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Advances in Brief

Abnormal Regulation of DNA Methyltransferase Expression during Colorectal Carcinogenesis[1]

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Abstract

Somatic changes in CpG dinucleotide methylation occur quite commonly in human cancer cell DNA. Relative to DNA from normal human colonic cells, DNA from human colorectal cancer cells typically displays regional CpG dinucleotide hypermethylation amid global CpG dinucleotide hypomethylation. The role of the maintenance DNA methyltransferase (DNMT1) in the acquisition of such abnormal CpG dinucleotide methylation changes in colorectal cancer cells remains controversial; in one study, 60–200-fold increases in *DNMT1* mRNA expression were detected in colorectal polyps and cancers relative to normal colonic tissue [W. S. El-Deiry *et al., Proc. Natl. Acad. Sci. USA, 88*: 3470–3474, 1991], whereas in another study, only small increases in *DNMT1* mRNA expression, commensurate with differences in cell proliferation accompanying colonic tumorigenesis, were observed [P. J. Lee *et al., Proc. Natl. Acad. Sci. USA, 93*: 10366–10370, 1996]. To definitively ascertain whether abnormal *DNMT1* expression might accompany human colorectal carcinogenesis, we subjected a series of normal and neoplastic colonic tissues to immunohistochemical staining using a polyclonal antiserum raised against a DNMT1 polypeptide. A concordance of DNMT1 expression with the expression of PCNA and other cell proliferation markers, such as Ki-67 and DNA topoisomerase IIα, was observed in normal colonic epithelial cells and in cells comprising other normal epithelia and lymphoid tissues. The polypeptide p21, which has been reported to undermine DNMT1 binding to proliferating cell nuclear antigen at DNA replication sites, was not expressed by normal colonic cells containing DNMT1 and other cell proliferation markers. In adenomatous polyps, although DNMT1 expression coincided with the expression of other cell proliferation markers, many DNMT1-expressing cells also expressed p21. The fidelity of DNMT1 expression was further undermined in colorectal carcinomas, in which a striking heterogeneity in DNMT1 expression, with some carcinoma cells containing very high DNMT1 levels and others containing very low DNMT1 levels, was observed. These results indicate that human colorectal carcinogenesis is accompanied by a progressive dysregulation of DNMT1 expression and suggest that abnormalities in DNMT1 expression may contribute to the abnormal CpG dinucleotide methylation changes characteristic of human colorectal carcinoma cell DNA.

Introduction

Although most self-complementary CpG dinucleotides in mammalian genomic DNA carry $^{5-m}$C in place of C, clusters of unmethylated CpG dinucleotides, termed "CpG islands," encompass the transcriptional regulatory regions of many genes (1). In normal somatic cells, the precise patterns of CpG dinucleotide methylation

in the genome can be maintained with fidelity through DNA replication and mitosis via the action of a maintenance DNA methyltransferase. However, human cancer cells typically contain DNA with abnormal CpG dinucleotide methylation patterns (2–4). Most often, cancer cell DNA exhibits increases in CpG dinucleotide methylation at certain specific CpG island sequences, accompanied by generalized decreases in CpG dinucleotide methylation at most other sites. Such alterations in DNA methylation can lead to changes in gene function subject to selection during neoplastic transformation and malignant progression, *e.g.,* CpG island DNA methylation changes can result in gene inactivation via a silencing of gene transcription.

How do cancer cells acquire abnormal CpG dinucleotide methylation patterns? One mechanistic hypothesis focuses on a malfunctioning of the DNA methyltransferase DNMT1. Tumorigenic cells have long been known to have high DNA methyltransferase levels (5). In *in vitro* model systems, overexpression of DNA methyltransferases has been shown to cause increased CpG dinucleotide methylation (6) and to trigger transformation (7, 8). Recently, increased DNA methyltransferase expression was demonstrated to be an essential molecular step in c-*fos*-mediated transformation *in vitro* (9). DNA methyltransferase activity may also be essential for transformation *in vivo*; mice carrying defective *Apc* genes at high risk for developing colonic adenomas develop fewer polyps when carrying disrupted alleles for *Dnmt1*, a gene encoding a DNA methyltransferase likely capable of both maintenance and de novo CpG dinucleotide methylation (10, 11). Recent data have also provided a clue as to how decreased methylation of CpG dinucleotides might occur, despite the expression or overexpression of a DNA methyltransferase (12). To carryout its maintenance DNA methylation function, DNMT1 must be recruited to sites of DNA replication by binding to PCNA[3] (13–15). Chuang *et al.* (12) have reported that the cell cycle regulatory polypeptide p21 disrupts DNMT1 binding to PCNA. Perhaps, if p21 is inappropriately expressed or induced by DNA damage or other stress, maintenance DNA methylation activity may be disrupted (16).

DNA methylation changes, including generalized CpG dinucleotide hypomethylation and specific CpG island hypermethylation, stereotypically accompany human colorectal carcinogenesis (17–23). El-Deiry *et al.* (24) reported that increases in *DNMT1* mRNA expression also tended to accompany colorectal carcinogenesis. When compared with *DNMT1* levels present in normal colonic epithelium, using a reverse transcription-PCR assay technique, as much as a 60-fold increase in *DNMT1* mRNA was detected in adenomatous polyp lesions, and as much as a 200-fold increase in *DNMT1* mRNA was detected in carcinoma lesions. In a follow-up study, more modest increases in DNA methyltransferase activity in colonic neoplasms *versus* normal colonic epithelium were noted (25). Lee *et al.* (26) subsequently disputed these findings. Using an

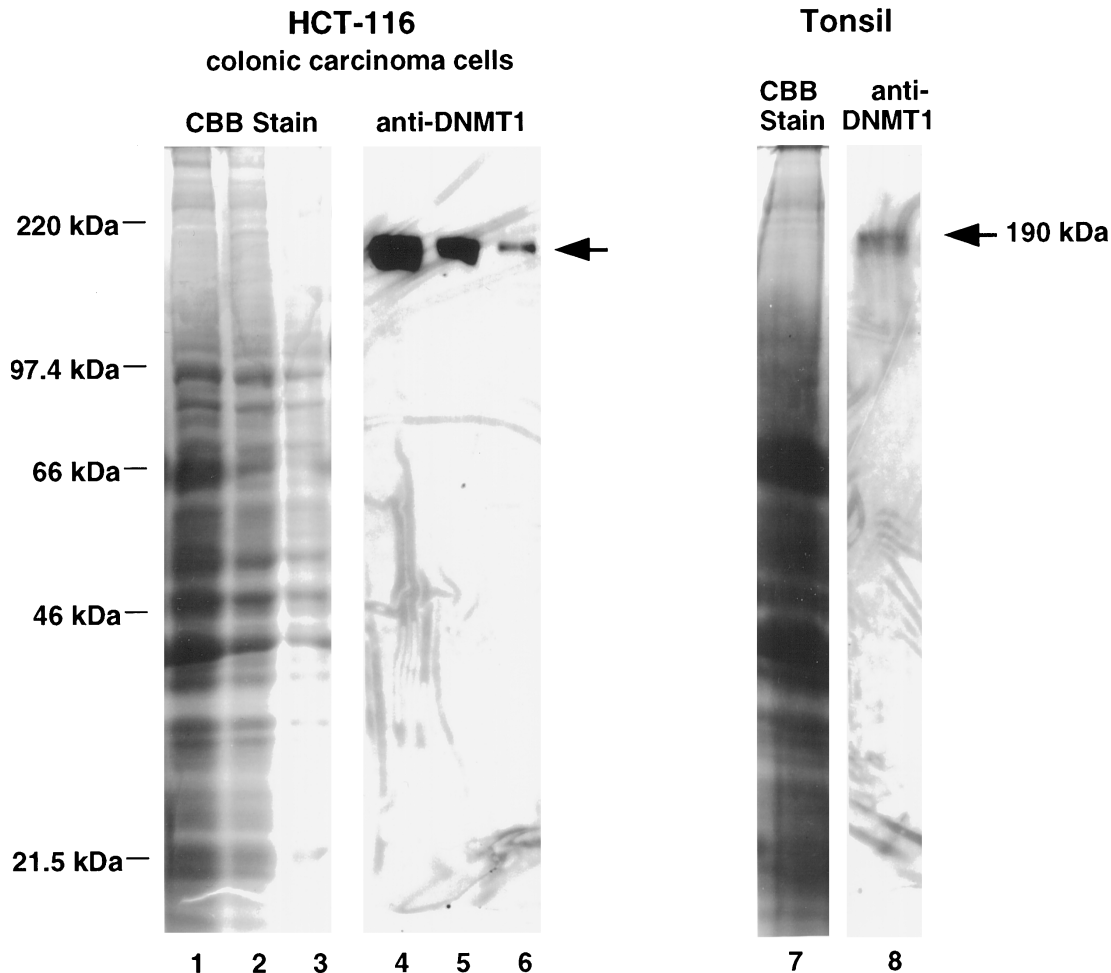[3] The abbreviation used is: PCNA, proliferating cell nuclear antigen.

Fig. 1. Immunoblot analysis of protein extracts from HCT-116 colonic carcinoma cells and from human tonsil tissue for DNMT1 expression using anti-DNMT1 antibodies. Protein extracts were electrophoresed on polyacrylamide gels in the presence of SDS and stained with Coomassie brilliant blue (*CBB*) or transferred to nitrocellulose filters and stained with anti-DNMT1 antibodies (*anti-DNMT1*) as described in "Materials and Methods." *Lanes 1* and *4,* 400 μg of HCT-116 protein extract; *Lanes 2* and *5,* 200 μg of HCT-116 protein extract; *Lanes 3* and *6,* 100 μg of HCT-116 protein extract; *Lanes 7* and *8,* 2 mg of tonsil protein extract. A single polypeptide of $M_r \approx 190,000$ was detected in each extract.

RNase protection technique targeting a different region of *DNMT1* mRNA, only slight increases in *DNMT1* mRNA levels in colonic neoplasms, commensurate with increases in cell proliferation, were observed. To definitively ascertain whether abnormal *DNMT1* expression might accompany human colorectal carcinogenesis, we subjected a series of normal and neoplastic colonic tissues to immunohistochemical staining for DNMT1 polypeptides. We report here that human colorectal carcinogenesis is accompanied by a progressive dysregulation of DNMT1 expression and propose that abnormalities in DNMT1 expression likely contribute to the abnormal CpG dinucleotide methylation changes characteristic of human colorectal carcinoma cell DNA.

Materials and Methods

Normal and Neoplastic Colonic Tissues. Normal and neoplastic formalin-fixed, paraffin-embedded tissues were obtained from the surgical pathology archives of the Johns Hopkins Hospital. Specimens of normal colonic tissue, colonic adenoma tissue, and invasive colonic carcinoma tissue were removed at colectomy for colorectal cancer or at endoscopy for colorectal polyps. Other normal tissues, including specimens of tonsil, uterine cervix, stomach, and small intestine, were also removed as part of surgical procedures. At the time of removal, all tissue specimens were immediately immersed in 10% neutral buffered formalin and subsequently embedded into paraffin blocks. Additional tonsil tissue was snap frozen and subsequently stored at −70°C for protein extraction (see below).

Immunohistochemical Staining. For immunohistochemical staining analyses, tissue sections cut from the paraffin blocks were first deparaffinized and rehydrated and subjected to specific antibody staining for various antigens using the Biotek Techmate 1000 robotic immunostainer (Ventana Medical Systems, Tuscon, AZ). All primary antibody incubations were conducted at room temperature for 45 min; biotinylated secondary antibody incubations were carried out at room temperature for 30 min. Histochemical localization was accomplished using avidin-biotin horseradish peroxidase complex (Vectastain ABC; Vector Laboratories, Incorporated, Burlingame, CA) with 3,3′-diaminobenzidine tetrahydrochloride as the chromagen. After immunohistochemical staining, tissue sections were counterstained with hematoxylin.

Antibodies used for immunohistochemical staining included antibodies recognizing DNMT1, PCNA (mouse monoclonal clone #24 used at a dilution of 1:250; Transduction Labs, Lexington, KY), p21 (mouse monoclonal anti-WAF1 Ab-1 used at a dilution of 1:50; PharMingen, San Diego, CA), Ki-67 [mouse monoclonal MIB-1 (27) used at a dilution of 1:100; Immunotech, Miami, FL], and topoisomerase IIα (mouse monoclonal AB-1 used at a dilution of 1:100; Calbiochem, San Diego, CA). The DNMT1 antibodies used were an affinity-purified IgG preparation isolated from rabbit antiserum raised against a polypeptide N-MADANSPPKPLSKPRT-PRRS-C (derived from human DNMT1) at Research Genetics, Inc. (Huntsville, AL).

Immunoblot Analysis. Total protein extracts were prepared from frozen (−70°C) human tonsil tissue and from HCT-116 human colonic adenocarcinoma cells propagated *in vitro* in McCoy's growth medium supplemented
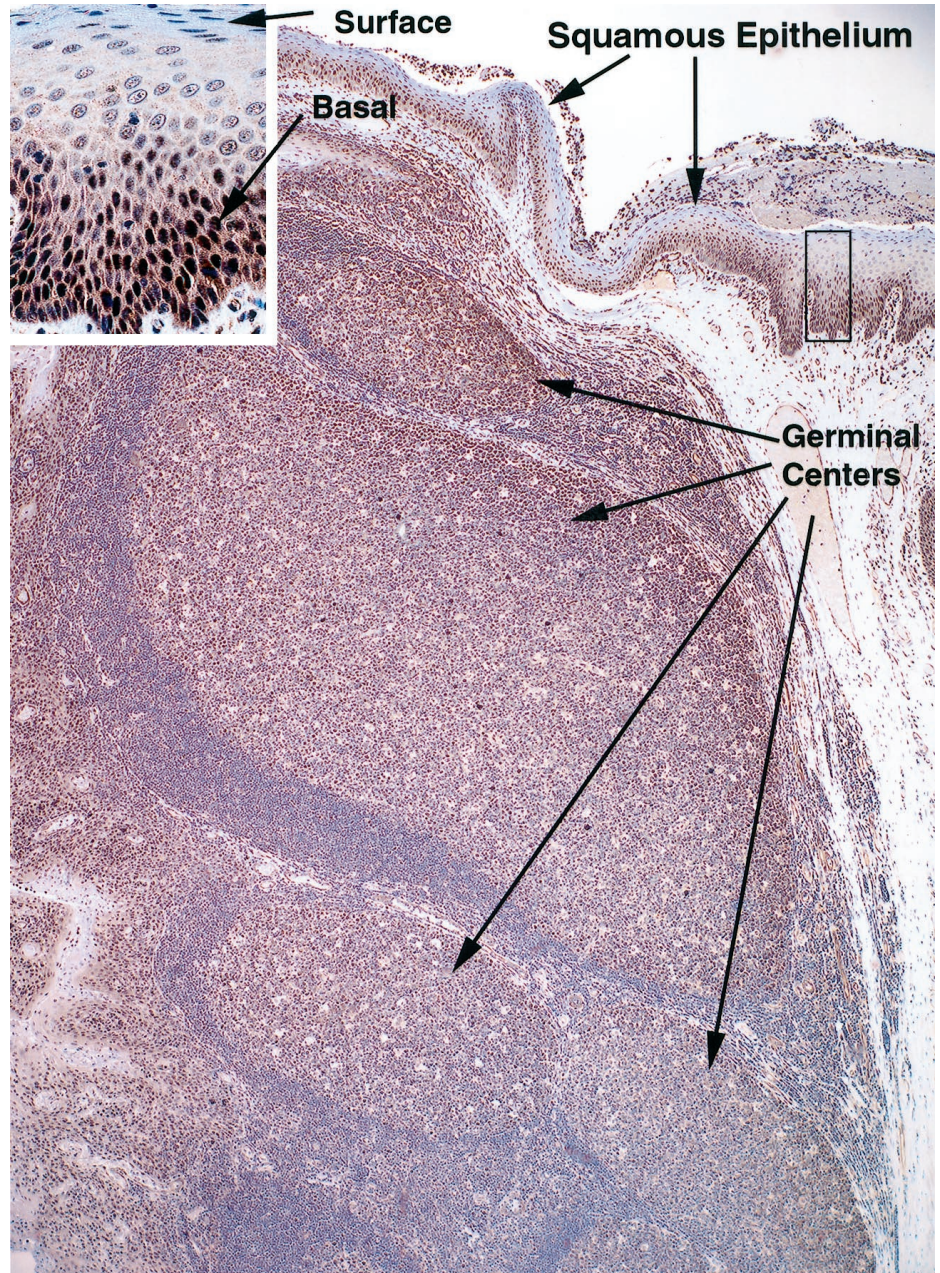
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Fig. 2. Immunohistochemical staining for DNMT1 in human tonsil tissue. Lymphoid cells in germinal centers and basal epithelial cells in respiratory epithelium expressed significant levels of DNMT1. Similar staining patterns were observed using antibodies directed against PCNA, Ki-67, and topoisomerase IIα.

with 10% FCS (Life Technologies, Inc., Gaithersburg, MD). Protein extraction was accomplished by homogenization at 4°C in a lysis buffer containing 1% NP40, 150 mM NaCl, and 1 mM EDTA in 50 mM Tris-HCl at pH 7.5 supplemented with a protease inhibitor mixture that included 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml E64, and 100 μg/ml phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO). The resultant homogenates were clarified by centrifugation at 10,000 rpm in a Beckman microfuge for 10 min, and the protein extract supernatants were stored at −70°C. The protein concentration of each extract was estimated using the BCA assay (Pierce, Rockford, IL). For immunoblot analysis, protein extracts were electrophoresed on 6.5% polyacrylamide gels in the presence of SDS and then transferred to nitrocellulose filters (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) electrophoretically. The nitrocellulose membrane blots were then treated with a blocking solution (5% nonfat dry milk and 0.05% Tween 20 in TBS) overnight at 4°C before being exposed to anti-DNMT1 antibodies at a concentration of 2 μg/ml in 5% nonfat dry milk and 0.05% Tween 20 in TBS for 2 h at room temperature. After extensive washing with TBS containing 0.05% Tween 20, the anti-DNMT1 antibodies were detected using horseradish peroxidase-conjugated anti-rabbit antibodies (Amersham Pharmacia Biotech) and a chemiluminescence substrate (SuperSignal West Pico chemiluminescence substrate; Pierce) in a manner similar to that described previously (28). Alternatively, SDS-polyacrylamide gels containing electrophoretically separated protein extracts were stained with Coomassie blue (Sigma).

Results and Discussion

Previous data have suggested a tight coupling between normal somatic cell proliferation and DNA methyltransferase expression (13, 14, 29–32). Analysis of *Dnmt1* expression and activity in mouse 3T3 cells has demonstrated that as growth-arrested cells are stimulated to progress from G_0 into S-phase, an increase in *Dnmt1* mRNA levels and Dnmt enzyme activity occurs (30). However, nuclear run-on transcription assays suggested that *Dnmt1* was transcribed in both growth-arrested and growth-stimulated cells, indicating that the proliferation-dependent increases in *Dnmt1*

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mRNA levels were likely the result of a posttranscriptional mechanism, such as differential mRNA stability (30). Perhaps because *DNMT1* mRNA abundance may be subject to active mRNA degradation, two independent studies of *DNMT1* mRNA levels in normal and neoplastic human colonic tissues, using assays targeting different regions of the *DNMT1* mRNA template, yielded remarkably different results (24, 26).

For our study, we used antibodies raised against a human DNMT1-derived polypeptide to directly detect the enzyme in normal and neoplastic cells and tissues. When the antibodies were used for immunoblot analysis of protein extracts from normal tonsil tissue and from HCT-116 colonic carcinoma cells propagated *in vitro*, a single polypeptide of $M_r \approx 190,000$ was detected in each extract (Fig. 1). When the same antibodies were used in immunohistochemical staining studies of a variety of normal tissues, including tonsil (Fig. 2), respiratory epithelium (Fig. 2), uterine cervix, stomach, small intestine, and colon (Fig. 3), a tight concordance of DNMT1 expression with the expression of PCNA was evident. A fraction of cells in the colon that appeared to express DNMT1 and PCNA also expressed other DNA proliferation markers such as Ki-67 (27) and DNA topoisomerase IIα (Refs. 33 and 34; not shown).

Analysis of DNMT1 expression in normal colonic epithelium also disclosed that the crypt epithelial cells that expressed DNMT1 and PCNA did not express p21, whereas the noncrypt epithelial cells, which tended not to express DNMT1 or other cell proliferation markers, expressed high levels of p21 (Fig. 3). In adenomatous polyps, Wu *et al.* (35) have reported abnormal p21 expression in cells expressing markers of proliferation such as Ki-67. In our study, we also found abnormal coexpression of p21 and proliferative markers in adenomatous polyps. Furthermore, many cells comprising adenomatous polyps appeared to express both DNMT1 and p21 (Fig. 4). A possible consequence of the coexpression of DNMT1 and p21 in proliferating colonic polyp cells may be an undermining of maintenance DNA methyltransferase activity, via p21 disruption of DNMT1 and PCNA interactions at replication foci (12–14), that might lead to global hypomethylation. Occasional cells in colonic polyps expressing DNMT1 appeared also to contain slightly higher levels of the enzyme than normal crypt cells expressing DNMT1 in adjacent colonic mucosa. This elevated expression of DNMT1 in these rare adenoma cells might tend to lead to hypermethylation of some regions of adenoma cell DNA (6).

DNMT1 expression in colorectal carcinomas appeared strikingly abnormal (Fig. 5). Carcinoma cells exhibiting very high and very low levels of immunohistochemical staining for DNMT1 levels present in the same cancer tissue were detected. In addition, p21 also appeared to be haphazardly expressed (not shown). Clearly, the dysregulated expression of DNMT1 in colorectal carcinoma cells could result in a dysregulation of the fidelity of CpG dinucleotide methylation, with cells expressing low levels of DNMT1 or coexpressing DNMT1 and p21 prone to hypomethylation and cells expressing high levels of DNMT1 prone to hypermethylation. In any case, all of the DNMT1 immunohistochemistry data collected for this study revealed that a progressive dysregulation of DNMT1 expression appeared to accompany colorectal carcinogenesis (Fig. 6).

Maintenance of the fidelity of CpG dinucleotide methylation patterns through DNA replication likely requires the tightly regulated expression of a maintenance methyltransferase in replicating cells and the tightly controlled function of the enzyme near the DNA replication fork (15). We propose that dysregulation of DNMT1 expression, such as that seen during human colorectal carcinogenesis, might lead to
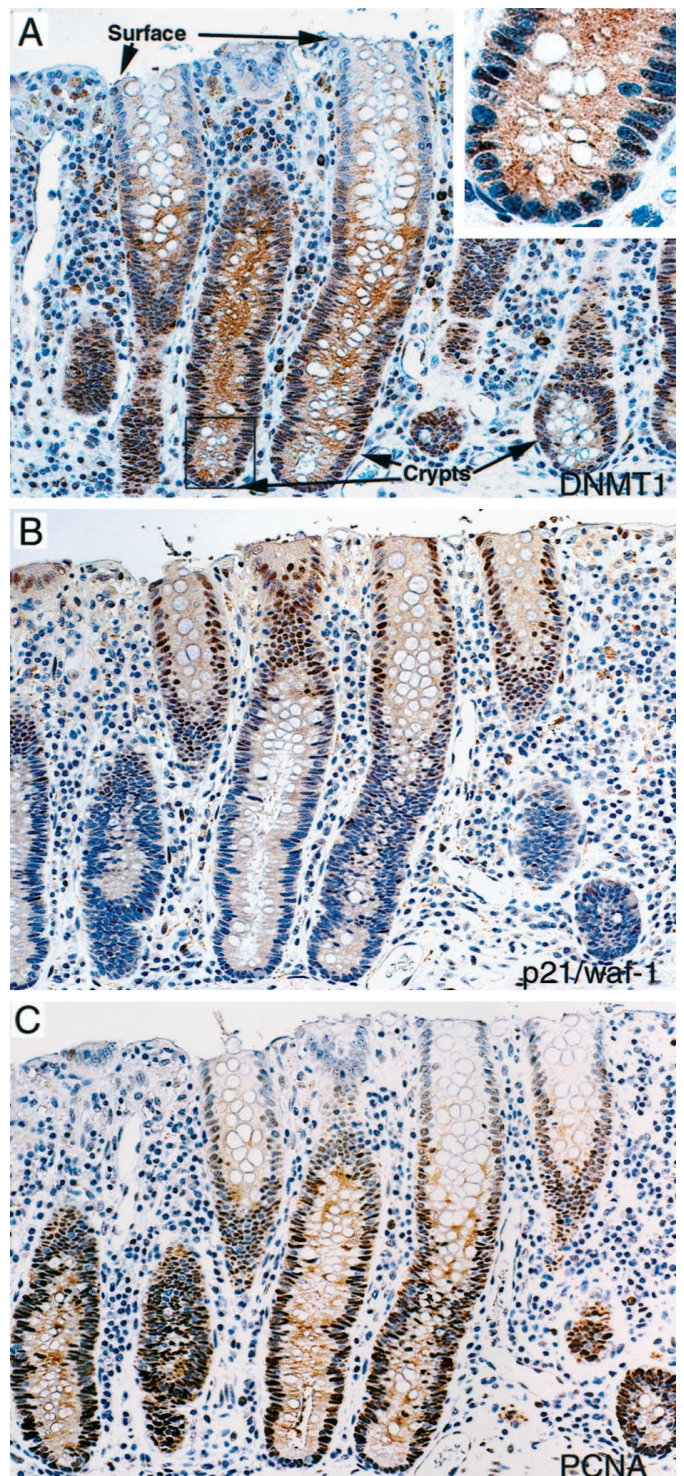

Fig. 3. Immunohistochemical staining for DNMT1, PCNA, and p21 in normal colonic epithelium. Crypt cells expressed DNMT1 and PCNA but not p21; surface cells expressed p21 but not DNMT1 and PCNA.

inadequate maintenance of normal CpG dinucleotide methylation patterns and to abnormal *de novo* methylation changes. Although the mechanisms by which the control of DNMT1 expression becomes altered in neoplastic colorectal carcinoma cells have not been identified, studies of the mouse *Dnmt1* regulation have suggested that ras signaling may trigger transcriptional activation (36, 37). Further insights into the normal and abnormal regulation of *Dnmt1* and *DNMT1* await more precise definition and characterization of the gene regulatory region (38).

Fig. 4. Immunohistochemical staining for DNMT1, PCNA, and p21 in colonic adenoma tissue. Compared with normal colonic epithelium (see Fig. 3), an increased fraction of cells expressed DNMT1 and PCNA. In addition, some DNMT1-expressing cells also appeared to contain p21.
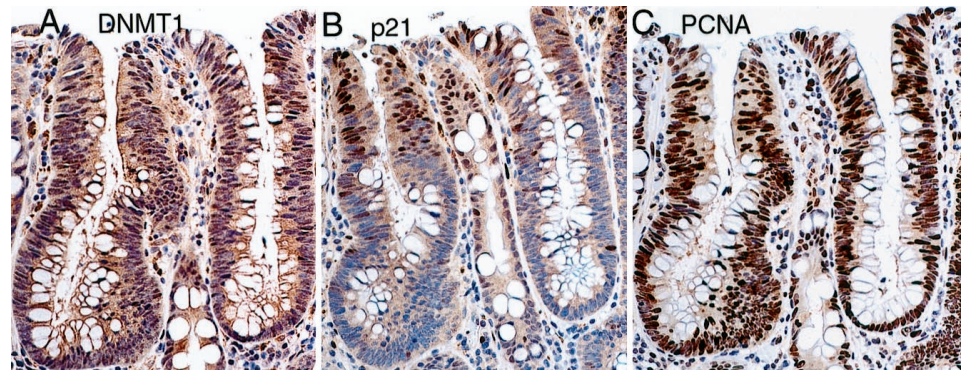
Our finding of a progressive dysregulation of DNMT1 expression accompanying colorectal carcinogenesis supports the general set of mechanistic hypotheses that emphasize a malfunctioning of a maintenance DNA methyltransferase in the acquisition of abnormal CpG dinucleotide patterns characteristic of neoplastic transformation and malignant progression. Maintenance methyltransferase malfunction may not be the only mechanism by which abnormal CpG dinucleotide methylation occurs in cancer cells, however. The recent identification of additional mammalian DNA methyltransferases (39), capable of *de novo* CpG dinucleotide methylation *in vitro*, and of a mammalian $^{5-m}$CpG demethylase (40), capable of reducing CpG dinucleotide methylation *in vivo*, offers additional possible mechanistic explanations for the acquisition of abnormal CpG dinucleotide methylation changes in cancer cells as well. Abnormal regulation of additional *de novo* DNA methyltransferases and of $^{5-m}$CpG demethylases in neoplastic cells could only further undermine the fidelity of CpG dinucleotide pattern maintenance.
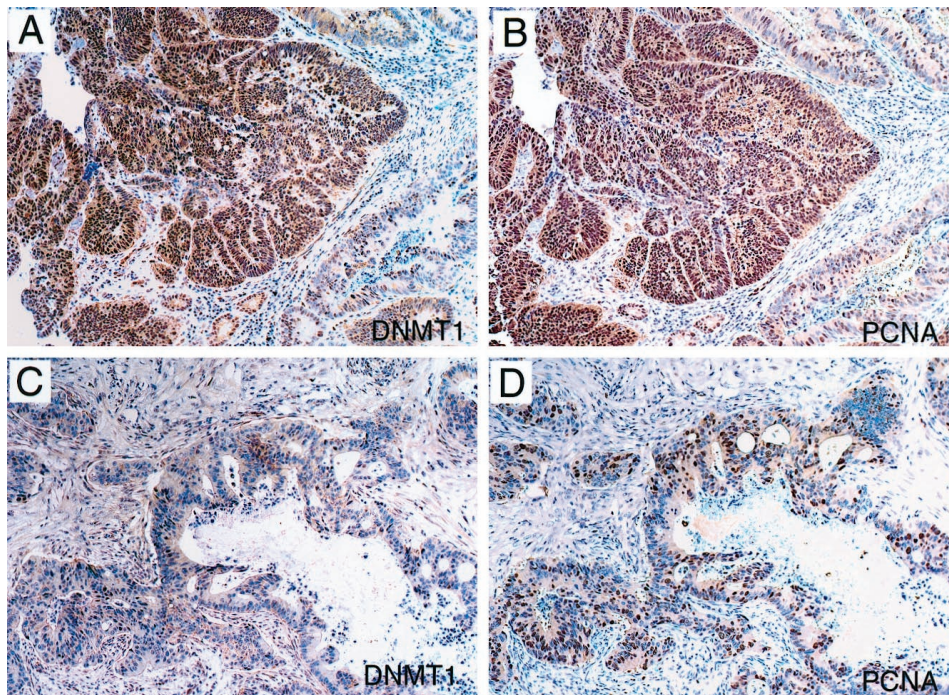

Fig. 5. Immunohistochemical staining for DNMT1 and PCNA in colonic carcinoma tissue. Carcinoma cells expressing very high and very low levels of DNMT1 and PCNA were evident. (*A* and *B*) and (*C* and *D*), two different regions of the same carcinoma.
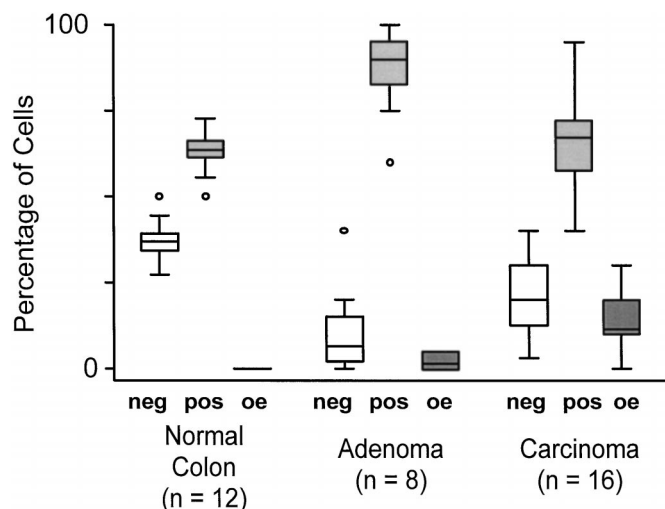
Fig. 6. Progressive dysregulation of DNMT1 expression during colorectal carcinogenesis. Using tissue sections stained with anti-DNMT1 antibodies, the fractions of epithelial cells not expressing DNMT1 (*neg*), expressing physiological levels of DNMT1 (levels present in normal colonic crypt cells; *pos*), or expressing very high DNMT1 levels (greater than present in normal colonic crypt cells; overexpressed or *oe*) were estimated for normal colonic epithelium (*Normal Colon*; $n = 12$), for colonic adenomas (*Adenoma*; $n = 8$), and for colonic carcinomas (*Carcinoma*; $n = 16$). The percentage of cells at each DNMT1 expression level for each group is displayed. *Central bars* within *boxes,* median percentages. *Upper bars* of the *boxes,* upper quartile; *lower bars* of the *boxes,* lower quartile. *Bars above* and *below boxes,* 95% confidence limits. ○, outlier cases. Statistical analyses were accomplished using STATA 5.0 software for Windows 95. For the appearance of cells expressing high levels of DNMT1 (*oe*), a trend of increasing numbers of such cells associated with the progression from normal colonic epithelium to colonic adenoma to colonic carcinoma (estimated as the Pearson product-moment correlation coefficient, *r*, of 0.729 with $P < 0.0001$), was detected.

Acknowledgments

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