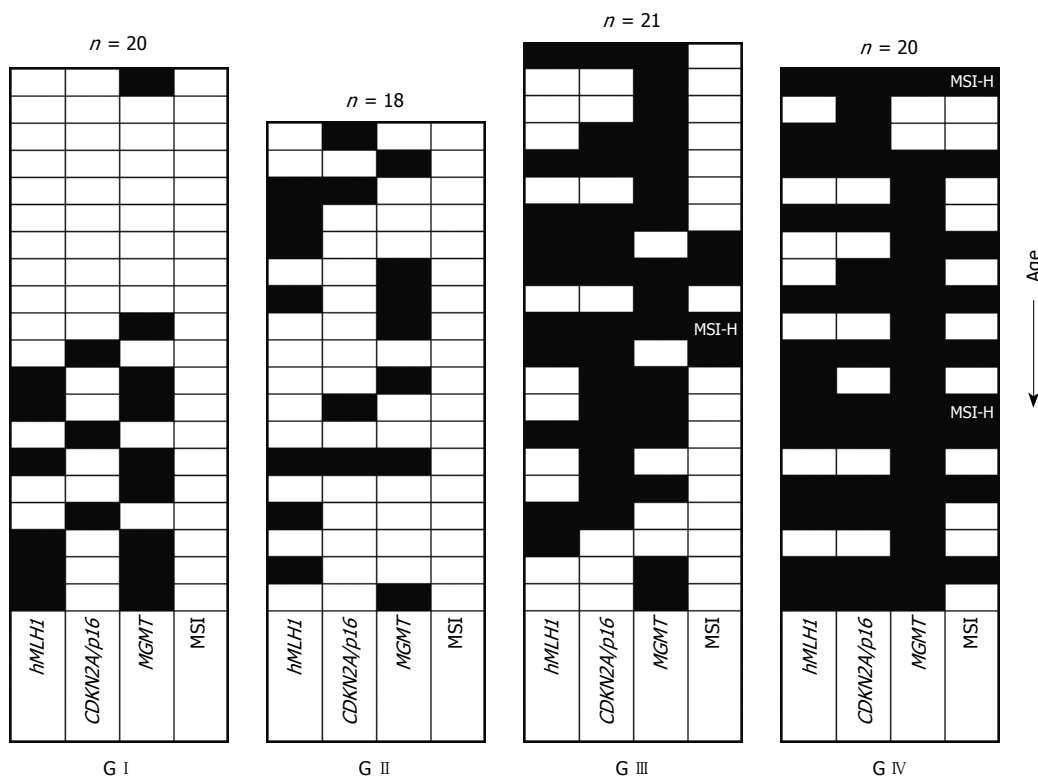

Figure 1 CpG island methylation at three loci and the microsatellite instability phenotype in G I (*n* = 20), G II (*n* = 18), G III (*n* = 21) and G IV (*n* = 20). The rows represent individual cases and the dark boxes indicate methylation or microsatellite instability (MSI) phenotype; the three high-level MSI (MSI-H) cases are also indicated.

also in part tissue/site specific. Another explanation for this could also be a low incidence of circulating cells having methylated promoters. In any case, this will require further investigation. The frequency of promoter methylation for each locus, as well as the number of methylated loci, increased from group G I to G IV (Table 2 and Figure 1), with *MGMT* showing a higher ratio. All of the methylated tissue samples also displayed evidence of unmethylated *hMLH1*, *CDKN2A/p16*, and *MGMT*, indicating that only one of the alleles is methylated or that only a part of the tissue contained cells that carried the methylated allele of the examined promoters (Figure 2).

It must be noted that *hMLH1* showed similar levels of promoter methylation between groups G II and G III (39% *vs* 48%, *P* = 0.576) in contrast to *CDKN2A/p16* and *MGMT*, which showed clearly increased levels of methylation in group G III (22% *vs* 67%, *P* = 0.008, and

39% *vs* 76%, *P* = 0.025, respectively), suggesting that *hMLH1* promoter methylation is an early phenomenon in comparison to polyp formation, while methylation of *CDKN2A/p16* and *MGMT* is correlated to the progression of polyps to more tumorigenic cases.

CDKN2A/p16 methylation was usually accompanied by methylation of another locus in tumorous and highly tumorigenic tissues (13/14 for G III and G IV), in contrast to the normal and low tumorigenic tissues (0/3 for GI and 2/4 for G II respectively). *MGMT* follows a rather different pattern, being in many cases the only methylated locus (5/7, 6/16, and 5/18 for G II, G III and G IV, respectively). The pattern for *hMLH1* is even more complicated (Figure 1).

Of the 18 tubular adenoma patients examined 14 (77%) showed methylation in at least one of the three tested loci, while four (22%) showed no evidence of pro-
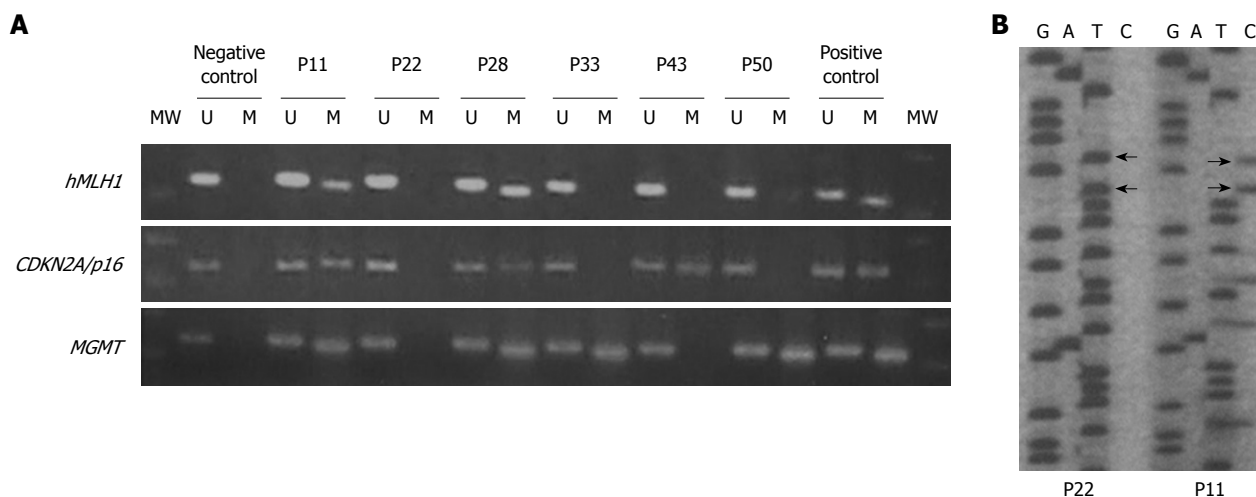
Figure 2 Methylation-specific polymerase chain reaction and sequence analysis. A: Methylation specific polymerase chain reaction (PCR) for *hMLH1*, *CDKN2A/p16* and *MGMT* promoter methylation. In this panel, the MSI-H patients P11 and P28 show promoter hypermethylation for *hMLH1*, *CDKN2A/p16*, and *MGMT*. The low-level MSI (MSI-L) patient P33 shows promoter hypermethylation for *MGMT*. The MSI stable (MSS) patient P22 does not show promoter hypermethylation for any gene, whereas the MSS patient P43 shows promoter hypermethylation only for *CDKN2A/p16*.The healthy MSS patient P50 shows promoter hypermethylation only for *MGMT*; B: Sequence analysis of methylation-specific PCR (MSP) PCR products of *hMLH1* promoter. The sequence analysis of MSP products for patients P22 (unmethylated) and P11 (methylated) reveals the complete transition of non-methylated cytosines to thymine (indicated by arrows) after bisulfite treatment. There also cytosines that are partially methylated, as indicated from the co-existence of T and C. The sequence from the bottom to top: 5'-TGG tGT TTG AtG TtG TGT TtG tGG GTA GT-3' P22 (unmethylated); 5'-TGG t(C)GT TTG At(C)G Tt(C)G TGT TCG CGG GTA GT-3' P11 (methylated); 5'-TGG CGT TTG ACG TCG TGT TCG CGG GTA GT-3' (wild type). Lower case letters represent thymines derived from unmethylated cytosines, the letters in parenthesis represent partially methylated cytosines and the bolds the CpG islands. MW: Molecular weight; M: Methylated promoter; U: Unmethylated promoter.

moter methylation, and only one (6%) showed methylation in all loci (Figure 1). All 21 G Ⅲ, as well as the 20 G Ⅳ patients (100%), showed promoter methylation in at least one locus. In particular, 6/21 (28%) and 11/20 (55%) for group G Ⅲ and G Ⅳ, respectively, were methylated in all loci, suggesting a potentially crucial role of methylation in the progression of CRC.

MSI status and hMLH1, CDKN2A/p16, and MGMT promoter methylation

According to our introductory remarks concerning the linkage between methylation status and MSI phenotype, we analysed the MSI status using the five Bethesda markers recommended by the NCI workshop[13]. Of the 79 samples examined, 66 (83%) were MSS, 10 (13%) were MSI-L and three (4%) were MSI-H (Figure 1). Only the G Ⅲ and G Ⅳ patients showed MSI, which reached 14% (3/21) for MSI-L and 5% (1/21) for MSI-H for the patient group G Ⅲ, whereas the corresponding percentages were 35% (7/20) and 10% (2/20) for MSI-L and MSI-H, respectively, in the G Ⅳ patient group.

Of the five Bethesda markers used for MSI analysis, BAT25 and BAT26 showed instability only in the MSI-H patients. On the other hand, the MSI-L phenotype was exclusively restricted to dinucleotide markers D2S123, D5S346, and D17S250 (3, 4 and 3 cases respectively). In the 13 cases with MSI-H or MSI-L, the following positive rates were obtained: 23% (3/13) in BAT26, 23% (3/13) in BAT25, 46% (6/13) in D2S123, 54% (7/13) in D5S346, and 46% (6/13) in D17S250, which is in accordance with previous references for cancer patients[15], as well as for patients with adenomatous polyps[16,17]. In a larger cohort of MSI-H cancer patients, Arnold *et al*[18] showed that the

specificity of Bethesda markers was best for BAT26 and BAT25, with 99% and 95%, respectively. It is important to note that simultaneous *hMLH1*, *CDKN2A/p16*, and *MGMT* promoter methylation was present in 8/9 G Ⅳ patients, with the MSI phenotype as also being present in 2/4 patients of group G Ⅲ. All MSI-H patients showed methylation in all loci. From the remaining three patients, the two in group G Ⅲ showed methylation in two promoters (*hMLH1* and *CDKN2A/p16*) and the one G Ⅳ patient only in the *MGMT* promoter. The latter case also showed instability for the D2S123 marker. In the previous work of Arnold *et al*[18], all Bethesda markers, except the dinucleotide repeat D2S123, had a high detection rate (up to 90%) for the combination of MSI-H cancers and *hMLH1* methylation. This suggests that MSI-H cancers might originate from different pathways, e.g. one being caused by silencing the *hMLH1* gene and others by as yet unrecognized mechanisms.

DISCUSSION

The transformation of normal colon epithelial cells to adenomas, and then to cancer, is believed to be an evolutionary process in which neoplastic cells acquire heritable genetic and epigenetic alterations that drive the process of carcinogenesis[19]. Gene promoter hypermethylation is increasingly recognized to play an important role in cancer development through silencing gene transcription. It is also likely that the genetic and epigenetic alterations co-operate to promote tumor formation, and that the detection of colon polyps or adenomas that present aberrant promoter methylation might identify colonic epithelium that is at significant risk of acquiring genetic alterations

that will lead to colon tumor formation[20].

A number of studies have investigated the concordant methylation of multiple genes in colon adenoma[10,21,22]. Recently, aberrant promoter methylation found in different polyp forms[23,24] serves for a better understanding of aberrant CpG island methylation in the polyp/adenoma/carcinoma sequence of colorectal tumorigenesis. Thus, CIMP has been identified during several key stages in colon tumorigenesis, including aberrant crypt foci (ACF, the earliest identifiable neoplastic lesions in the colon), hyperplastic, tubular and tubulovillous/villous polyps, sporadic serrated adenomas, and tumors[3,10,22,25,26], suggesting that DNA methylation might be a pivotal event in the development of CRC. Furthermore, the aberrant methylation of *MGMT* and *hMLH1* has been shown to silence some genes and to result in cancer promoting events, such as an MSI phenotype or *k-RAS* mutation[27,28].

The molecular genetics of colorectal neoplasms have been studied extensively, but few studies have addressed the clinical and pathological associations in prospectively defined patient populations. Therefore, we studied specific promoter-methylation in a cohort that underwent colonoscopy including healthy individuals, polyp-bearing patients (more or less tumorigenic), as well as CRC patients.

Although we employed the MSP assay to detect methylated *hMLH1*, *CDKN2A/p16*, and *MGMT* DNA in the blood samples of patients of our cohort, we were able to detect methylated DNA in blood samples only for the *MGMT* promoter in all patient groups, including those with CRC. We note that Grady *et al*[11] used MSP in a study of 20 patients, detected methylated *hMLH1* DNA in the serum in 30% of patients with sporadic MSI colon cancer. This difference with our results might be attributed to the small number of MSI cancer patients of our cohort or to a different methylation site on the promoter.

We also observed increasing frequencies of promoter methylation as well as an increased number of methylated loci moving from group G Ⅰ to group G Ⅳ, for the three genes examined, which is in accordance with the data of Lee *et al*[29]. They also observed a stepwise increase in the number and frequency of methylated genes through the stages of multistep colorectal carcinogenesis in a study including twelve loci.

Adenomas with a villous component are generally larger than tubular adenomas and have been associated with a higher risk of CRC[30-32]. A number of studies have reported higher rates of methylation in adenomas with tubulovillous or villous histology (TVAs and VAs)[10,23,24,33,34] as compared to tubular adenomas (TA). Our study also demonstrated that methylation occurs more frequently in patients with TVAs/VAs (G Ⅲ) in comparison to patients with TAs for *MGMT* and *CDKN2A/p16* (statistically significant). By contrast, for *hMLH1,* the two groups showed a similar ratio. An increase in *MGMT* methylation has been demonstrated by other studies: 38% to 61% by Petko *et al*[23], 30% to 65% by Kim *et al*[33], and 37% to 87% by Kakar *et al*[24]. The higher rates of *MGMT* meth-

ylation in our study in comparison to previous ones could be attributed in part to the use of fresh tissue samples instead of paraffin-embedded tissue samples. Our results also showed an increased frequency of CDKN2A/p16 comparable with that of Petko *et al*[23], who found a ratio of 10% in the methylation of *CDKN2A/p16* for HPs and up to 30% for the adenomatous cases.

Furthermore, in our study, methylation, at least in one locus, reached 100% for TVAs/VAs and cancer patients, which is a higher value than that shown by Rashid *et al*[10].

It is likely that several different methylation pathways operate in CRC progression. Methylation of *MGMT* and *CDKN2A/p16* loci that are found to increase from TAs to TVA/VA could be a result of the adenoma to carcinoma progression, whereas methylation of *hMLH1* might be an initial step strongly associated with MSI-mediated carcinogenesis. The higher frequency of methylation in all loci for cancer patients compared to TVAs/VAs patients, as well as the increase in methylation from TAs to TVAs/VAs for *MGMT* and *CDKN2A/p16* supports this hypothesis.

The fact that MSI is evident in TVAs/VAs and that MSI and aberrant promoter methylation are observed simultaneously, suggests that MSI and hypermethylation are dependent on each other. As it has been shown before, that among 10% to 15% of the patients with colon cancer who have MSI, approximately 70% to 80% exhibit epigenetic gene silencing of the mismatch repair gene, *hMLH1*[6,27,35]. Moreover, a minor fraction of MSI-L and MSS cancers also appear to be methylated at the *hMLHl* promoter[36]. In our study, all MSI-H patients and 9/10 MSI-L patients showed simultaneous promoter methylation of *hMLH1,* while the remaining one MSI-L patient showed methylation of *MGMT*. However, not all MSI-L patients show inactivation of *MGMT* by promoter methylation, as we demonstrated for two of the G Ⅲ patients with the MSI-L phenotype (Figure 2).

The present study used MSP for detecting methylated alleles. MSP is a qualitative assay and does not provide quantitative information. Thus, the methylation detected by the MSP assay might not reflect gene expression, because the assay can detect only one methylated allele among 1000 unmethylated ones, and thus the vast majority of tumor cells may not harbor CpG island methylation of the given gene. The present study also shows that the methylated alleles of certain genes are present at an early stage of tumorigenesis, and that the number of genes with methylated alleles increases along the polyp/adenoma/carcinoma sequence, with *MGMT* being the most methylated gene.

In conclusion, our data indicates that aberrant CpG island hypermethylation occurs early and accumulates during multistep colorectal carcinogenesis, and that a temporal order exists in the methylation of tumor related genes. Furthermore, MSI is tightly connected to *hMLH1* as well as to *MGMT* promoter methylation, indicating that inactivation and/or overloading of the mismatch repair system might have a crucial role in driving CRC progression.

COMMENTS

Background

Colorectal carcinogenesis is a multistep process in which the progressive accumulation of genetic and epigenetic changes leads to a malignant transformation of normal epithelial cells to adenoma and, moreover, to cancer of the colon. CpG island DNA methylation, the most extensively studied epigenetic alteration in neoplasms, is characterized by the concordant methylation of the promoter region of many tumor suppressor and DNA repair genes, such as *hMLH1*, *CDKN2A/p16*, and *MGMT*, although their influence on disease progression remains inconclusive.

Research frontiers

Epigenetic changes usually begin very early in carcinogenesis, they are potentially reversible, and they can be thought of as one hit of the two-hits required for inactivation of carcinogenesis-related genes. For this reason, detection of aberrant methylation is important for early diagnosis, prognosis, and subsequent treatment of patients affected by this disease.

Innovations and breakthroughs

The frequency of promoter methylation for each promoter locus increases in the healthy tissue/adenoma/carcinoma sequence. *MGMT* shows the highest frequency in each group. *MGMT* and *CDKN2A/p16* present a statistically significant increase in promoter methylation between the less and more tumorigenic form of colorectal adenomas (tubular *vs* tubullovillous and villous adenomas). All tubulovillous/villous adenomas bearing patients, as well as all colorectal cancer patients, showed promoter methylation in at least one of the examined loci. These findings suggest a potentially crucial role for methylation in the polyp/adenoma to cancer progression in colorectal carcinogenesis. Microsatellite instability (MSI) and methylation seem to be dependent on each other, as simultaneous *hMLH1*, *CDKN2A/p16*, and *MGMT* promoter methylation was present in 8/9 colorectal cancer patients showing the MSI phenotype.

Applications

The results presented here show that a series of genetic and epigenetic molecular changes drives the next step during tumorigenesis in colorectal cancers, which results in a different molecular profile for each step. This underlines the need for a detailed record of the molecular situation, in order to establish the most efficient treatment for the patient. Prospective studies supplemented by the conventional study of prognostic factors, could improve the quality and accuracy of patients' prognosis and aid design of more efficient treatments.

Terminology

Epigenetic changes: Heritable changes in gene structure that do not include the changes in DNA sequence. CpG islands: CpG rich areas located in the promoter regions of many genes. CpG island methylation: The addition of a methyl group to a cytosine residue that lies next to guanine within CpG dinucleotides. Aberrant *de novo* methylation of CpG islands within the promoter region might lead to silencing of gene transcription through a complex process involving chromatin condensation and histone deacetylation. MSI is a change in the length of DNA microsatellites due to the insertion or deletion of repeating units (usually 1-5 nucleotides long), caused by defects in mismatch repair genes (*MLH1*, *MSH2*, or *MSH6*, and others) or methylation of the *MLH1* promoter.

Peer review

In this study, Psofaki *et al* investigated promoter methylation of *hMLH1*, *p16* and *MGMT*, as well as MSI, in a cohort of samples from patients underwent colonoscopy. They found accumulation of promoter methylation events during colorectal tumor progression. Promoter methylation and MSI seem to be dependent on each other. These results confirm that epigenetic inactivation is an important mechanism of tumor suppressor gene silencing. The study was well designed and the results are interesting. The manuscript is well written and includes potentially interesting findings.

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