SPANDIDOS PUBLICATIONS

O^6-methylguanine-DNA methyltransferase promoter hypermethylation in colorectal carcinogenesis

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Received August 16, 2006; Accepted September 28, 2006

Abstract. Epigenetic alterations have been reported in colorectal neoplasia which can either complement or in some cases be predisposed to genetic alterations such as K-*ras* mutations. We examined the promoter methylation status of the *CDKN2A* and O^6-methylguanine-DNA methyltransferase (*MGMT*) genes, after sodium bisulfite conversion and DNA amplification with methylation specific PCR. Moreover, we searched for G to A transitions in codons 12 and 13 of the K-*ras* oncogene in normal colorectal mucosae, aberrant crypt foci (ACF, early premalignant lesions) and carcinomas. *CDKN2A* hypermethylation was an infrequent event in ACF (2 of 26, 7.7%). On the contrary, *MGMT* hypermethylation was found in the normal mucosae (3 of the 12 samples, 25%), in 14 of the 26 ACF (53.8%) and in 7 of the 9 (77.8%) carcinomas examined. K-*ras* mutations were evident in 6 ACF (23%) and in 3 carcinomas (33.3%), mostly associated with *MGMT* promoter hypermethylation. These findings strongly support the hypothesis that epigenetic mechanisms play an important role in the early steps of colorectal carcinogenesis.

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Abbreviations: ACF, aberrant crypt focus/foci; *MGMT*, O^6-methylguanine-DNA methyltransferase; PCR, polymerase chain reaction; MSP, methylation-specific PCR; MASA, mutant allele-specific amplification

Key words: aberrant crypt foci, O^6-methylguanine-DNA methyltransferase promoter hypermethylation, colorectal cancer, colorectal carcinogenesis, K-*ras* mutation

Introduction

Aberrant crypt foci (ACF) are the earliest morphological step in the development of colorectal cancer (1,2). A large body of evidence has been produced in support of this contention, including the presence of some genetic alterations in ACF occurring at the later stages of colorectal carcinogenesis (3), even in hereditary cases (4). Most of these alterations are mutations or chromosomal deletions affecting tumor suppressor genes or genes controlling DNA mismatch-repair, causing the loss of function of these genes. Epigenetic mechanisms have also been reported in colorectal carcinogenesis. In particular, transcriptional silencing through the hypermethylation of the CpG islands in the promoter regions of several genes has been demonstrated in colorectal cancer (5), adenomas (6), and even in human ACF (7,8). Furthermore, the hypermethylation of the CpG islands in the promoter region of *hMLH1* underlies most sporadic colorectal cancers with microsatellite instability, and it has also been found in normal mucosae as an age-related phenomenon (9). K-*ras* activating mutations, mainly at codon 12, occur frequently in colorectal cancer and in other tumors, and in some instances, they correlate with the silencing of O^6-methylguanine-DNA methyltransferase (*MGMT*) through promoter hypermethylation (10,11). K-*ras* mutations have been found also at the stage of ACF (12). It is noteworthy that the transgenic overexpression of human *MGMT* inhibits the development of azoxymethane-induced ACF and the occurrence of specific K-*ras* mutations in mice (13), suggesting an important role for this pathway in colorectal tumorigenesis. *CDKN2A* (*p16*) is a tumor suppressor gene, whose inactivation leads to the deregulation of cell proliferation and to genomic instability. In some cases it has been shown to be silenced by promoter hypermethylation even at the early stages of tumor progression (14). Thus, we evaluated whether, and to what extent, *p16* and *MGMT* were silenced by promoter hypermethylation in human ACF using a real-time PCR technique, and whether such events had functional consequences on the K-*ras* oncogene.

Table I. Features of patients and ACF examined.

Patient no.	Sex/age	Disease	ACF no.	Site[a]	ACF multiplicity[b]	Shape of crypt openings in ACF[c]
1	F 78	Cancer	1	Left colon	150	S
2	M 75	Sigmoiditis	2	Left colon	250	S
3	F 79	Cancer	3	Left colon	150	R
			4		40	R
			5		130	R
4	M 75	Cancer	6	Rectum	50	R
			7		40	R
			8		40	R
			9		80	R
5	F 48	Cancer	10	Rectum	10	S
6	M 83	Cancer	11	Right colon	50	S
7	M 72	Diverticulitis	12	Left colon	70	E
			13		40	R
			14		120	S
			15		50	R
8	M 69	Adenoma	16	Right colon	70	R
9	M 55	Cancer	17	Left colon	70	R
			18		80	R
10	M 45	Cancer	19	Rectum	100	E
11	M 53	Cancer	20	Rectum	120	S
			21		70	S
			22		100	S
			23		130	S
			24		90	S
12	M 94	Cancer	25	Right colon	100	E
			26		400	R

[a]The site of colorectal mucosa examined in search for ACF. Right colon = cecum, ascending colon, hepatic flexure, transverse colon, splenic flexure; left colon = descending colon and sigmoid colon; rectum = rectosigmoid junction and rectum. [b]Number of crypts per ACF. [c]S, serrated; R, round; E, elongated (15).

Materials and methods

Sample collection. We collected specimens of colorectal mucosa from 12 patients after surgery for colorectal cancer or benign diseases of the large bowel (Table I). Nine patients had colorectal carcinoma at the time of surgery. The remaining three patients had no cancer in the resected specimen. Two of them (nos. 7 and 8) however, had an adenoma, one of whose had been removed 3 months prior to surgery, and the other one at the time of surgery. Informed consent for the use of the colonic samples was obtained from the patients. The protocol of the study was reviewed by the local ethics committee. The mucosal surface was examined under a dissecting microscope after staining with methylene-blue, as previously described (15). When ACF were identified, the number of crypts and the prevalent shape of the crypt luminal openings in ACF were recorded, and then dissected, avoiding as many normal surrounding crypts as possible, and stored at -80˚C until the analysis. The number of crypts in ACF has been referred to as crypt multiplicity, which is considered an estimate of the size of ACF. The prevalent shape of the crypt openings in ACF was divided into 3 main features: round (R),

 S), and elongated (E). These 3 shapes are strictly the histology of ACF (15). In particular, the round or serrated luminal openings identify nondysplastic ACF, whereas the elongated ones define dysplastic ACF. For each patient at least 1 sample of normal mucosa was also taken. A total of 26 ACF were available for the analysis, as shown in Table I. For all the patients with cancer, carcinoma samples were also taken at surgery. Also available, were adenoma and hyperplastic polyp samples which were taken from two patients and one patient, respectively.

Cell lines. The colon cancer cell lines, CO115, GP5-D, HCT116 and LS174T, were kindly provided by The Zurich Cancer Network.

Nucleic acid isolation. Genomic DNA was extracted with the QUIamp DNA mini kit (Qiagen) according to the manufacturer's instructions.

Sodium bisulfite conversion. Sodium bisulfite conversion of the genomic DNA was performed as previously described (16), with minor modifications. Briefly, DNA (2 μg) was diluted in 50 μl distilled water and then denaturated by a final concentration of 0.3 M NaOH for 20 min at 37˚C. Thirty microliters 10 mM hydroquinone (Sigma) and 520 μl 3.46M sodium bisulfite (Sigma) at pH 5.0 were then added, and the samples were incubated at 50˚C for 16-18 h. Subsequently, the DNA was purified using the Wizard DNA Clean-Up system (Promega), following the manufacturer's protocol. The DNA was then desulfonated with 0.3 M NaOH for 20 min at 37˚C, neutralized with 18 μl 10 M ammonium acetate, precipitated with ethanol and finally resuspended in 20 μl water.

Methylation-specific PCR (MSP). The modified DNA was subjected to methylation-specific PCR (MSP) with allele-specific primers complementary to the modified or unmodified sequences of the gene promoters studied. For *CDKN2A* (*p16*) the following primers were designed: *p16* methylation-specific, 5' GTTAACGTTGGTTTTGGCGA 3' (sense) and 5' ACGCTATAAACGAATACTCGAA 3' (antisense); and *p16* nonmethylation-specific, 5' TGTTAATGTT GGTTTTGGTGAG 3'(sense) and 5' CACAATATCTTT CCAAACAAAAAAACA 3' (antisense) (amplicon location from -323 to -167 for the methylated reaction and from -324 to -143 for the unmethylated from the ATG transcription start codon; GenBank accession no. X94154). For *MGMT* promoter analysis the following primers were used: the methylation-specific 5' AGGCGTCGTTCGGTTTGTATCG 3' (sense) and 5' CGCCGAACCTAAAACAATCTACG 3' (antisense); and the unmethylation-specific 5' GGTGGAAGTTGG GAAGGTGTTG 3' (sense) and 5' CAACACATACCCA ATACAACAACACCA 3' (antisense) with products located from 703 to 831 for the methylated reaction and from 689 to 853 for the unmethylated in the *MGMT* promoter sequence (GenBank accession no. X61657).

Real-time PCR was performed by the Roche LightCycler system using the QuantiTect SYBR-Green kit (Qiagen) according to the manufacturer's instructions (0.5 μM of each primer and 2 μl modified DNA in a 20 μl final reaction volume). Real-time cycler conditions were as follows: after an initial activation step (95˚C for 15 min), 55 cycles (94˚C for 15 sec; 60˚C for the methylated reactions and 58˚C for the unmethylated, for 20 sec; 72˚C for 10 sec) followed by a melting curve analysis step. The specificity of the reactions was confirmed by using positive controls for the methylated reactions for both the gene promoters examined; we used the fully-methylated DNA in the *MGMT* promoter from the CO115 colorectal cancer cell line and the hemimethylated DNA in the *CDKN2A* promoter from the HCT116 colorectal cancer cell line (17). DNA from normal lymphocytes was used as a negative control.

MGMT gene promoter bisulfite sequencing. Bisulfite sequencing was performed in several ACF and in the CO115 and GP5D colorectal cancer cell lines. A region from 666 to 1127 in the *MGMT* promoter sequence (GenBank accession no. X61657), which contains 63 CpG sites, was amplified from the modified DNA by PCR using HotStarTaqDNA polymerase (Qiagen) with the following primers: GGGAA TTCGTAAATTAAGGTATAGAGTTTTAGG (sense) and TGTGGATCCCAAACACTCACCAAATC (antisense). The PCR conditions were as follows: one cycle of denaturing at 95˚C for 15 min, followed by 35 cycles at 94˚C for 30 sec, 51˚C for 30 sec, and 72˚C for 1 min before a final extension at 72˚C for 10 min. The PCR products were cloned into the pGEM-T7 Vector (Promega) and the clones were sequenced by the Applied Biosystems PRISM dye terminator cycle sequencing method.

Detection of G to A transitions in K-ras codons 12 and 13. Mutant allele-specific amplification (MASA) was performed by real-time PCR in 20 μl reaction volumes using the QuantiTect SYBR-Green kit (Qiagen), 50 ng template and 0.5 μM of each allele-specific primer, whose sequences were previously described (11). The PCR conditions were the same as those used for the methylation analysis except for the annealing temperatures (62˚C for the detection of G to A transitions in the first base of codon 12, 63˚C for the second base of codon 12 and the first base of codon 13, and 64˚C for the second base in codon 13) for a total of 50 cycles of amplification. The positive controls for the transitions in the second base of codons 12 and 13 were DNA from the LS174T and HCT116 colorectal cancer cell lines, respectively. DNA from normal lymphocytes was used as a negative control.

Results

In order to be able to detect even low levels of genetic and epigenetic alterations in normal mucosae and in ACF, we chose an allele-specific amplification approach, which by the use of the real-time PCR technology, could guarantee high sensitivity and specificity (Fig. 1). Moreover, the positivity of a sample was validated by melting curve analyses including positive controls and the right lengths of the amplicons were examined by agarose gel electrophoresis (Fig. 2). In addition, *MGMT* promoter bisulfite sequencing was performed in several ACF and in the control colorectal cell lines (Fig. 3). The results of the methylation analysis of *CDKN2A* (*p16*) and the *MGMT* gene promoters and the detection of G to A

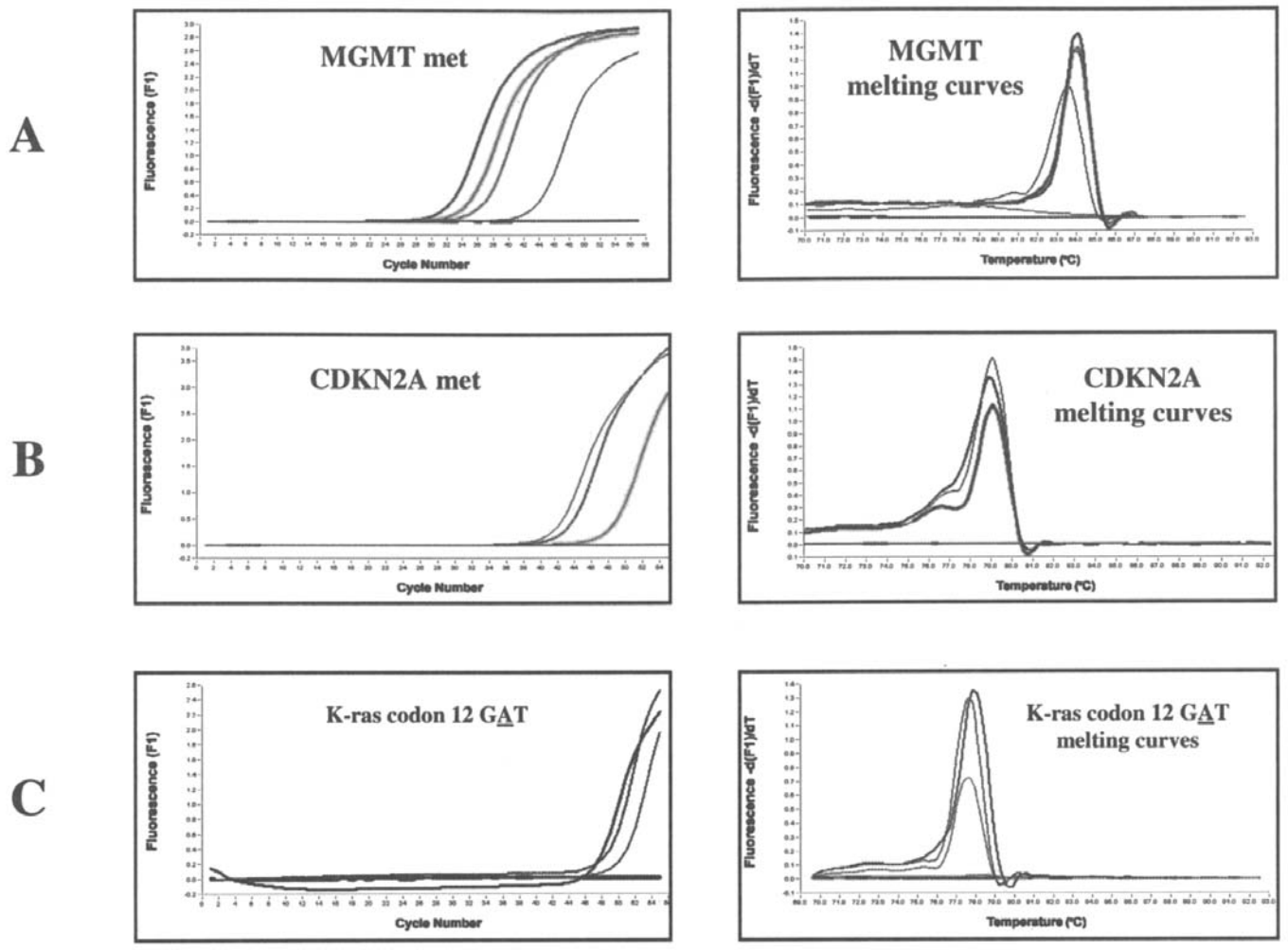

Figure 1. Amplification profile (on the left) and melting curve analysis (on the right) of allele-specific amplifications performed by real-time PCR. The positivity of the samples and controls is shown by the increasing of the fluorescence signals, while the negative samples and controls remain at the baseline. (Panel A) *MGMT* promoter amplification with methylated allele-specific primers. (Panel B) *CDKN2A* (*p16*) promoter methylated products. (Panel C) MASA of samples with G to A transitions in codon 12 (second base) of the K-*ras* oncogene.

transitions in codons 12 and 13 of the K-*ras* oncogene are shown in Table II. *CDKN2A* hypermethylation was observed in only 2 ACF (7.7%), in 3 of the 9 carcinomas (33.3%), and in none of the normal mucosa and polyp samples. On the contrary, the *MGMT* promoter was methylated in more than half of the ACF analyzed (14 of 26, 53.8%) and in 3 of the 7 patients with ACF methylated in *MGMT* this epigenetic change was also present in the normal mucosae. As far as K-*ras* mutational analysis is concerned, G to A transitions were found in 6 ACF (23%): one in the first base of codon 12, 4 in the second base of codon 12, and one in the second base of codon 13. Five of these ACF also showed *MGMT* promoter hypermethylation (Table II). No mutation was found in the first base of codon 13 in ACF. *MGMT* promoter hypermethylation was also found in 7 of the 9 carcinomas examined (77.8%), and in two of them a G to A transition in the second base of codon 12 was also present. One carcinoma (patient no. 12) showed a K-*ras* mutation but no *MGMT* promoter methylation. Finally, the two adenomas examined showed a K-*ras* mutation in the second base of codon 13, but only one of them had concomitant *MGMT* promoter hypermethylation.

Discussion

The data presented are consistent with *MGMT* promoter hypermethylation being an early event in colorectal carcinogenesis. *MGMT* protects cells from the mutagenic and cytotoxic effects of environmental alkylating carcinogens by transferring the mutagenic methyl group from the O^6-position of guanine to a cysteine residue at its active site (18). If this alkyl adduct cannot be removed as a consequence of *MGMT* inactivity, O^6-methylguanine will mispair with thymine during replication, resulting in a G to A transition. Consequently, we searched for mutations of this kind in codons 12 and 13 of the K-*ras* oncogene. In our study, the specificity of *MGMT* promoter hypermethylation detection was obtained using allele-specific primers in a region previously shown to be heavily methylated and associated with local heterochromatinization of the transcription start site in the *MGMT*-silenced cells (19). The percentage of ACF with *MGMT* promoter hypermethylation was much higher (53.8%) than that reported by Chan *et al* (12%) who first described the changes in methylation in the promoter region of some genes in ACF (7), while similar percentages were

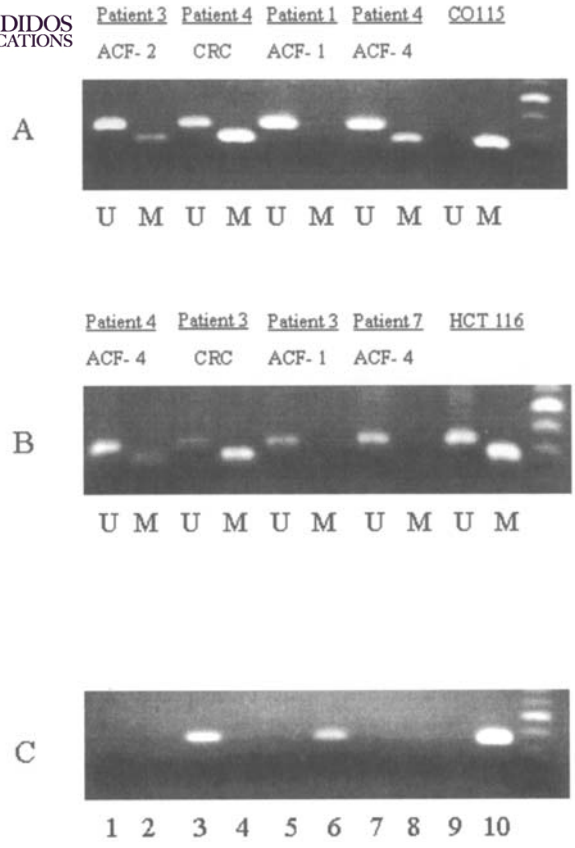

Figure 2. (Panel A) Examples of *MGMT* promoter methylation analysis in ACF and colorectal cancers (CRC). The presence of a visible PCR product in the lanes U, indicates the presence of unmethylated alleles in the sample, while a product in lanes M indicates the presence of *MGMT* promoter methylated alleles. The colorectal cancer cell line CO115, fully-methylated in the *MGMT* promoter, was used as the positive control fo the reactions (product only in lane M). (Panel B) *CDKN2A* (*p16*) promoter methylation analysis. In this case the hemimethylated DNA in the *CDKN2A* promoter from the HCT116 colorectal cancer cell line was used as the control (visible PCR products in lanes U and M). (Panel C) Detection of G to A transitions in codon 12 (second base) of the K-*ras* oncogene by MASA. Visible products in lanes 3 (patient no. 4, CRC) and 6 (patient no. 3, ACF-1) indicate the presence of the GAT mutation in these samples, as confirmed by the amplification of the positive control in lane 10 (the LS174T colorectal cancer cell line).

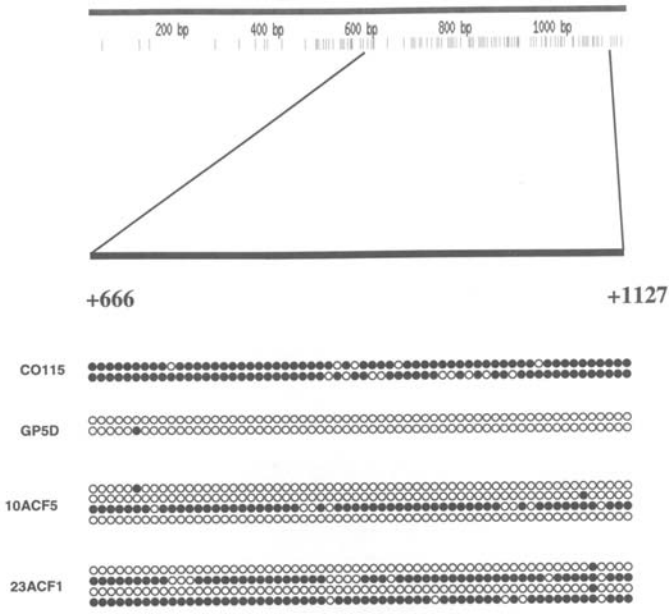

Figure 3. Bisulfite genomic sequencing of the *MGMT* gene promoter. Top, a schematic depiction of the *MGMT* promoter with CpG dinucleotides (short vertical lines). The methylation status of the individually cloned DNA fragments of two colorectal cell lines and two ACF is shown. Each row represents one sequenced allele. Each circle represents a CpG dinucleotide. Filled circle, methylation; open circle, no methylation.

found for *CDKN2A* (*p16*) promoter hypermethylation (7.7% vs 4%). This discrepancy could be due to the older median age of the patients in our study (68.8 vs 54.5 years), and also to the absence of patients with familial adenomatous polyposis, who were included in Chan's study (12). In the present study, the mean age of the patients with hypermethylated *MGMT* in the normal mucosa and/or in at least one ACF was ten years older than that of the patients with no *MGMT* methylation. Thus, *MGMT* promoter hypermethylation is an age-related phenomenon, as it has also been shown for the *hMLH1* promoter (9), and for other gene promoters (20), even in normal mucosae. A recent study supports this evidence showing the correlation of the *MGMT* methylation level with age in the normal colonic mucosae of patients with cancers silenced in this gene (21). The authors of this study suggested a field effect molecularly defined by the epigenetic inactivation of *MGMT* from which some sporadic colorectal cancers may arise. We show that ACF may have not only molecular but also morphological features to satisfy the definition of a field effect in carcinogenesis (22). In our study *MGMT* promoter hypermethylation was not dependent on ACF size (Table I, crypt multiplicity) or histology (Table I, shape of crypt openings in ACF) (15). In fact, even a 10-crypt ACF (Table I, ACF no. 10), and a nondysplastic ACF (serrated and round luminal openings) were *MGMT*-hypermethylated. *MGMT* hypermethylation was also demonstrated in ACF from patients with benign diseases of the large intestine (Table II, patients no. 2, 7 and 8).

We propose a possible 'field effect' due to this epigenetic alteration, which could be found in the same patient not only in cancer and in ACF but, in some cases, even in the normal colonic mucosa. In this context, *MGMT*-silenced cells are predisposed, in the presence of environmental alkylating carcinogens, to acquire somatic mutations (G to A transitions) that can affect not only K-*ras* but also *p53* (23), and potentially many other genes whose gain or loss of function may be responsible for the transition from normal mucosa, through ACF, to adenoma and carcinoma. In this study, K-*ras* mutations were found almost exclusively in the lesions with *MGMT* promoter hypermethylation. However, *MGMT* silencing was not a condition per se which invariably led to K-*ras* mutations. In fact, only 5 of the 13 (38.4%) *MGMT*-hypermethylated ACF and 2 of the 7 (28.6%) *MGMT*-hypermethylated carcinomas showed this alteration. The frequency of K-*ras* mutations found in ACF in the present study was lower than those reported in other investigations, however, considering only G to A transitions, it is in the range of previous studies in humans (12,24-26). Our data support the association between *MGMT* promoter hypermethylation and G to A transitions in K-*ras* in colorectal tumorigenesis (10). However, this association could be a weak one, as recently reported by Halford *et al* (27).

Table II. *CDKN2A* and *MGMT* promoter hypermethylation.

Patient no.	Lesion	*CDKN2A* promoter hypermethylation	*MGMT* promoter hypermethylation	K-*ras* codon 12 AGT	K-*ras* codon 12 GAT	K-*ras* codon 13 AGC	K-*ras* codon 13 GAC
1	NM	-	-	-	-	-	-
	ACF-1	-	-	-	-	-	-
	K	-	+	-	-	-	-
2	NM	-	-	-	-	-	-
	ACF-1	-	+	-	-	-	-
3	NM	-	+	-	-	-	-
	ACF-1	-	+	-	+	-	-
	ACF-2	-	+	-	-	-	-
	ACF-3	-	+	-	-	-	-
	K	+	+	-	-	-	-
4	NM	-	-	-	-	-	-
	ACF-1	-	-	-	-	-	-
	ACF-2	-	+	-	-	-	-
	ACF-3	-	+	-	-	-	-
	ACF-4	+	+	-	+	-	-
	K	+	+	-	+	-	-
5	NM	-	+	-	-	-	-
	ACF-1	-	+	-	-	-	-
	K	-	+	-	-	-	-
6	NM	-	-	-	-	-	-
	ACF-1	-	-	-	-	-	-
	K	-	+	-	-	-	-
7	NM	-	+	-	-	-	-
	ACF-1	-	+	-	+	-	-
	ACF-2	-	+	-	-	-	-
	ACF-3	-	+	-	-	-	-
	ACF-4	-	+	-	-	-	-
8	NM	-	-	-	-	-	-
	ACF-1	+	+	-	+	-	-
	Ad	-	+	-	-	-	+
9	NM	-	-	-	-	-	-
	ACF-1	-	-	+	-	-	-
	ACF-2	-	-	-	-	-	-
	K	-	+	-	-	-	-
10	NM	-	-	-	-	-	-
	ACF-1	-	-	-	-	-	-
	K	-	+	-	+	-	-
11	NM	-	-	-	-	-	-
	ACF-1	-	-	-	-	-	-
	ACF-2	-	-	-	-	-	-
	ACF-3	-	-	-	-	-	-
	ACF-4	-	-	-	-	-	-
	ACF-5	-	-	-	-	-	-
	Hyper	-	-	-	-	-	-
	K	-	-	-	-	-	-
12	NM	-	-	-	-	-	-
	ACF-1	-	-	-	-	-	-
	ACF-2	-	+	-	-	-	+
	Ad	-	-	-	-	-	+
	K	+	-	+	-	-	-

The G to A transition was also examined in codon 12 (wild-type GGT), codon 13 (wild-type GGC) of K-*ras* in normal colorectal mucosae (NM), ACF, adenomas (Ad), hyperplastic polyps (Hyper), and carcinomas (K) of the large bowel.

clusion, the results of the present study suggest that silencing is an early and age-related event in colorectal carcinogenesis, being present even in normal colonic mucosae. However, the mechanisms that trigger the preferentially *de novo* methylation of some promoter genes are still poorly understood. These epigenetic changes can lead to the functional activation of other genes, including K-*ras*, thus starting the early genetic changes necessary for cancer development. Further studies are needed in order to elucidate the very early alterations which underlie colonic cell transformation, and in particular, not only the qualitative, but also the quantitative assessment of the methylation status in normal-appearing mucosae by the use of the real-time PCR technology.

Acknowledgements

This study was supported by grants from the Italian Ministry of University (MIUR), the Italian Association for Cancer Research (AIRC), and the Associazione Angela Serra per la Ricerca sul Cancro di Modena.

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