Evaluation of global DNA hypomethylation in human colon cancer tissues by immunohistochemistry and image analysis

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Abstract

***Background*—Global hypomethylation of DNA is frequently observed in human tumours. This alteration is detected in early adenomas in colorectal tumorigenesis. Information is currently acquired after extraction of DNA from tissues, digestion with nucleases, and analysis by reverse phase chromatography, or treatment with restriction enzymes followed by gel electrophoresis analysis and Southern hybridisation with radiolabelled probes.**

***Aims*—The purpose of our work was to evaluate the global methylation status of DNA in malignant lesions without loosing the histopathological features of the samples.**

***Patients*—The investigation was performed on paired normal-tumour tissues from 13 patients undergoing surgical resection of colorectal adenocarcinomas.**

***Methods*—Antibodies raised against 5-methylcytidine can be used to label methyl rich regions in interphase nuclei. This technique was adapted to the study of paraffin embedded tissues and an immunohistochemical method was developed to assess the global methylation status of individual nuclei while preserving cell morphology and tissue architecture. Computer assisted quantification of the staining intensity was performed on malignant and normal zones of human colon tissues to test the correlation between the immunolabelling signal and the respective histological patterns observed.**

***Results*—Qualitative and quantitative differences were observed and measured between the normal and malignant part of each sample. Morphologically altered nuclei displayed densely labelled spots within faintly labelled areas whereas normal nuclei were darker and uniformly stained. Image analysis allowed calculation of the average integrated optical density of the nuclei in both types of tissues, demonstrating a constant and significantly lower intensity for the former type of cells.**
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Keywords: colon adenocarcinoma; DNA hypomethylation; immunohistochemistry

Loss of methyl groups in DNA is frequently observed in carcinogenesis.[1] An average global hypomethylation rate of 8–10% has been observed in colon adenomas or adenocarcinomas[2 3] and a strong correlation between the malignant phenotype and DNA methylation status was demonstrated in colorectal cancer cells.[4–8] It is known that 5-methylcytidine (5-MeCyd), a spontaneous frequent site of C to T mutation,[9 10] is involved in the control of gene expression,[11] in carcinogenesis,[12 13] and in tumour progression.[14] Therefore, global DNA hypomethylation could induce expression of proto-oncogenes[15–17] whereas hypermethylation could silence tumour suppressor genes.[18–21]

Global methylation of DNA is usually quantified by chromatography[1] whereas the methylation status of specific genes is currently studied by molecular hybridisation or genomic sequencing.[22] These sophisticated techniques require extraction of DNA and do not allow the cell integrity to be preserved. An immunochemical approach was developed using monoclonal antibodies that recognise the presence of a methyl group on the carbon 5 of cytidine, to investigate DNA methylation in situ, which allowed computer assisted quantification of global methylation to be performed on interphase nuclei in several cell types, on a cell by cell basis by microscopy.[23–26] The present work describes the immunohistochemical method set up to visualise and quantify the signal in paraffin embedded tissue sections. Colon biopsies were obtained which allowed us to compare the neoplastic areas with normal tissue and to verify that it was possible to correlate the histopathological pattern with the immunostaining intensity of the nuclear compartment. Preliminary results reported here indicate that differences in the respective immunolabelling intensities of nuclei between normal and malignant cells can be visualised and quantified in situ, and related to the morphological alterations observed.

Methods

Paraffin embedded, formalin fixed biopsies from 13 patients were obtained and the malignant lesions were compared with normal tissue from the same patient from a distant site. Sections (4 μm) were dewaxed and then rehydrated and rinsed in phosphate buffered saline (PBS). After antigen retrieval in a microwave oven set at full power (720 W) for 10 minutes

Abbreviations used in this paper: PBS, phosphate buffered saline; PBST, PBS containing 0.1% Tween 20; HPLC, high pressure liquid chromatography; 5-MeCyt, 5-methylcytosine; 5-MeCyd, 5-methylcytidine; PBS-BSA, PBS-bovine serum albumin.

in pH 3.4 citrate buffer, the slides were immersed in 2 N HCl for two hours at 37°C and washed in PBS. Sections were covered with 100 µl of hybridoma supernatant containing anti-5-MeCyd monoclonal antibody (5 µg/ml) and incubated for one hour at 37°C. This monoclonal antibody was obtained and characterised as previously described.[27] Its presence was revealed by incubation with a biotinylated goat antimouse secondary antibody (Dako, Glostrup, Denmark) diluted 1/200 in PBS containing 0.1% bovine serum albumin (BSA) (PBS-BSA) for one hour at room temperature. Samples were reacted over 30 minutes with streptavidin-peroxidase (Dako) diluted 1/100 in PBS-BSA. Sections were then treated with diaminobenzidine (Dako) for five minutes. Between each incubation step the slides were washed three times with PBS containing 0.1% Tween 20 (PBST). Pictures were taken on colour positive films under the same illuminating conditions for each sample. The optical density of the nuclei of epithelial cells was measured in 20 microscopic fields belonging to the malignant portion of the sample and in 20 fields of the normal tissue, for each patient, with a Leica Quantimet Analysis System (100× objective).

To establish the specificity and validity of the immunolabelling, two types of experiments were performed.

DOT BLOTS

DNA from *Xanthomonas oryzae* bacteriophage XP-12 (a generous gift of Dr M Ehrlich) has almost all of its cytosine replaced by 5-methylcytosine (5-MeCyt). A solution of this DNA (100 ng/µl) was diluted with increasing amounts of non-methylated λ phage DNA (Sigma, La Verpillière, France) in pH 8.0 TE buffer (10 mM Tris, 1 mM EDTA) so as to obtain mixtures of methylated/non-methylated DNA ranging from 100% to 0%. Aliquots of 1 µl were dotted onto nitrocellulose membrane strips and incubated successively with a saturating solution (blocking reagent; Boehringer-Mannheim, Meylan, France) for 30 minutes at 20°C, and then with anti-5-MeCyd monoclonal antibodies (hybridoma supernatant diluted by 25% in 0.15 M NaCl-10 mM malic acid, using 250 µl/cm^2 of support) over two hours at room temperature. After rinsing with PBST, binding of monoclonal antibodies was detected with antimouse immunoglobulins conjugated to alkaline phosphatase (Life Technologies, Cergy, France), diluted 1/2000 and NBT/BCIP as substrate (Boehringer-Mannheim).

IMMUNOBLOTTING OF GENOMIC DNAs

DNA was extracted from four pairs of normal and malignant tissues with phenol-chloroform, and digested for 16 hours at 37°C with MspI and HpaII restriction enzymes (Boehringer Mannheim) at 10 units/µg of DNA. DNA samples were loaded onto 1% agarose gels. After electrophoresis DNA fragments were transferred under alkaline conditions onto Hybond N membranes (Amersham). After transfer, the gel was re-exposed to UV light to verify that no DNA remained in agarose. Immunodetection of 5-MeCyd was then performed as described for dot blots.

The method used to estimate methylation status was the same as that described by other authors with ethidium bromide stained agarose gels[28] or with ^{32}P labelled DNA.[8 29] Briefly, density profiles obtained after scanning allowed calculation of the ratios between the signal recorded in the low molecular weight zone corresponding to satellite DNA and the signal recorded in the total lane, giving a value representative of the sensitivity of the sample to digestion by restriction enzymes. Images of immunoblots were recorded with a Kodak DCS 200 digital camera. Gray level based intensity measurements were performed on each dot and along each lane using SigmaScan/Image software (Jandel Scientific GmbH, Erkrath, Germany).

Results

Figure 1 illustrates the difference in staining intensity between normal mucosa (fig 1A) and the adenocarcinomatous zone (fig 1B) in the paired samples of one patient. Immunostaining is localised in the nuclei of the cells. The staining pattern of pleomorphic nuclei in the neoplastic area was distinctly different from that observed in the normal counterpart. The morphologically altered nuclei displayed densely labelled spots within faintly labelled areas whereas normal nuclei were darker and uniformly stained. This difference in staining intensity between the two types of cells was confirmed by image analysis, as shown in table 1, in which the average integrated optical den-

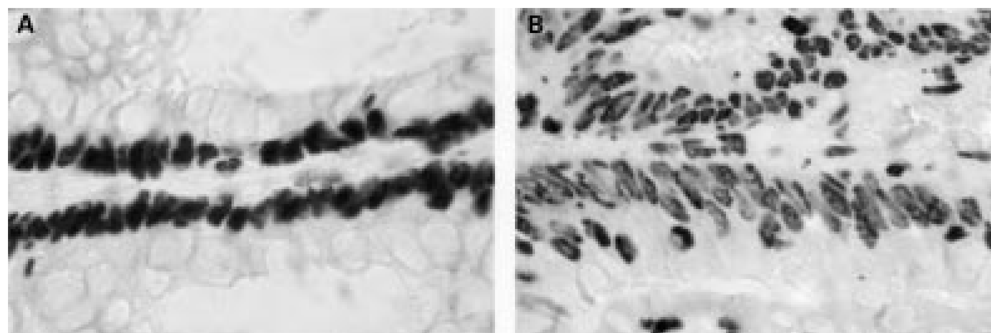

Figure 1 Indirect immunoperoxidase labelling. Example of dense nuclear labelling in normal colon tissue (A) and heterogeneous moderately stained nuclei in well differentiated colorectal adenocarcinoma (B). Before printing, colour slides were converted to black and white images without alteration of the range of density.

Table 1 Nuclei densities after immunostaining with anti-5-MeCyd antibodies in colon biopsies. Paraffin embedded sections from paired tissues were processed as described in methods. Numbers indicate average optical density (mean (2SD)) for each sample

Case No	*Location*	*Age*	*Dukes' stage*	*Differentiation*	*OD tumour*	*OD normal*	*Difference*
1	Right colon	84	D	WD	0.796 (0.039)	1.014 (0.039)	21.4%
2	Right colon	63	D	WD	0.766 (0.013)	0.997 (0.026)	23.2%
3	Right colon	75	D	MD	0.816 (0.041)	1.025 (0.035)	20.0%
4	Right colon	85	C	MD	0.773 (0.019)	1.021 (0.046)	24.3%
5	Right colon	69	D	WD	0.792 (0.041)	1.030 (0.038)	23.0%
6	Left colon	50	B	MD	0.764 (0.033)	1.019 (0.048)	25.1%
7	Left colon	58	C	WD	0.739 (0.051)	1.021 (0.031)	27.6%
8	Left colon	64	C	PD	0.751 (0.039)	1.018 (0.021)	26.2%
9	Left colon	60	B	WD	0.723 (0.026)	1.026 (0.026)	29.5%
10	Left colon	77	B	WD	0.837 (0.037)	1.010 (0.032)	17.1%
11	Left colon	74	A	WD	0.784 (0.043)	1.006 (0.024)	22.1%
12	Left colon	91	B	WD	0.818 (0.055)	1.027 (0.039)	20.4%
13	Left colon	62	C	MD	0.787 (0.480)	1.009 (0.032)	22.0%

OD, optical density; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.
Dukes classification: Dukes CE. The classification was established according to *J Pathol Bacteriol* 1932;**35**:323–44.

sity of the nuclei in the neoplastic zone and that of nuclei in the normal tissue are reported for each patient, demonstrating a constant and significantly lower intensity for the former type of cell (Student's *t* test: $p<0.05$). The possibility that this discrepancy could be due to a difference in the accessibility of DNA can be ruled out for the following reasons.

(1) 5-MeCyd rich regions are mainly present in the compacted heterochromatin compartment[23 25] which should then be less accessible and less labelled than the open euchromatin. The exact opposite pattern of staining is observed.

(2) In a recently published study,[30] labelling mouse embryos with anti-DNA antibodies gave rise to the same signal in the paternal and maternal pronuclei whereas the anti-5-MeCyd signal was markedly reduced in the female genome. Therefore, the differences observed in the present work cannot be attributed to accessibility.

Results obtained on dot blots are illustrated by fig 2. Diluting XP-12 phage DNA with non-methylated λ phage DNA yielded a progressively decreasing signal, a limit being approached with 3% of methylated DNA. This result confirms the specificity of the antibodies for methylated DNA.

Immunodetection was then applied to the analysis of DNA samples obtained from normal and malignant tissues. Immunoblotting generated signals such as those shown in fig 3. This illustrates the results obtained using this method on human DNA. The staining patterns demonstrate that smears of various shapes and intensities can be observed before and after digestion, the longest smear being obtained, as expected, with Mspl. These results are comparable with those obtained by other authors who used radiolabelled probes for hybridisation.[6 29] The density profiles acquired by scanning illustrate the results obtained with a pair of DNA samples (normal tissue-malignant tissue) from patient No 8 in table 1. As Mspl is not sensitive to methylation, nearly identical indexes were obtained for each sample, as expected (*r* tumour/*r* control=1.09), whereas a lower sensitivity, regarding Hpall digestion, was observed for DNA from normal tissues than for DNA from the malignant lesion (*r* tumour/*r* control=1.194) confirming the hypomethylated status of the latter. Three other paired DNA samples were obtained from patients not included in the immunohistochemical study and gave similar results under the same conditions (table 2).

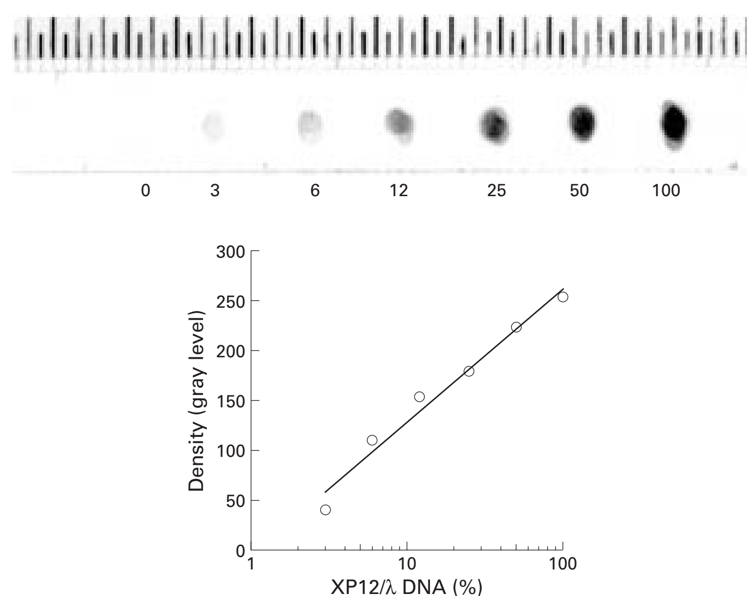

Figure 2 Dot blots of methylated DNA. Xp12 DNA was serially diluted with non-methylated λ phage DNA and 1 µl aliquots were dotted onto nitrocellulose, as described in materials and methods. Density levels were measured as described in materials and methods and plotted against percentage dilution.

Discussion

Visual inspection of paired normal-malignant tissues obtained from colon cancer patients indicated lower intensity staining with the anti-5-MeCyd antibody in these lesions. This feature was confirmed by image analysis performed on a series of 13 pairs of samples for which lower intensity staining of 17–29% was calculated in malignant samples compared with non-malignant counterparts. Similar results were obtained previously using the same technique for other types of malignant cells[24] and cell lines.[23 25 26] For colon adenocarcinomas, Feinberg and colleagues[3] detected 8–10% DNA hypomethylation after extraction of DNA, followed by nuclease digestion and high pressure liquid chromatography (HPLC) analysis. The immunohistochemical method

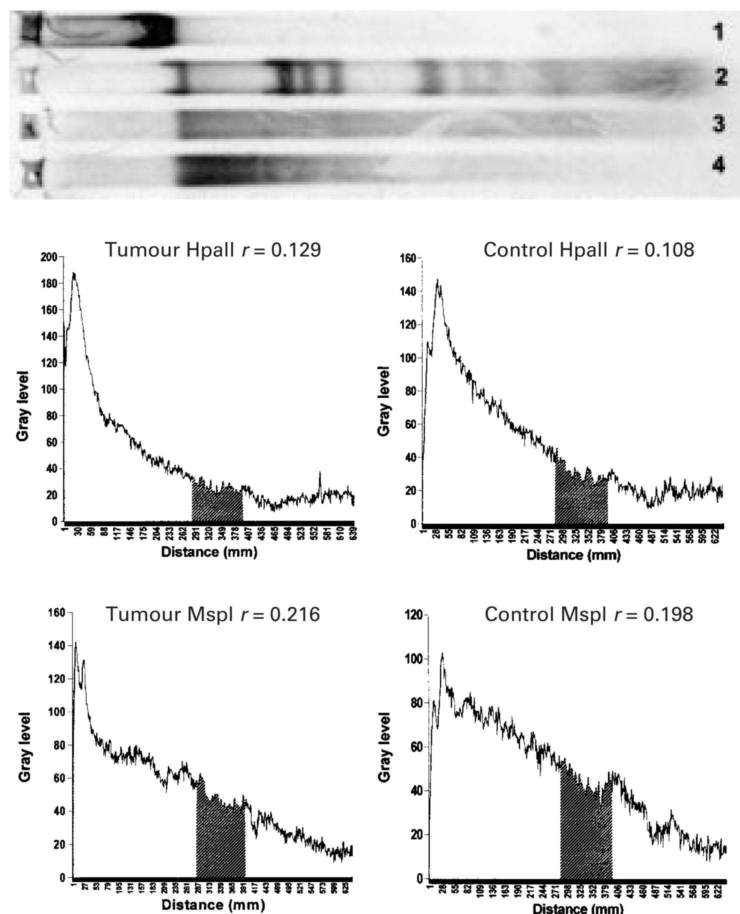

Figure 3 DNA methylation assessment by Southwestern blotting. Blots: Example of results observed by Southwestern blotting with anti-5-MeCyd antibodies. Lane 1: non-digested human DNA; lane 2: human DNA digested with Eco R1; lane 3: human DNA digested with Mspl; and lane 4: human DNA digested with Hpall. Density profiles: DNA was extracted from paired normal-malignant tissues, digested with Mspl or Hpall, and submitted to gel electrophoresis, transferred onto nitrocellulose, and incubated with anti-5-MeCyd antibodies, as described in methods. Density profiles were registered and analysed as described in methods. The shaded area indicates the portion of the lane corresponding to satellite DNA. The ratios r between the surface of this portion and the surface of the entire lane are indicative of the sensitivity of each sample to digestion.

Table 2 Assessment of global DNA methylation indexes by Southwestern blotting. Each immunoblot of HpaII digested DNA was scanned as described in methods. The ratio between the shaded area and the total area of each lane was taken as an index reflecting the sensitivity of the sample towards the restriction enzyme activity. Samples from patient No 8 were obtained from the same patient as in table 1. The three other pairs were from other patients not included in the set of samples studied by immunohistochemistry

Case No	*Localisation*	*Age*	*Dukes' stage*	*Differentiation*	*Southwestern blot index*
8	Left colon	64	C	PD	21.3
14	Left colon	60	A	WD	14.8
15	Right colon	55	D	MD	19.4
16	Left colon	76	B	WD	15.2

WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

used here allowed us to select and measure the signal in individual epithelial nuclei of the altered areas of the tissue and this feature may account for the difference between our results and those obtained using other methods but is in accordance with the global hypomethylated status of DNA in colon carcinomas reported by other authors.[31 32] Our results also showed that the low immunohistochemical signal was associated with a change in chromatin condensation. In pleomorphic nuclei, some areas of the nucleoplasm contained intensely stained spots. Whether these dense strongly stained regions correspond to localised hypermethylated sites remains to be proved. However, it is known that during cancer progression an increase in DNA methyltransferase activity is observed.[33 34] Simultaneously, large nuclear areas display a weak immunostaining signal which could reveal hypomethylated regions similar to those observed in leukaemic cells.[24] One can reasonably assume that these alterations reflect the instability of the genome and are probably due to disorganisation of chromatin compaction induced by modifications of the methylation pattern.

Alteration of DNA methylation patterns is frequently observed in numerous human tumours. The link between this post-replicative modification and control of gene expression as well as detection of frequent C to T mutations has led authors to build a genetic model of carcinogenesis and tumour progression, especially for colorectal cancer.[35 36] DNA global hypomethylation is an early event in colorectal tumorigenesis[37] and progression of the disease is associated with an increase in DNA methyltransferase activity and appearance of hypermethylated sites. These biochemical changes are paralleled by modifications in the histopathological patterns of colorectal tumours which include successive steps progressing from adenomas to carcinomas. Increasing epithelial dysplasia and cell dedifferentiation are considered markers of multistep carcinogenesis. Global DNA hypomethylation can be detected at the early adenoma stage but requires HPLC analysis, whereas investigations on specific genes rely on hybridisation with radiolabelled probes or DNA sequencing, which are not always available in routine laboratories. The immunohistochemical method described here allows the pathologist to collect useful information on the DNA methylation status of various regions in the biopsy, on a cell by cell basis, while preserving tissue architecture and cellular morphology.

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