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Hypermethylation of the p16^{INK4a} Promoter in Colectomy Specimens of Patients with Long-standing and Extensive Ulcerative Colitis[1,2]

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ABSTRACT

Functional inactivation of the *p16^{INK4a}* gene has been reported to be involved in the development of a variety of human malignancies. Recent evidence shows that transcriptional silencing as a consequence of hypermethylation of CpG islands is the predominant mechanism of *p16^{INK4a}* gene inactivation in sporadic colon cancer. This study sought to identify the significance of p16^{INK4a} methylation in the colonic epithelium of patients with long-standing ulcerative colitis. A total of 89 tissue samples was retrieved from three colectomy specimens. A methylation-specific PCR assay was applied. The methylation status was compared with histological findings and the flow cytometrically determined DNA index. Hypermethylation of the p16^{INK4a} promoter region was detected in 12.7% of samples that were negative for dysplasia. However, 70.0% of samples with dysplasia and all of the samples with carcinomatous lesions revealed hypermethylation. Hypermethylation of the *p16^{INK4a}* gene promoter was detected already in 40% of specimens with lesions indefinite for dysplasia and in 13.7% of samples with exclusively diploid cell populations. These results suggest that hypermethylation of the *p16^{INK4a}* promoter region is a frequent and early occurring event during the process of neoplastic progression in ulcerative colitis.

INTRODUCTION

The CDK[5] inhibitor p16^{INK4A} acts as a negative cell cycle regulator. The p16^{INK4A} protein binds to CDK4 and CDK6 and induces a G_1-phase arrest in the molecular machinery of the cell cycle by interfering with binary cyclinD-CDK4 complexes (1, 2). Thus, if the *p16^{INK4A}* gene or its transcript are impaired, one regulatory mechanism designated to block cell cycle progression is missing. An uncontrolled growth of genetically damaged cells may be promoted—a major characteristic of cancer—which made the *p16^{INK4A}* gene an excellent candidate as a tumor suppressor gene.

Similar to well-known mechanisms of inactivation of other tumor suppressor genes, it has been reported that functional loss of p16^{INK4A} frequently occurs as a consequence of LOH with somatic mutations of the remaining allele or homozygous deletions. LOH of the gene locus *9p21*, where the *p16* gene is localized, has actually been shown to be one of the most frequent genetic abnormalities in human neoplasia, only second to *p53* gene abnormalities (3). Functional loss of p16^{INK4A} has been reported for a variety of human neoplasia, among them esophageal and colorectal cancer (4, 5).

However, in contrast to the findings in cell culture experiments, 9p21 LOH with somatic mutations in the remaining *p16^{INK4A}* allele occurs at a relative low frequency in primary cancers (6, 7). Although showing functional defects of p16^{INK4A} quite commonly, certain neoplasias, such as breast and colon cancer, have been reported to display rarely homozygous deletions or point mutations of the *p16^{INK4A}* gene (2). This observation prompted the question whether functional loss of p16^{INK4A} activity is caused by alternative mechanisms in these CAs.

In addition to homozygous deletions or somatic mutations, DNA methylation has been suggested as an alternative mechanism of *p16^{INK4A}* tumor suppressor gene inactivation (5). DNA methylation in eukaryotic DNA is a normal postreplicative process and occurs at the 5-position of cytosine residues in the majority of CpG dinucleotides. This modification is associated with gene activity and is essential for normal mammalian development. However, discrete regions of CpG-rich sequences without methylation are clustered as CpG islands. These islands have been shown to be often associated with promoter regions of genes (8). Alterations of DNA methylation patterns in these regions have important regulatory effects on gene expression. Hypermethylation of CpG islands has been shown to be associated with structural alterations in chromatin and transcriptional repression (9). Recent evidence actually demonstrates that abnormal hypermethylation of CpG islands exists in a variety of human neoplasias such as hematological malignancies (10), esophageal adenocarcinomas (11), colorectal cancer (5, 12), and pancreatic CA (13). Transcriptional silencing as a consequence of hypermethylation has been shown to occur in several neoplasia-associated genes. For p16^{INK4A}, it has been demonstrated that functional loss due to promoter hypermethylation is a major mechanism of inactivation in some tumors (5, 10, 14, 15). Recent evidence suggests that promoter hypermethylation of the *p16^{INK4A}* gene is the predominant mechanism of functional p16^{INK4A} loss in sporadic colorectal cancer (5, 12).

It is commonly recognized that long-standing and extensive ulcerative colitis represents a precancerous condition, predisposing to the development of colorectal cancer (16). Periodic endoscopic examinations and the detection of dysplasia form the hallmarks of presently performed surveillance strategies. Mainly because the biomarker dysplasia has shortcomings, the controversy about the efficacy of these programs continues. Hence, there is a need for better markers that indicate an increased risk of neoplastic transformation earlier and in a more objective and reliable manner.

This study sought to get additional insight into the role of hypermethylation of the tumor suppressor gene *p16^{INK4A}* during the process of neoplastic progression in ulcerative colitis. In regard to a possible role as a biomarker of neoplastic transformation, the frequency and time of onset of this epigenetic lesion should be investigated. Hence, colectomy specimens of patients with long-standing ulcerative colitis and previously detected dysplastic lesions were mapped in regard to histomorphological lesions, DNA ploidy, and methylation status of the p16^{INK4A} promoter region.

PATIENTS, MATERIALS, AND METHODS

Patients and Samples. Tissue samples were retrieved from each of 24 to 34 positions throughout the whole colon per colectomy specimen from three patients with long-standing ulcerative pancolitis. The interval between the

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[5] The abbreviations used are: CDK, cyclin dependent kinase; LOH, loss of heterozygosity; NEG, no dysplasia; IND, lesion indefinite for dysplasia; LGD, low-grade dysplasia; HGD, high-grade dysplasia; CA, carcinoma; p16PHM, p16 promoter hypermethylation; AN, aneuploidy.

Table 1 *Number of investigated specimens according to histomorphological diagnosis for patients P1, P2, and P3 and number of aneuploid specimens and of specimens with p16PHM among investigated 89 specimens*

Patient	NEG	IND	LGD	HGD	CA	Total
P1	12	3	4	1	4	24
AN	1	2	3	1	4	11
p16PHM	1	2	4	1	4	12
P2	9	7	9	6		31
AN	6	6	8	4		24
p16PHM	6	2	7	2		17
P3	34					34
AN	0					0
p16PHM	0					0

positions was 2–3 cm. The samples were divided into two sections to facilitate comparisons between histological, flow cytometric, and methylation results. Although in two patients (P1, P2), surgery was recommended because of the previous endoscopic detection of dysplasia after a disease duration of 18 and 20 years, respectively, colectomy in the third patient (P3; disease duration 10 years) was performed because of clinical deterioration despite intensive medical treatment.

Histopathological Evaluation. One part from each location was routinely fixed in formalin, embedded in paraffin, and stained with H&E. Histological slides were reviewed by an experienced gastrointestinal pathologist (F. B.), who was blinded to clinical data as well as to the results of flow cytometric and methylation analysis. In accordance with the standardized classification of dysplasia in inflammatory bowel disease, samples were classified as negative for dysplasia, IND, LGD, HGD, or CA (17).

Flow Cytometry. The second part of each sample was frozen at −80°C in a DMSO-citrate buffer until flow cytometric analysis. Before analysis, the samples were rapidly thawed in a water bath to 37°C. Samples were gently ground in a 0.3-mm steel mesh with a small glass pestle (18). The resulting cell suspension was flushed with DMSO-citrate buffer into a test tube. Cell nuclei were isolated by a detergent-trypsin technique and stained with propidium iodide (19). To minimize aggregates, the suspension was passed several times through a syringe during the incubation period. A total of 10,000 nuclei per specimen were subsequently analyzed in a fluorescein-activated cell sorter (FACScan™ flow cytometer, Becton Dickinson, Heidelberg, Germany). The coefficient of variation of the G_1 full peak in all of the samples was 3.1% (SD, 1.3). Samples with more than one peak in the histogram were judged as aneuploid. For aneuploid DNA samples, a DNA index was calculated as the ratio of the abnormal G_0-G_1 mean peak channel number to the diploid G_0-G_1 mean peak channel number. DNA histograms were classifed without knowledge of the histological results.

Methylation-specific PCR Amplification. After flow cytometry, high molecular weight DNA was isolated from the remaining nuclei according to standard methods of proteinase K/SDS digestion, phenol/chloroform extraction, and ethanol precipitation. A slight modification of the protocol suggested by Herman *et al.* (20) was applied. In brief, DNA modification by bisulfite exclusively converts unmethylated cytosines to uracil. Subsequent PCR amplification with primers specific for unmethylated *versus* methylated DNA reveals the methylation status of investigated DNA sections. Initially 1 μg of DNA in a volume of 50 μl was denatured by NaOH (final concentration 0.2 M) for 20 min at 37°C. Then 30 μl of 10 mM hydroquinone (Sigma, Deisenhofen, Germany) and 520 μl of 3 M sodium bisulfate (Sigma) at pH 6.0 were added. Samples were incubated at 55°C for 21 h. Modified DNA was purified using the Qiagen-PCR-Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Finally, a second NaOH treatment was performed (20 min at room temperature; final concentration, 0.3 M). Modified and purified DNA was precipitated by ethanol overnight and resuspended in 100 μl of water. Primer pairs for PCR amplification have been described previously (20) and were purchased from MWG-Biotech (Ebersberg, Germany). A 100-μl volume of PCR mixture contained 10 μl buffer (10 mM Tris-HCl, 50 mM KCl, and 0.1% Triton X-100), 1 μl of $MgCl_2$ (50 mM), 1.5 μl of dNTPs (1.25 mM), 100 pM primers, 2 units of *Taq* polymerase (PAN-Systems, Aidenbach, Germany), and 0.1 μl of DNA. Amplifications were performed in a temperature cycler (Biotherma, Göttingen, Germany) for 35 cycles (at 95°C for 5 min, at annealing temperature for 90 s, and at 72°C for 60 s) concluded by a final 8-min extension at 72°C. A control without the addition of DNA was performed for each PCR set. Twenty μl of PCR product were loaded on nondenaturating polyacrylamide gels (8%) and visualized by silver staining. If a methylation-specific PCR product was detected, the whole procedure of sodium bisulfite pretreatment and methylation-specific PCR amplification was performed another time to minimize the possible influence of contamination or incomplete bisulfite treatment. Hypermethylation was

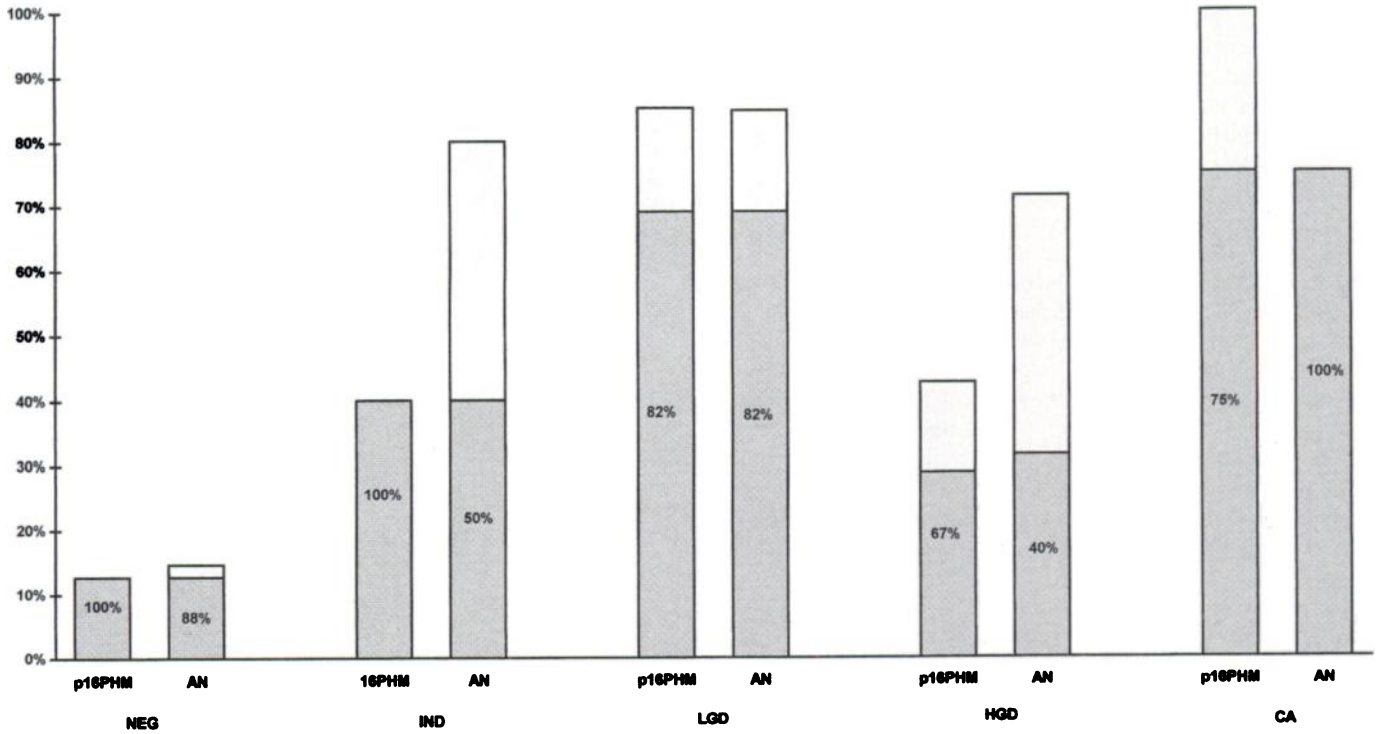

Fig. 1. The rate of detected *p16PHM* and of aneuploid cell populations (*AN*) relative to the grade of histomorphological lesions (*NEG*, *IND*, *LGD*, *HGD*, or *CA*). *Shaded areas* indicate the share of specimens with detected hypermethylation that simultaneously revealed AN or the share of specimens with AN that simultaneously revealed p16PHM, respectively.

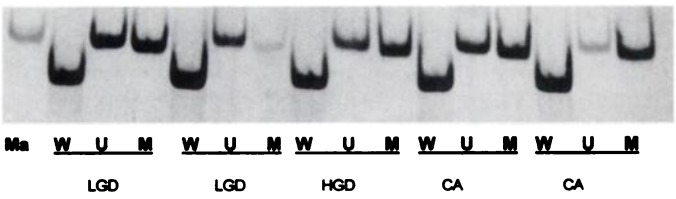

Fig. 2. Polyacrylamide gel, silver-stained, showing the PCR products for five different samples from patient P1. Each *first lane* (*W*) shows the amplification product for initial PCR with primers for promoter region 1 of p16^{INK4a}. Each *second lane* (*U*) shows products of PCR for unmethylated templates; each *third lane* (*M*) shows products of PCR for methylated templates. *Ma*, marker.

stated if at least two experiments had demonstrated an unequivocal amplification product of methylation-specific PCR.

Statistical Analysis. Statistical comparisons were performed using a χ^2 test.

RESULTS

A total of 89 tissue samples from 3 colectomy specimens of patients with ulcerative colitis was analyzed.

Thirty-four samples (38.2%) displayed dysplastic lesions; 10 samples showed lesions that were classified as IND; 13 samples revealed LGD; 7 revealed HGD; and 4 revealed carcinomatous lesions. The results of histomorphological analysis for each patient are given in Table 1.

A total of 35 specimens (39.3%) showed an aneuploid DNA index. Rates for detected AN in relation to the histomorphological diagnosis are demonstrated in Fig. 1. DNA AN was significantly correlated to the presence of dysplastic lesions (χ^2, 37.05; P = 0.0005; Table 1). The colectomy specimen of the patient without dysplastic lesions did not reveal aneuploid cell populations in any of the investigated locations, whereas 33.3% of the specimens without histomorphological lesions in patients P1 and P2 revealed the presence of aneuploid cell populations.

Initially performed DNA amplification with primers targeting the wild-type promoter region of the unmodificated *p16^{INK4A}* gene showed an unequivocal product in all of the specimens. Methylation of the promoter region of the *p16^{INK4A}* gene was detected in 29 specimens (32.6%; Fig. 2). In the patient without any dysplastic alteration throughout the colectomy specimen (P3), none of the 34 analyzed tissue samples showed a PCR product with methylation specific primers. Methylation was significantly associated with the presence of dysplasia and CA (χ^2, 25.84; P = 0.0005). Rates for detected p16PHM in relation to the histomorphological diagnosis are demonstrated in Table 1. With increasing severity of histomorphological lesions, a trend toward higher rates of promoter hypermethylation was observed. The relation of severity of histomorphological lesions and methylation status is demonstrated in Fig. 1. Fig. 3 schematically demonstrates the distribution of dysplasia, DNA ploidy, and methylation status of the *p16^{INK4A}* gene throughout the colectomy specimen of patient P1.

Ten (28.6%) of 35 samples showed DNA AN without the simultaneous detection of p16 promoter methylation. In 4 (13.7%) of 29 samples with p16 promoter methylation, no AN was found (Fig. 1).

DISCUSSION

It has been well documented that inactivation of the *p16^{INK4A}* gene is involved in disturbances of cell cycle regulation in a multitude of human malignancies. The loss of wild-type p16^{INK4A} expression by common mechanisms of gene inactivation as homozygous deletions or the LOH for a gene locus in association with point mutations of the remaining allele has been demonstrated for a variety of tumor-derived cell lines and some primary tumors (2, 5, 10, 15). However, in certain primary tumors including colon cancer, 9p21 LOH with somatic p16 mutations or homozygous deletions were only rarely found, whereas functional p16^{INK4A} loss seemed to be a common event (7). This triggered the search for alternative mechanisms of functional p16^{INK4A} repression. Recently, methylation of the promoter region was reported to be a predominant mechanism of inactivation of the tumor suppressor p16^{INK4A} in sporadic colon cancer (14).

Our study demonstrates for the first time a possible role of p16^{INK4A} promoter methylation for the process of neoplastic progression in ulcerative colitis. None of the samples retrieved from the patient without dysplastic lesions (P3) displayed methylation of the promoter region or AN; however, in the two patients with dysplasia (P1, P2), AN was present in 7 (33%) of 21 samples without dysplasia and in 19 (79%) of 24 samples with frank dysplasia or cancer. Methylation of the promoter region of p16^{INK4A} was present in 7 (33%) of 21 samples without dysplasia and in 18 (75%) of 24 samples with dysplasia or cancer. In colons with dysplastic lesions, the frequency detected of both p16^{INK4A} promoter methylation and AN increased with the severity of histomorphological lesions.

All carcinomatous samples that were analyzed revealed p16^{INK4A} promoter methylation. Thus, inactivation of the tumor suppressor gene *p16^{INK4A}* by hypermethylation of its promoter region obviously is a common epigenetic event also during the process of neoplastic transformation in ulcerative colitis. These data are in accordance with those results reported on the significance of p16^{INK4A} promoter methylation in sporadic colorectal cancer. Herman *et al.* (14) detected methylation of CpG islands in the promoter region of p16^{INK4A} in

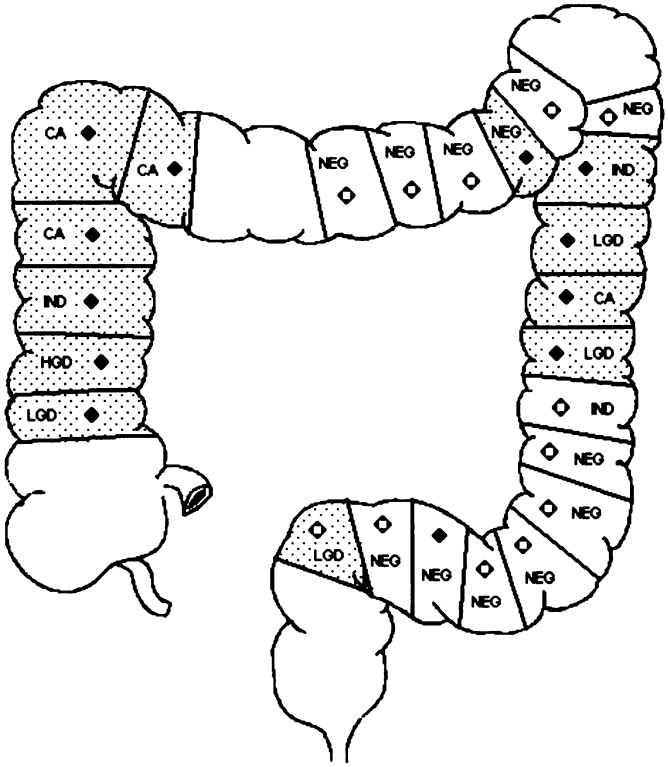

Fig. 3. Schematogram of the colectomy specimen of patient P1 demonstrating the methylation status of p16 promoter (*shaded*, detected hypermethylation) in relation to DNA ploidy (◆, aneuploid cell population; ◇, diploid cell population) and histological diagnosis (*NEG*, *IND*, *LGD*, *HGD*, or *CA*).

92.0% of tumor-derived cell lines of colon cancer and in 40% of primary sporadic colon CAs.

To our knowledge there is still no clear evidence in regard to the time of onset of p16INK4A abnormalities during the adenoma-carcinoma sequence in sporadic colon cancer. In our study, 40.0% of specimens with histomorphological lesions indefinite for dysplasia and 70% of specimens with dysplasia revealed a positive methylation status. Furthermore, in 33.3% of tissue samples without dysplasia, exclusively located in the two colectomy specimens with dysplasia or CA nearby, methylation was detected. In comparison, none of the 34 samples in the specimen without any dysplastic lesions showed a positive methylation status. These data suggest that p16INK4A promoter methylation is an early epigenetic event during the process of neoplastic progression in ulcerative colitis. Elegant studies by Reid and colleagues (4, 11, 21) have shown a similar situation for the development of adenocarcinoma in Barrett's esophagus. Allelic loss of 9p21 was found previously in neoplastic stages that were assessed as premalignant and that showed no AN (21). In regard to p16INK4A promoter hypermethylation, aneuploid cell populations were analyzed, and hypermethylation was detected in neoplastic stages classified as premalignant (11).

For some human malignancies, a prognostic significance of *p16INK4A* gene impairment has been reported. A correlation between loss of p16INK4A expression and a more aggressive tumor behavior or less favorable prognosis has been shown for melanoma, pancreatic CA, and lymphomas (22–24). Studies have shown that the detection of aneuploid cell populations in ulcerative colitis is of independent prognostic significance in regard to the development of ulcerative colitis-associated neoplasia (25). The discrepancy between the rates of detected AN and p16INK4A promoter hypermethylation in our study underlines the need to elucidate a possible prognostic significance of p16INK4A promoter hypermethylation in regard to the development of dysplasia or CA in ulcerative colitis.

In conclusion, our data suggest a major role of functional p16INK4A repression by promoter methylation in the process of neoplastic progression in ulcerative colitis. Methylation of the *p16INK4A* gene promoter was shown to be a common, likely, and early event during that process. These findings suggest that p16INK4A promoter methylation is a candidate biomarker in the surveillance of patients with ulcerative colitis and requires the evaluation of its prognostic significance in a longitudinal study.

REFERENCES

1. Serrano, M., Hannon, G. J., and Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK 4. Nature (Lond.), *366:* 704–707, 1993.
2. Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S., Johnson, B. E., and Skolnick, M. H. A cell cycle regulator potentially involved in genesis of many tumor types. Science (Washington DC), *246:* 436–440, 1994.
3. Sherr, C. J. Cancer cell cycles. Science (Washington DC), *274:* 1672–1677, 1996.
4. Barrett, M. T., Galipeau, P. C., Sanchez, C. A., Emond, M. J., and Reid, B. J. Determination of the frequency of loss of heterozygosity in esophageal adenocarcinoma by cell sorting, whole genome amplification and microsatellite polymorphisms. Oncogene, *12:* 1873–1878, 1996.
5. Gonzalez-Zulueta, M., Bender, C. M., Yang, A. S., Nguyen, T., Beart, R. W., Van Tornout, J. M., and Jones, P. A. Methylation of the 5′ CpG island of the *p16/CDKN2* tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. Cancer Res., *55:* 4531–4535, 1995.
6. Spruck, C. H., Gonzalez-Zulueta, M., Shibata, A., Simoneau, A. R., Lin, M-F., Gonzales, F., Tsai, Y., and Jones, P. A. p16 gene in uncultured tumors. Nature (Lond.), *370:* 183–184, 1994.
7. Cairns, P., Polascik, T. J., Eby, Y., Tokino, K., Califano, J., Merlo, A., Mao, L., Herath, J., Jenkins, R., Westra, W., Rutter, J., Buckler, A., Gabrielson, E., Tockman, M., Cho, K. R., Hedrick, L., Bova, G. S., Isaacs, W., Koch, W., Schwab, D., and Sidransky, D. Frequency of homozygous deletion at p16/CDKN2 in primary human tumors. Nat. Genet., *11:* 210–212, 1995.
8. Li, E., Beard, C., and Jaenisch, R. Role for DNA methylation in genomic imprinting. Nature (Lond.), *366:* 362–365, 1993.
9. Costello, J. F., Berger, M. S., Huang, H-J. S., and Cavenee W. K. Silencing of *p16/CDKN2* expression in human gliomas by methylation and chromatin condensation. Cancer Res., *56:* 2405–2410, 1996.
10. Herman, J. G., Civin, C. I., Issa, J. P., Collector, M. I. Sharkis, S. J., and Baylin, S. B. Distinct patterns of inactivation of *p15INK4B* and *p16INK4A* characterize the major types of hematological malignancies. Cancer Res., *57:* 837–841, 1997.
11. Wong, D. J., Barrett, M. T., Stöger, R., Emond, M. J., and Reid, B. J. *p16INK4a* promoter is hypermethylated at a high frequency in esophageal adenocarcinoma. Cancer Res., *57:* 2619–2622, 1997.
12. Ahuja, N., Mohan, A. L., Li, Q., Stolker J. M., Herman J. G., Hamilton, S. R., Baylin, S. B., and Issa, J-P. J. Association between CpG island methylation and microsatellite instability in colorectal cancer. Cancer Res., *57:* 3370–3374, 1997.
13. Schutte, M., Hruban, R. H., Geradts, J., Maynard, R., Hilgers, W., Rabindran, S. K., Moskaluk C. A., Hahn, S. A., Schwarte-Waldhoff, I., Schmiegel, W., Baylin, S. B., Kern, S. E., and Herman, J. G. Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. Cancer Res., *57:* 3126–3130, 1997.
14. Herman, J. G., Merlo, A., Mao, L., Lapidus, R. G., Issa, J. P., Davidson, N. E., Sidransky, D., and Baylin, S. B. Inactivation of the *CDKN2/p16/MTS1* gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res., *55:* 4525–4530, 1995.
15. Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P. C., Baylin, S. B., and Sidransky, D. 5′ CpG island methylation is associated with transcriptional silencing of the tumor suppressor p16/CDKN2/MTS1 in human cancers. Nat. Med., *1:* 686–692, 1995.
16. Ekbom, A. Cancer in inflammatory bowel disease. Curr. Opin. Gastroen., *11:* 316–320, 1995.
17. Riddell, R. H., Goldman, H., Ransohoff, D. F., Appelman, H. D., Fenoglio, C. M., Haggitt, R. C., Ahren, C., Correa, P., Hamilton, S. R., and Morson, B. C. Dysplasia in inflammatory bowel disease: standardized classification with provisional clinical applications. Hum. Pathol., *14:* 931–966, 1983.
18. Porschen, R., Robin, U., Schumacher, A., Schauseil, S., Borchard, F., Hengels, K. J., and Strohmeyer, G. DNA aneuploidy in Crohn's disease and ulcerative colitis: results of a comparative flow cytometric study. Gut, *33:* 663–667, 1992.
19. Vindelov, L. L., Christensen, I. J, and Nissen, N. I. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry, *3:* 323–327, 1983.
20. Herman, J. G., Graff, J. R., Myöhänen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA, *93:* 9821–9826, 1996.
21. Barrett, M. T., Sanchez, C. A., Galipeau, P. C., Neshat, K., Emond, M., and Reid, B. J. Allelic loss of 9p21 and mutation of the CDKN2/p16 gene develop as early lesions during neoplastic progression in Barrett's esophagus. Oncogene, *13:* 1867–1873, 1996.
22. Hu, Y. X., Watanabe, H., Ohtsubo, K., Yamaguchi, Y., Ha, A., Okai, T., and Sawabu, N. Frequent loss of p16 expression and its correlation with clinicopathological parameters in pancreatic carcinoma. Clin. Cancer Res., *3:* 1473–1477, 1997.
23. Pinyol, M., Hernandez, L., Cazorla, M., Balbin, M., Jares, P., Fernandez, P. L., Montserrat, E., Cardesa, A., Lopez-Otin, C., and Campo, E. Deletions and loss of expression of p16INK4A and p21Waf1 genes are associated with aggressive variants of mantle cell lymphomas. Blood, *89:* 272–280, 1997.
24. Reed, J. A., Loganzo, F., Shea, C. R., Walker, G. J., Flores, J. F., Glendening, J. M., Bogdany, J. K., Shiel, M. J., Haluska, F. G., Fountain, J. W., and Albino, A. P. Loss of expression of the *p16*/cyclin-dependent kinase inhibitor 2 tumor suppressor gene in melanocytic lesions correlates with invasive stage of tumor progression. Cancer Res., *55:* 2713–2718, 1995.
25. Rubin, C. E., Haggitt, R. C., Burmer, G. C., Brentnall, T. A., Stevens, A. C., Levine, D. S., Dean, P. J., Kimmey, M., Perera, D., and Rabinovitch, P. DNA aneuploidy in colonic biopsies predicts future development of dysplasia in ulcerative colitis. Gastroenterology, *103:* 1611–1620, 1992.

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