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Advances in Brief

Hypermethylation Can Selectively Silence Individual *p16^{ink4A}* Alleles in Neoplasia[1]

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Abstract

Inactivation of *p16^{ink4A}* and other tumor suppressor genes has been associated with promoter region hypermethylation in neoplasia. However, direct proof for aberrant DNA methylation as an independent event for loss of gene function has been difficult to obtain. We addressed this question in the colon carcinoma cell line HCT116, which contains one allele of *p16^{ink4A}* with a coding region frameshift mutation and one wild-type allele. Neither allele contains a mutation in the proximal promoter region. The promoter of the wild-type allele, but not the mutant allele, is hypermethylated, and only the mutant allele is expressed. Transcription from the methylated/wild-type allele was restored after cell treatment with the demethylating agent 5-aza-2′-deoxycytidine. Thus, in neoplastic cells, stable allele-specific loss of transcription may arise from aberrant methylation of a nonmutated promoter region, identifying hypermethylation as a direct mechanism for tumor suppressor gene inactivation.

Introduction

Inactivation of the *p16^{ink4A}* cyclin-dependent kinase inhibitor is one of the most commonly observed abnormalities in human cancer, and inactivation of the cyclin D-p16-Rb pathway could indeed be required for progression of most tumor types (1). Whereas genetic mechanisms, including point mutations (2–4) and, more commonly, homozygous deletions (2, 5), often inactivate *p16^{ink4A}* in tumors, loss of *p16^{ink4A}* expression and several other tumor suppressor genes can also be associated with promoter region hypermethylation (6–13). This latter change accompanies a transcriptional repression that seems to be an alternative to coding region mutations for loss of gene function (6, 10, 14). However, molecular evidence that promoter region hypermethylation is directly associated with transcriptional loss has been difficult to establish. We now show that in the same cell, individual alleles of *p16^{ink4A}* are transcriptionally silenced by abnormal DNA methylation, demonstrating that DNA methylation can lead directly to loss of transcription, rather than simply being a mark for this process.

Materials and Methods

Southern Analysis and Cell Culture. Methylation analysis of the 5′ CpG island of p16 was performed exactly as described previously (7). Quantitation was carried out using ImageQuant software after exposure using a PhosphorImager (Molecular Dynamics). Treatment with 5-aza-2′-deoxycytidine was at a concentration of 1 μM for 5 days as described previously (10).

MSP.[3] Bisulfite treatment was as described previously (15). Unmethylated and methylated alleles of *p16^{ink4A}* were specifically amplified from bisulfite-modified DNA with an anchored upstream primer U1 (5′-TTTTTAGAGGATTTGAGGGATAG) and methylation-specific antisense primers p16-U2 and p16-M2 (15), respectively. A nested PCR was performed with biotinylated antisense primer BIO U/M (5′-AATCRACCTCCRACCRTAACTATT) and sense primer U2N (5′-GATTTGAGGGATAGGGTAGGAGG) to produce single-stranded templates for DNA sequencing (16). Biotinylated PCR products were isolated using streptavidin-coated magnetic beads (Dynal AB, Oslo, Norway). Sequencing reactions were performed with Sequenase DNA sequencing kit (United States Biotechnology) after strand separation with alkali treatment. A nested, methylation nonspecific primer, p16seq (5′-TTTTGTTAGTATTAGGAGGAAGA), was used in sequencing.

Genomic sequencing of normal colon was performed with two sets of primers that do not contain any CpG sites: U1 (15) and L1 (5′-CTACCTAATTCCAATTCCCCTACA) for the first PCR; and U2N (15) and L2BIO (5′-TCCAATTCCCCTACAAACTTC) for the nested PCR. Primer p16seq was used as a sequencing primer (15).

cDNA Analysis. cDNA synthesis was performed as described previously (10). Primers 5′-TGGAGCCTTCGGCTGACT and 5′-GGGACCTTCCGCGGCCAT from exon 1 and exon 2, respectively, were used to amplify a product of 428 bp. Primers for the nested PCR, ex1a (5′-TTCGGCTGACTGGCTGGC) and ex1b (5′-CTGGATCGGCCTCCGAC), were both from exon 1. The lower-strand primer was labeled with biotin to facilitate solid-phase DNA sequencing of the amplified products. Primer ex1a was used as a sequencing primer.

Promoter Sequencing. Primers P1A (5′-TAGCTCCCTCCCCATTTTCCTAT) and P1B (5′-ACCCTCTACCCACCTGGATCG) were used to amplify a 1068-bp product of the *p16^{ink4A}* promoter region (−905 to +163 from ATG) followed by nested PCR with primers P2A (5′-TTTTCCTATCTGCCTACAGGC-3′) and P2B (5′-GGCCTCCGACCGTAACTAT) that amplify a product of 1034 bp. The lower-strand primer in the nested PCR was biotinylated. Sequencing was performed with primers P2A, SeqA1 (5′-TGAACCCCGCGCTCCTG), SeqA (5′-TGGCAGTTAGGAGGTTGT), SeqB (5′-TTCGCTAAGTGCTCGGAGT), and SeqC (5′-AGGAGGGGCTGGCTGGTC).

Results and Discussion

In studies of *p16^{ink4A}* alterations in human neoplasia, we encountered a human colon cancer cell line with an unusual pattern of hypermethylation. Whereas most colon cancer cell lines contain only hypermethylated *p16^{ink4A}* alleles (7), cell line HCT116 exhibits 50% methylation at the promoter region CpG island of *p16^{ink4A}* (Fig. 1*a*). Previously, a frameshift mutation in the first exon of *p16^{ink4A}* was reported in HCT116, resulting in a premature stop codon (17). This cell line provided an excellent model to study the primary consequences of promoter region hypermethylation for inactivation of *p16^{ink4A}*.

To determine whether the 50% methylation reflected allele-specific methylation of *p16^{ink4A}*, we used MSP (15) to specifically amplify unmethylated and methylated alleles of the *p16^{ink4A}* promoter region CpG island in HCT116. MSP allows amplification of alleles dependent on methylation status, because bisulfite treatment of DNA changes unmethylated, but not methylated, cytosines to uracil (18). In Fig. 1*b*, the presence of both unmethylated and methylated alleles is demonstrated for HCT116. Sequencing of these PCR products revealed that only the nonmutant allele CpG island was hypermethylated, and that every CpG site in this region had undergone this change (Fig. 2*A*). In contrast, products amplified with primers specific for unmethylated DNA contained only alleles with the previously described (17) frameshift mutation in exon 1, but they had no methylation at any CpG site

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[3] The abbreviation used is: MSP, methylation-specific PCR.

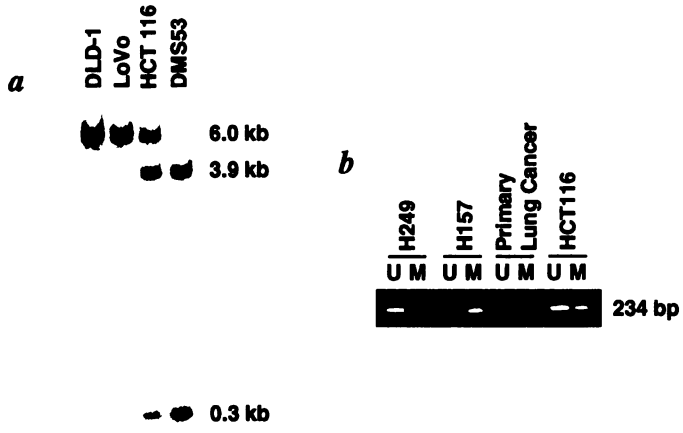

Fig. 1. Partial methylation of $p16^{ink4A}$ in HCT116. *a*, Southern analysis of DNA from cancer cell lines restricted with *Hin*dIII and *Sac*II. The presence of the 6-kb fragment indicates methylation of CpGs within both *Sac*II restriction sites (Ref. 7; Fig. 2*D*). *b*, MSP with bisulfite modification and amplification (15). H249 is a lung cancer cell line with only unmethylated $p16^{ink4A}$ alleles, whereas H157 is a lung cancer cell line with only methylated $p16^{ink4A}$ alleles (10). The primary lung cancer sample contains both unmethylated and methylated $p16^{ink4A}$ alleles (15). The presence of a PCR product indicates either unmethylated (*U*) or methylated (*M*) alleles in the DNA samples. *U* represents reactions using p16-U2 primers specific for unmethylated CpG sites; *M* represents reactions using p16-M2 primers specific for methylated CpG sites (15).

in this region (Fig. 2*B*). Genomic sequencing of normal colon shows no methylation of the $p16^{ink4A}$ promoter and only the wild-type sequence (Fig. 2*C*).

Point mutations of $p16^{ink4A}$ are unusual in colon cancer. The frameshift mutation in HCT116 may have resulted from mutation in the mismatch repair gene hMLH1, which induces a high rate of frameshift mutations in HCT116 cells (19). This raises the possibility that hypermethylation of the $p16^{ink4A}$ allele with a wild-type coding region might be associated with mutation in the functional promoter. Hypermethylation might arise secondarily through impaired transcription or loss of key elements, such as Sp1 binding sequences, which may protect CpG islands from methylation (20–22). To examine this possibility, we sequenced the entire basal promoter (23) of $p16^{ink4A}$ in HCT116, three colon cancer cell lines with methylated $p16^{ink4A}$ alleles (COLO320, SW48, and RKO), and a small cell lung carcinoma cell line (NCI H69) with unmethylated $p16^{ink4A}$ alleles (7, 10). All Sp1 sites (Fig. 2*D*) were unaltered in all cell lines. No mutations were found in $p16^{ink4A}$ alleles in the 0.9-kb promoter region for HCT116 or COLO320 cell lines. RKO, SW48, and H69 cells contained alleles with the same promoter region sequences as those in the HCT116 and COLO320 cell lines, but they also had alleles with an apparent polymorphism (A/G) at position −191 from the translation start site (data not shown). Because this polymorphism was present in tumors with unmethylated $p16^{ink4A}$ alleles (H69) and in those with methylated $p16^{ink4A}$ alleles (SW48 and RKO), this G/A polymorphism does not correlate with hypermethylation of the promoter.

We next examined the expression pattern of the methylated and unmethylated $p16^{ink4A}$ alleles in HCT116. Direct sequencing of reverse transcription-PCR products revealed that only the frameshift mutant allele is expressed (Fig. 3). To evaluate whether hypermethylation plays a direct role in selective silencing of the nonmutant allele, we treated HCT116 cells with the demethylating agent 5-aza-2′-deoxycytidine. Within 5 days, expression was apparent from both the mutant and the nonmutant allele (Fig. 3).

The present study provides the most definitive evidence to date that promoter region hypermethylation can selectively target individual tumor suppressor gene alleles. Previous studies have shown that partial activation of hypermethylated tumor suppressor genes can be achieved in neoplastic cells by reducing the aberrant methylation after treatment of cells with 5-aza-2′-deoxycytidine (6–10). These results implied but were not conclusive for a direct role for promoter region hypermethylation in the transcriptional silencing. Allele-specific hypermethylation had been proposed for inactivation of the retinoblastoma gene in primary retinoblastoma (13). However, the transcriptional consequence of the hypermethylated allele was not studied, and inactivation of the other, unmethylated allele was not demonstrated. Thus, that study did not provide evidence for allele-specific inactivation of *Rb*. Our results now clearly show that in a single tumor cell, $p16^{ink4A}$ alleles can be inactivated by two distinct mechanisms: (*a*) point mutation of one allele; and (*b*) promoter region hypermethylation of the other. Because the HCT116 cell line is capable of transcribing $p16^{ink4A}$, as demonstrated by the expression of the mutant $p16^{ink4A}$ allele, inactivation of the nonmutant allele is not the result of impaired transcriptional capacity, but the consequence of promoter region hypermethylation.

In HCT116, the simultaneous presence of nonmethylated and methylated $p16^{ink4A}$ alleles is similar to the promoters of several imprinted genes. In imprinted genes, transcription is silenced from one of two alleles in each cell through local control of the promoter by a parentally determined mechanism. Hypermethylation in the promoter region of such genes is critical at least for maintenance of transcriptional repression (24). There is no evidence, however, that $p16^{ink4A}$ is

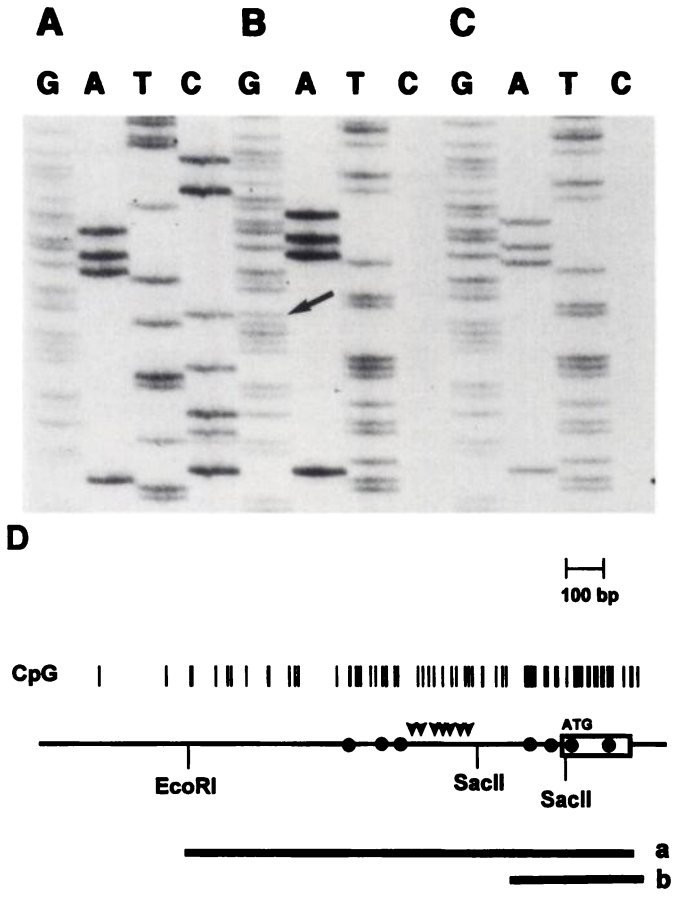

Fig. 2. Direct solid-phase sequencing of $p16^{ink4A}$-specific PCR products amplified from HCT116 cells using primers specific either for methylated (*A*) or unmethylated (*B*) cytosines. *C*, genomic sequencing of normal colon. The *arrow* in *B* points to a 1-bp insertion of a G residue resulting in a frameshift in exon 1. *D*, representation of the $p16^{ink4A}$ promoter and exon 1 region. CpG sites are shown above. *Arrows*, transcription start sites (23); *circles*, potential Sp1 binding sites. The coding region is depicted by a *box*, and the *Sac*II sites used for Southern analysis are indicated. Shown below are the promoter region (*a*) beginning from *Eco*RI, sequenced to exclude mutations, and the region analyzed by bisulfite-modified genomic sequencing (*b*).

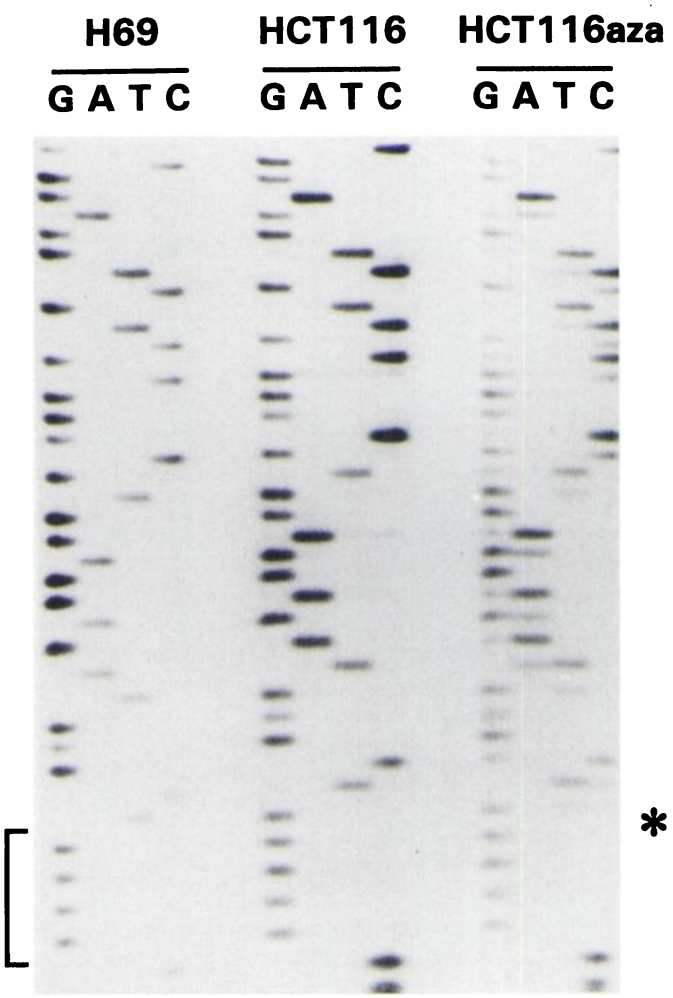

Fig. 3. Direct solid-phase sequencing of $p16^{ink4A}$-specific reverse transcription-PCR products from cell lines NCI H69, HCT116, and HCT 116 after treatment with 5-aza-2′-deoxycytidine (HCT116aza). *, the position of the frameshift in cDNA from cell line HCT116. After 5-aza-2′-deoxycytidine treatment, both wild-type and mutant alleles of $p16^{ink4A}$ are expressed.

imprinted, and no evidence of promoter region methylation of $p16^{ink4A}$ is found in normal tissues, including colon (Fig. 2*C*). Therefore, the inactivation of the nonmutant allele in HCT116 is a tumor-specific event.

The precise mechanisms leading to abnormal hypermethylation of CpG islands in neoplastic cells are poorly understood. Cell culture may lead to an increase in hypermethylated CpG islands (25). However, despite extended cell passage, the mutant $p16^{ink4A}$ allele in cell line HCT116 remains unmethylated, whereas the nonmutant allele is densely hypermethylated. Thus, each independent inactivation event, mutation of one allele and hypermethylation of the other, is selected for and independently maintained full loss of $p16^{ink4A}$ tumor suppressor function. Aberrant DNA methylation as a direct inactivating event for loss of gene function is likely to affect other genes critical for tumor initiation and/or progression. Further study of the mechanisms underlying the direct transcriptional silencing will be important not only for these genes but may also further our understanding of imprinted genes and the inactivation of the X chromosome.

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