Virchows Arch (1999) 434:57–62

ORIGINAL ARTICLE

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5′-Cytosine DNA-methyltransferase mRNA levels in hereditary colon carcinoma

Received: 20 July 1998 / Accepted: 21 September 1998

Abstract DNA methylation plays an important part in the regulation of gene expression. Alterations in DNA methylation in tumours have been reported and have been used to generate hypotheses about mutagenesis and silencing of tumour suppressor genes. However, the underlying mechanism is still poorly understood, and conflicting data on the levels of overexpression of 5′-cytosine DNA methyltransferase in sporadic colon carcinoma have been published. We used a competitive RT-PCR assay for quantification of mRNA of 5′-cytosine DNA methyltransferase in colon biopsies obtained from patients with hereditary colon carcinoma syndromes and compared the results with those obtained in a control group. No significant difference was found between the flat mucosa of FAP patients and the mucosa of the control group. In FAP and HNPCC patients, the 5′-cytosine DNA methyltransferase mRNA levels of adenomas were significantly higher ($P<0.05$) than of flat mucosa in the same group, but both showed great variability from patient to patient. Our findings suggest that the mRNA levels of methyltransferase cannot be used as predictive marker for screening in families affected by hereditary colon carcinoma.

Key words mRNA Quantification · RT-PCR · Competitive RT-PCR · Hereditary colon carcinoma · FAP · HNPCC

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Introduction

Familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) are among the most frequent inherited cancer syndromes and are transmitted in an autosomal dominant manner. Patients affected by FAP bear a mutation at locus 5q21 in the *APC* gene (for reviews see [15, 41]). FAP is characterized by the occurrence of hundreds of adenomatous polyps, mainly in the colon and rectum, in early adolescence and, if untreated, development of carcinoma [8]. In patients with HNPCC, a mutation in genes responsible for the repair of DNA mismatch errors is present and is associated with microsatellite instability (for reviews, see [14, 15, 25, 27]). Clinical features are characterized by the early age at onset of colon carcinoma and additional extracolonic carcinomas (for reviews see [25]). Despite the term nonpolyposis, adenomas occur in HNPCC and must be seen as representing the premalignant lesion. It is generally accepted that colon carcinogenesis follows a multistep pathway involving mutations or deletions in several genes and also changes in the methylation levels of the DNA [15].

There is growing evidence that DNA methylation plays an important part in the regulation of gene expression and in cancer development. The methylation of DNA in eukaryotes is mostly restricted to the dinucleotide cytosine guanidine (CpG) [5, 32]. CpG islands are areas of clustered CpG dinucleotides, are frequently located in promoters of constitutively expressed genes, and are generally unmethylated [2]. However the methylation of the cytosine residues in CpG islands can suppress subsequent gene expression. Changes of the DNA methylation state have been reported to occur in various human tumours (for reviews, see [16, 17, 18, 38]). It is believed that the methylation of cytosine residues alters the conformation of the DNA double helix [10, 43] and thereby modulates the affinity of DNA-binding proteins. The 5′-cytosine DNA methyltransferase (MTase; EC 2.1.1.37) that has been isolated and cloned from several species [3] is the enzyme for maintenance methylation of DNA

during the DNA replication, but de novo methylating activity has also been reported in vitro [21]. The expression of the MTase is tightly regulated by the cell cycle [39, 40] and is controlled by the proto-oncogenes *ras* and c-*jun* [26]. The MTase is essential for development and differentiation, since fetal development of null mutant mice is retarded and the embryos die at day 19 of gestation. However, heterozygous mice that have reduced MTase levels show no apparent growth or change in phenotype [22]. Apparently these mice display a lower incidence in colorectal cancers against a Min–/– (multiple intestinal neoplasia mouse) genetic background. This effect is even more pronounced in combination with administration of 5-aza-deoxycytidine [17].

Expression levels of MTase during colon carcinogenesis have attracted attention following the report by el-Deiry et al. [7] of an 18- to 200 fold overexpression detected by using a RT-PCR assay. Further, they presented data indicating the occurrence of a field effect in the normal appearing mucosa of colon carcinoma patients. Later on, however, the same group reported only a 5.4-fold overexpression of the MTase [12], and similar data were published by Schmutte et al. [35]. Lee et al. [19] found no significant differences when they applied a quantitative RNase protection assay using histone H4 mRNA as reference. All these data were obtained for sporadic colon carcinoma. Recently, studies on CpG methylation in HNPCC [1, 20] revealed an association between hypermethylation and microsatellite instability.

In the present study, we have investigated MTase mRNA levels in biopsies of flat mucosa of FAP and HNPCC patients and of as human colon carcinoma cell lines harbouring mutations in the *APC* and DNA mismatch repair genes. Our aim was to determine whether increased levels of MTase mRNA can be detected which would be indicative of a field effect in FAP and HNPCC patients and could potentially be used as a predictive marker before the onset of clinical manifestations of these inherited colon carcinoma syndromes. For this purpose, we applied a method for accurate quantification of small amounts of RNA. Competitive RT-PCR was chosen because of its high sensitivity, reproducibility and accuracy [9, 31, 36].

Materials and methods

Biopsy samples were obtained during endoscopy and immediately frozen in liquid nitrogen. The control group consisted of 19 individuals (average age 38 years; range 14–70 years) from each of whom a single biopsy was taken. They showed no abnormalities on endoscopy and had no family background of hereditary colorectal cancer syndromes. The FAP group was made up of 19 patients (average age 33 years; range 12–64 years) with clinically manifest FAP and positive family background. From 17 of the FAP patients a single biopsy from flat mucosa was obtained, and from 2 patients two biopsies each. Additional biopsies from adenomas were taken from 7 individuals. The HNPCC group consisted of 9 patients (average age 43 years; range 24–64 years) fulfilling the appropriate clinical criteria [15, 25]. A biopsy from the flat mucosa was obtained from each patient, and a biopsy was also taken from an adenoma in 3 patients.

Tissue samples were obtained from a total colectomy specimen of a 71-year-old female patient suffering from sporadic colon carcinoma. Samples from the carcinoma, a polyp and mucosa with a macroscopically normal appearance were immediately frozen in liquid nitrogen.

The following cell lines and media were used: HCT116, HCT116a, HCT116b [4] in RPMI1640 containing 10% FBS, 5.3 mM L-Glu, 1.5 mM pyruvate, 62.5 mM L-Ser, 83.3 mM L-Asn, 0.6% BME vitamins, 0.6% BME-AA, 1.25% nonessential MEM-AA, 1% penicillin/streptomycin; SW480, SW620 (ATCC) in Leibowitz L-15, 10% FBS, 0.2% sodium bicarbonate; KM12 (provided by I.J. Fidler, Houston, Tex) in Eagle's MEM with Hanks' salts, 5% FBS, 1% nonessential MEM-AA, 1% BME vitamins, 0.2% sodium bicarbonate, 1% penicillin/streptomycin; and HT29H (provided by D. Louvard, Paris, France) in DMEM, 4.5 g/l glucose, 10% FBS. Cells were passaged before reaching confluence. All culture reagents were purchased from Gibco BRL (Gaithersburg, Md.).

Total RNA was extracted from snap-frozen colon biopsy samples or from cultured cells using TRI Reagent and Microcarrier Gel-TR (MRC, Cincinnati, Ohio) according to the manufacturer's protocol. To evaluate the quality of the isolated RNA, 1 µg total RNA was separated in a denaturing formaldehyde-agarose gel, transferred to a nylon membrane (Hybond N+, Amersham, Cleveland, Ohio) by capillary transfer and stained with methylene blue [34]. The intensity of the 28S and 18S ribosomal RNA bands was used as a qualitative measure for the integrity of the isolated RNA.

Construction of the competitor for the human MTase

A C-terminal portion of the human MTase gene 467 bp in size was amplified by RT-PCR using RNA isolated from a small cell lung carcinoma cell line (NCI-H69, ATCC, Rockville, Md.) and the following primer pair: 5′-GAGCTCGAGCTCGTGCGGGAGTGTGCCCGC-3′, 5′-GGTACCGGTACCCCACTCATACAGTGGTAGATTTG-3′. The PCR product was cloned into the SacI/KpnI restriction sites of the pBluescript KS+ vector (Stratagene, La Jolla, Calif.). The identity and the orientation of the subcloned DNA fragment of the MTase (pMT460) were verified by DNA sequence analysis. An oligonucleotide cassette of 60 bp (5′-AGCTTATCGATGTCGACTCGAGCCCGGGCTCTAGAGGATCCTGC AGAATTCGATATCAAGCT-3′) was then introduced at the unique *Hind*III restriction site of the subcloned MTase fragment. The correct insertion of the oligonucleotide cassette was confirmed by DNA sequence analysis. The purified plasmid DNA containing the internal standard for the human MTase was linearized at the 3'-end with KpnI endonuclease and then subjected to in vitro transcription using T7 RNA polymerase (Promega, Madison, Wis.) followed by treatment with RNase-free Dnase I (Boehringer, Rotkreuz, Switzerland). The concentration of the internal standard competitor RNA was determined by measuring the absorbance at 260 nm. The presence of plasmid DNA was checked by PCR amplification of the newly transcribed RNA without reverse transcription. The working concentrations of RNA competitor were prepared in Formazol in 10-fold dilution steps with addition of 1 µg yeast tRNA (Stratagene) prior to each dilution.

Competitive RT-PCR assay

To eliminate possible experimental bias in the assay, all samples were blinded for their source. The amount of specific gene transcripts (mRNA) was measured by a competitive RT-PCR assay: 0.2 µg total cellular RNA and varying amounts of competitor RNA (10 fg to 100 pg) were added to give a final volume of 10 µl, heated to 65°C for 5 min and immediately placed on ice. The RT master mix (10 mM Tris-HCl, pH 8.8, 5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 15 U AMV reverse transcriptase (Promega), 1 U rRNasin (Promega), oligo$(dT)_{16–18}$ primer (Promega), 20 µM downstream PCR primer 5′-TTTAAAATCCAGAATGCACAAAG-3′) was added to give a final volume of 20 µl and incubated for 1 h at 42°C. The reverse transcription was terminated by raising the reaction temperature to 95°C for 5 min. Then 5 µl of this reverse transcription reaction and a PCR master mix (10 mM Tris-HCl, pH 9.0 [25°C], 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM dNTP, 0.5 µM PCR

primers 5′-GGCTCTTCGGCAACATCCTG-3′, 5′-TTTAAAATC-CAGAATGCACAAAG-3′, 0.2 U SuperTaq; Stehlin AG, Basel, Switzerland) were added to a final volume of 50 µl and overlaid with a drop of mineral oil (Sigma Chemicals, St. Louis, Mo.). The PCR reaction was carried out in a Perkin Elmer DNA Thermal Cycler TC-1 or TC-480 using the following cycle conditions: 1×95°/2 min, 50°/2 min; 3×94°/1.5 min, 50°/1.5 min, 72°/2 min; 37×94°/1 min, 50°/1 min, 72°/1 min; 1×72°/10 min. This resulted in the formation of PCR products 352 bp (native PCR product) and 410 bp (competitor PCR product) in size, which were separated by gel electrophoresis and visualized using an UV transilluminator. The DNA bands were scanned using a CS-1 CCD camera (Cybertech, Berlin, Germany) and quantified using Wincam Version 2.1 software package (Cybertech). The log of the ratio of native MTase PCR product to competitor PCR product was plotted against the log of the initially spiked competitor RNA. Using linear regression analysis, the amount of mRNA was determined from the point of equal molar ratios of the native MTase PCR product and competitor PCR product (Fig. 1). The results were obtained by at least two independent quantifications. The average level of MTase mRNA of each sample was normalized against the relative ratio of intensities of methylene blue stained 18S and 28S ribosomal RNA bands.

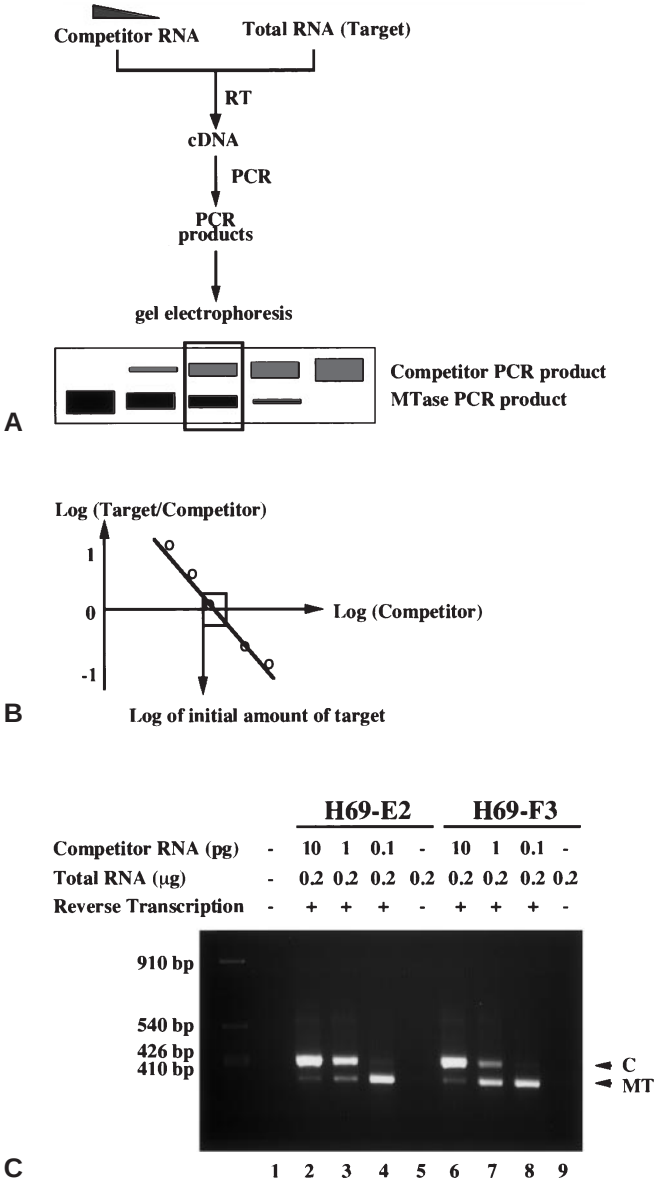

Fig. 1 **A** Flow chart diagram of competitive RT-PCR. The *lower part* is a schematic illustration showing the separation of competitor and native PCR product by gel electrophoresis (see also **C**). The *boxed region* indicates the point of equilibrium between spiked competitor RNA and target mRNA amounts. **B** Calculation of target mRNA amounts from multiple independent competition reactions by linear regression analysis (see "Materials and methods"). **C** Example of a competitive RT-PCR assay for MTase mRNA. Total RNA of two different small cell lung carcinoma lines was mixed with increasing amounts of competitor RNA, and then subjected to the MTase mRNA assay. The PCR products were electrophoretically separated in ethidium-stained agarose, bands visualized by UV-light, scanned and quantified (see "Materials and methods" section). The following controls were performed: *lane 1* PCR negative control, *lanes 5 and 9* PCR of RNA without prior reverse transcription, demonstrating absence of contaminating genomic DNA (*C* competitor PCR product, *MT* MTase PCR product)

MTase enzyme activity microassay

The MTase enzyme activity assay was performed according to Issa et al. [12]. In brief, the cultured cells and tissue samples were homogenized. The homogenates were then incubated with poly(dI-dC) as synthetic substrate and *S*-adenosyl-[methyl-^{3}H]methionine as methyl donor. After termination of the methyl transfer reaction, the labelled oligonucleotides were purified and the radioactivity incorporated was determined by scintillation counting.

Results

Competitive RT-PCR assay for human MTase

In order to establish a competitive PCR assay, a C-terminal fragment 467 bp in size of the human MTase gene was amplified by PCR, subcloned into a vector and modified by inserting a 60-bp oligonucleotide cassette to yield the competitor. The identities of the subcloned MTase fragment and the competitor were verified by DNA sequence analysis (data not shown). The amplification efficiencies of both native and competitor DNA were determined by coamplifying a mixture containing equal amounts of target DNA [28] and found to be equal over 35 and 40 PCR cycles (data not shown). Reverse transcription of RNA to cDNA may be subject to variation from reaction to reaction. To avoid such variations the competitor was added as cRNA to the total RNA and the mixture subjected to reverse transcription followed by PCR amplification (Fig. 1). The assay proved to be linear over more than an order of magnitude of total RNA (0.05–1 µg; Fig. 2).

In order to validate the competitive RT-PCR assay, MTase mRNA and the MTase enzyme activity of both cultured cells and frozen human tissue samples were determined in parallel. For both the cultured colon carcinoma cells and the colon tissues, a good correlation between MTase mRNA and enzyme activity was observed (Table 1). For instance, values for both increased in parallel from normal appearing mucosa to adenoma and carcinoma [7, 12, 19, 35]. From these results, we concluded that the PCR-based MTase mRNA assay was a reliable and sensitive method that can be applied to colon biopsies.

Table 1 MTase mRNA and enzyme activity in cultured cells and tissue

	MTase mRNA[a] (pg/µg total RNA)	Ratio	MTase activity[a] (dpm)	Ratio
Cultured cells[b]				
HCT116	14.5±0.8	*1.0*	127±14	*1.0*
HCT116a	6.5±0.2	0.4	71±5	0.6
HCT116b	23.5±2.5	1.6	180±7	1.4
Frozen tissue[c]				
Flat mucosa	1.5±0.3	*1.0*	43±14	*1.0*
Adenoma	6.5±0.8	4.3	103±12	2.4
Carcinoma	13.5±0.5	9.0	207±18	4.8

[a] Expressed as mean±SE
[b] The HCT116 cells were established from a sporadic colon carcinoma and were fractionated into the subpopulations HCT116a and HCT116b (Brattain et al. 1981)
[c] The tissue samples were obtained from a female patient (71 years old) suffering from sporadic colon carcinoma (see "Materials and methods" section)

Table 2 MTase mRNA levels in human colon

Patient groups	Flat mucosa		Adenoma	
	No. of biopsies	(pg/µg total RNA)[a]	No. biopsies	(pg/µg total RNA)[a]
Control group	19	3.4±1.6[b, c]	–	–
FAP	21	4.0±2.5[b, d]	7	13.8±13.9[d]
HNPCC	9	4.4±2.6[c, e]	3	24.9±0.9[e]

[a] Expressed as mean±SE
[b, c] Designate pairs of groups statistically not different (Wilcoxon-Mann-Whitney test; $P > 0.05$)
[d, e] Designate pairs of groups statistically different (Wilcoxon-Mann-Whitney test, $P < 0.05$)

Table 3 MTase mRNA level of various cell lines

Cell line	Origin	MTase mRNA[a] (pg/µg total RNA)
HCT116	Carcinoma	14.5± 0.7
HCT116a	Carcinoma	6.5± 0.2
HCT116b	Carcinoma	23.5± 2.5
SW480	Carcinoma	41.9± 0.6
SW620	Carcinoma	37.9± 0.9
KM12C	Carcinoma	30.4± 3.3
KM12L4	Carcinoma	31.3± 8.2
KM12SM	Carcinoma	42.2±10.7
AA/C1/75	Adenoma	20.8± 4.3
AA/10C	Adenoma	30.4± 2.3
AA/23	Adenoma	51.3± 0.7
HT29H	Carcinoma	73.5± 5.9

[a] Expressed as mean±SE

MTase mRNA in hereditary colon carcinoma

All samples were blinded for their source and the code was broken after all assays had been performed and analyzed. The MTase mRNA levels were determined in biopsies from patients with FAP and HNPCC, and compared with the control group (Table 2, Fig. 3). The level of MTase mRNA in the flat mucosa of patients with FAP did not differ statistically from the control level ($P > 0.05$). Similarly, samples of flat mucosa of HNPCC patients and control mucosa were not statistically different (Table 2; Fig. 3). In addition, the values showed great variability both in flat mucosa (2.1 to 12.7 pg MTase mRNA/µg total RNA) and in adenomas (1.8–50.3 pg MTase mRNA/µg total RNA). Values for control mucosa also varied (1.0–7.9 pg MTase mRNA/µg total RNA). Thus, it was not possible to establish a measure for a normal MTase mRNA level, and consequently there was no threshold level that would be clinically useful. The MTase mRNA levels were significantly higher in the adenomas of FAP and HNPCC (3-fold in FAP and 6-fold in HNPCC) than in flat mucosa (Table 2).

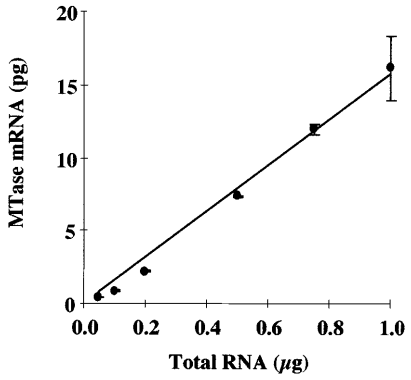

Fig. 2 Demonstration of the linearity of the competitive RT-PCR assay for human MTase mRNA. Increasing amounts of total RNA isolated from a colon carcinoma were subjected to quantification by competitive RT-PCR. The spiked competitors were varied from 10 fg to 100 pg per RT-PCR reaction. The PCR products were separated and visualized on a ethidium bromide-stained 2.5% agarose gel. The ratio of competitor to native MTase PCR product was determined using the Wincam V2.1 image scanning software. The mRNA expression level was calculated by linear regression analysis of the competitor to native PCR product ratio at three different competitor concentrations. The results are expressed as the average of at least two individual assays per total RNA quantity ±SD

MTase mRNA in cell lines

The HCT116 cell lines harbour a mutation in one of the DNA mismatch repair genes [29, 30], and SW480 and SW620 lines carry an *APC* mutation [33, 37]. The AA/C1/75 and AA/10 C cell lines represent FAP adenoma-derived polyclonal and monoclonal nontumorigenic cell lines, respectively, whereas the AA/23 is a chemically transformed tumorigenic line [42]. The genotypes of KM12 and HT29H cell lines were not available. The mRNA levels of MTase in various cell lines derived from carcinomas and an FAP adenoma are detailed in Table 3 and are generally higher than those measured in normal colonic mucosa and flat mucosa of FAP and HNPCC (Table 2). From our analysis it also became evident that the MTase mRNA levels vary between the cell lines by one order of magnitude. In the FAP adenoma-derived cell lines, MTase mRNA was higher in the chemically transformed, tumorigenic line AA/23 than in the nontumorigenic AA/C1/75 and AA/10 C lines (Table 3).

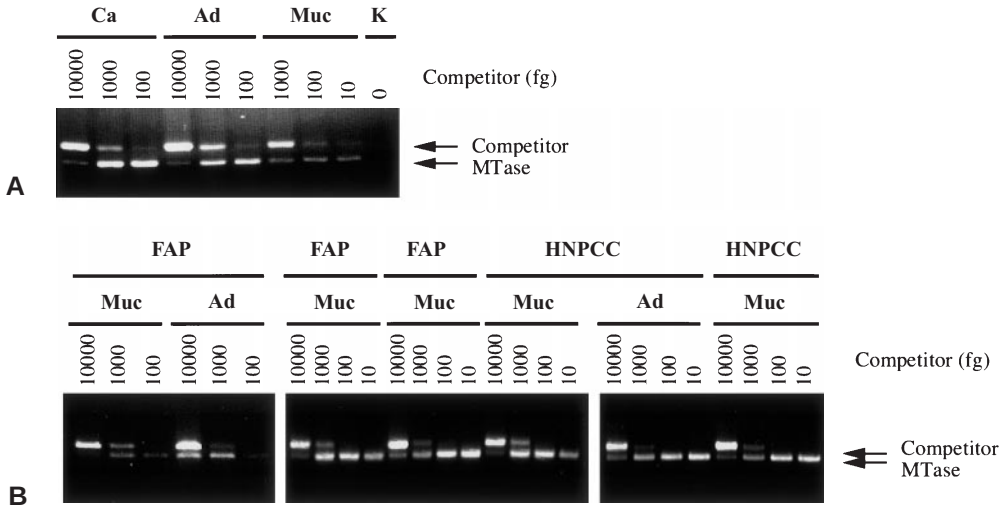

Fig. 3 A, B Competitive RT-PCR of MTase of human colon tissue. **A** Specimens obtained from a sporadic colon carcinoma. **B** Colon biopsy samples. (*Ca* carcinoma, *Ad* adenoma, *Muc* mucosa, *K* control PCR with no DNA added, *FAP* samples taken from patients with familial adenomatous polyposis, *HNPCC* samples taken from patients with hereditary nonpolyposis colon carcinoma). For further details, see "Materials and methods" section

Discussion

Various investigators have studied MTase expression in sporadic colon carcinoma [7,12, 19, 35]. A major objective of our study was to compare the MTase mRNA levels in colonic mucosa of healthy subjects and in the flat mucosa of patients affected by hereditary colon carcinoma syndromes and to evaluate its possible value as a predictive marker. For this purpose, we have investigated MTase mRNA levels in biopsy samples of FAP and HNPCC by applying a quantitative RT-PCR method.

The levels of MTase mRNA we found in adenomas in hereditary colon cancer were similar to those reported for sporadic adenomas [19, 35]. However, we noted considerable variations from patient to patient in the levels of MTase mRNA in our cases. Similarly, MTase mRNA levels of the studied human colon carcinoma cell lines varied widely. A closer examination of the previously published data on both MTase mRNA [7, 19] and enzyme activity [12] showed similar variations. The discrepancies in the MTase mRNA levels reported in the literature [7, 12, 19, 35] may be due to the different methods used. In our studies, we used a sensitive competitive RT-PCR assay for measuring MTase mRNA in small amounts of total RNA isolated from colon biopsies. In order to prevent erroneous data due to RNA degradation in the samples, biopsies were immediately snap-frozen and a small fragment of MTase (352 nucleotides) was amplified by RT-PCR. Under our conditions no RNA degradation was observed and the assay proved to be highly reproducible. Furthermore, the possibility that variations in results might be due to differing cellular composition of the studied samples can be excluded, since in our assay MTase mRNA determinations are related to total RNA. Hence, we believe that our measurements reflect the endogenous amounts of MTase mRNA of the samples analysed and represent individual differences in the patients and cell lines examined.

We were unable to detect statistically significant differences in MTase mRNA levels between normal control mucosa and the flat mucosa of the patients with FAP or HNPCC. Lee et al. [19], comparing normal colonic mucosa with apparently normal mucosa of sporadic colon carcinoma patients, also reported no differences in the MTase mRNA levels. Thus, in contrast to an earlier report [7], no field effect was detectable in the flat mucosa of hereditary colon carcinoma. Hence, our data exclude the use of MTase mRNA measurements as a molecular diagnostic marker in patients at risk of inherited colon carcinoma. In this context, recent studies by Hiltunen et al. [11] on hypermethylation of the *APC* gene promoter region of colorectal carcinoma are very informative. They found hypermethylation in carcinomas but not in normal mucosa or in adenomas, and concluded that hypermethylation may be a rather late event during malignant progression. Lee et al. [19] demonstrated that apparently elevated MTase mRNA levels were due to increased cellular proliferation. Increased cellular proliferation and widening of the proliferative zone in flat mucosa of manifest FAP in comparison to mucosa of healthy subjects is well established [6, 13, 23, 24]. Thus, it may be that the elevated, but statistically not significantly elevated, MTase mRNA levels in flat mucosa of FAP found in the present study reflected this increased proliferation. Moreover, we cannot exclude the possibility that the significantly elevated values for adenomas (compared with flat mucosa) in both FAP and HNPCC resulted from increased proliferation [19].

Two recent studies have convincingly shown that local hypermethylation occurs not only in cells of FAP harbouring a mutation in the *APC* gene, but even more drastically in cells with defects in genes of the DNA mismatch repair system found in HNPCC. Lengauer et al. [20] studied tissue culture cells of known genotypes, and Ahuja et al. [1], samples of colon cancer patients. Both studies reveal enhanced hypermethylation of CpG in regulatory regions of investigated gene loci in cells originating from HNPCC patients. In our small HNPCC group higher levels of MTase mRNA were found than in samples from adenomas of patients in the FAP group. More studies will be necessary to clarify the implications of MTase expression, DNA methylation and tumour progression in FAP and HNPCC.

Acknowledgements We thank Dr. R.J. Scott (Institute of Genetics, University of Basel) for *APC* mutation analysis. The KM12 cell lines were kindly provided by Dr. J.I. Fidler (MD Anderson

Cancer Center, Houston, Tex.) and the HT29H cell line by Dr. D. Louvard (Institute Curie, Paris). The RNA of the AA cell lines was graciously provided by Dr. C. Paraskeva (Department of Pathology and Microbiology, School of Medical Sciences, Bristol, UK). This work was supported by the Cancer League of Canton Zürich, the Kamillo Eisner Foundation, Hergiswil, the Swiss Cancer League and the Hochschulstiftung Zürich.

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