Inactivation of the CDKN2/p16/MTS1 Gene is Frequently Associated with Aberrant DNA Methylation in all Common Human Cancers

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Advances in Brief

Inactivation of the *CDKN2/p16/MTS1* Gene Is Frequently Associated with Aberrant DNA Methylation in All Common Human Cancers[1]

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Abstract

The tumor suppressor gene *CDKN2/p16/MTS1*, located on chromosome 9p21, is frequently inactivated in many human cancers through homozygous deletion. Recently, we have reported another pathway of inactivation that involves loss of transcription associated with *de novo* methylation of a 5′ CpG island of *CDKN2/p16* in lung cancers, gliomas, and head and neck squamous cell carcinomas. We now show that this aberrant CpG island methylation also occurs frequently in cell lines of breast cancer (33%), prostate cancer (60%), renal cancer (23%), and colon cancer (92%) and is associated with loss of transcription. Primary tumors of the breast (31%) and colon (40%) also displayed *de novo* methylation of this CpG island. This alteration of p16 in colon cancer was particularly striking, since inactivation does not occur through homozygous deletion in this tumor type. Our data show that in tumors, *de novo* methylation of the 5′ CpG island is a frequent mode of inactivation of *CDKN2/p16* and also firmly demonstrate that *CDKN2/p16* is one of the most frequently altered genes in human neoplasia.

Introduction

Frequent LOH[3] and homozygous deletion of chromosome 9p21 has suggested the presence of tumor suppressor genes in this region. Localization of an inhibitor of the cyclin D/CDK4 complex (CDKN2/p16) to 9p21, along with frequent homozygous deletions of this gene in human cancer cell lines (1, 2), suggested that *p16* might be the target gene. Since the initial reports of this homozygous deletion, numerous studies have shown varying, but in general much less frequent, abnormalities of *p16* in primary tumors of these cancers (3–8). For example, although the rate of homozygous deletions ranged from 40–60% of breast cancer cell lines (1, 8), neither homozygous deletion or point mutations were observed frequently (8) in primary breast carcinomas. Furthermore, certain common neoplasms, such as prostate and colon cancer, have not been found to harbor homozygous deletions in established cell lines (1, 4).

In recent studies, our group has focused on hypermethylation of gene promoter regions called CpG islands, which is associated with loss of transcription (9), as demonstrated for the *VHL* gene in human renal cell cancer (10). We have shown recently that for some neoplasms (non-small cell lung cancer, gliomas, and head and neck squamous cell carcinoma), *de novo* methylation of the 5′ CpG island of *p16* was a frequent abnormality of this gene (11). This aberrant methylation was correlated with loss of expression of the normal *p16* or *VHL* mRNA, and reactivation of each gene was obtained by treatment of aberrantly methylated cell lines with the demethylating agent, 5-deoxyazacytidine (10, 11), suggesting that this as an alternate mechanism of inactivation.

In the present study, we report that hypermethylation of the 5′CpG island of CDKN2/p16 is frequent in cell lines and primary tumors of other common human neoplasms. Furthermore, inactivation through DNA methylation can occur not only in neoplasms where homozygous deletion is frequent in cell lines (breast, renal cell) but also in those which are not commonly associated with loss of *p16* through homozygous deletion (colon and prostate). Our findings establish that hypermethylation of the *p16* gene promoter region is a common abnormality of *p16* in human cancer.

Materials and Methods

Southern Hybridization. Five μg of genomic DNA was digested overnight according to conditions specified by the manufacturer (New England Biolabs), followed by ethanol precipitation. Restricted DNA was fractionated on a 1% agarose gel and transferred to Zeta-Probe nylon membranes (Bio-Rad). Filters were hybridized with a random, prime-labeled, 340-bp PCR product that included exon 1 (Fig. 1), as described (11), at 65°C overnight, followed by stringent washes including a final wash at 70°C for 30 min with 0.1× SSC-0.1% SDS. The blots were then exposed in a phosphorimager (Molecular Dynamics).

RT-PCR. RT-PCR was performed as described previously (11) from 6 μg of total RNA used to generate cDNA. These cDNA products were then amplified by PCR, using primers for exons 1 and 2 of *p16* yielding a 428-bp product. To control for cDNA quality, β-actin was used as a control, as described previously (12). PCR products were analyzed on 1.5% agarose gels and stained with ethidium bromide.

LOH. DNA from the 13 colon cancer cell lines was analyzed for heterozygosity by amplification of dinucleotide repeat containing sequences using PCR and conditions described previously (13). The primers *D9S156, IFNA, D9S171, D9S126,* and *D9S200* were obtained from Research Genetics (Huntsville, AL). The absence of the second allele in all five highly polymorphic markers on 9p was used as statistical evidence for LOH (14).

Results and Discussion

Exon 1 of *p16* lies in a typical CpG island (15), which is unmethylated in all normal tissues tested (11). Restriction with a nonmethylation-sensitive restriction enzyme, such as *Hin*dIII or *Eco*RI, provides a convenient flanking cut which, when combined with a methylation-sensitive enzyme (such as *Eag*I, *Sac*II, or *Sma*I), allows rapid determination of the methylation status of this CpG island. We first used this approach to study breast and renal cancer cell lines, which have frequent homozygous deletion of *p16* (1). Southern analysis confirmed homozygous deletions of *p16* in human breast cancer cell lines MDA-MB-231 and MCF-7 (1) and Hs578t (8), since hybridization with exon 1 (Fig. 2*A*) or exon 2 of *p16* produced no visible bands, while control probes revealed adequate high molecular weight DNA (data not shown). However, in two breast cancer cell lines (ZR-75–1 and T47D), restriction with flanking enzymes and either of

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[3] The abbreviations used are: LOH, loss of heterozygosity; *Rb*, retinoblastoma gene; *VHL*, von Hippel-Lindau gene; RT-PCR, reverse transcription PCR.

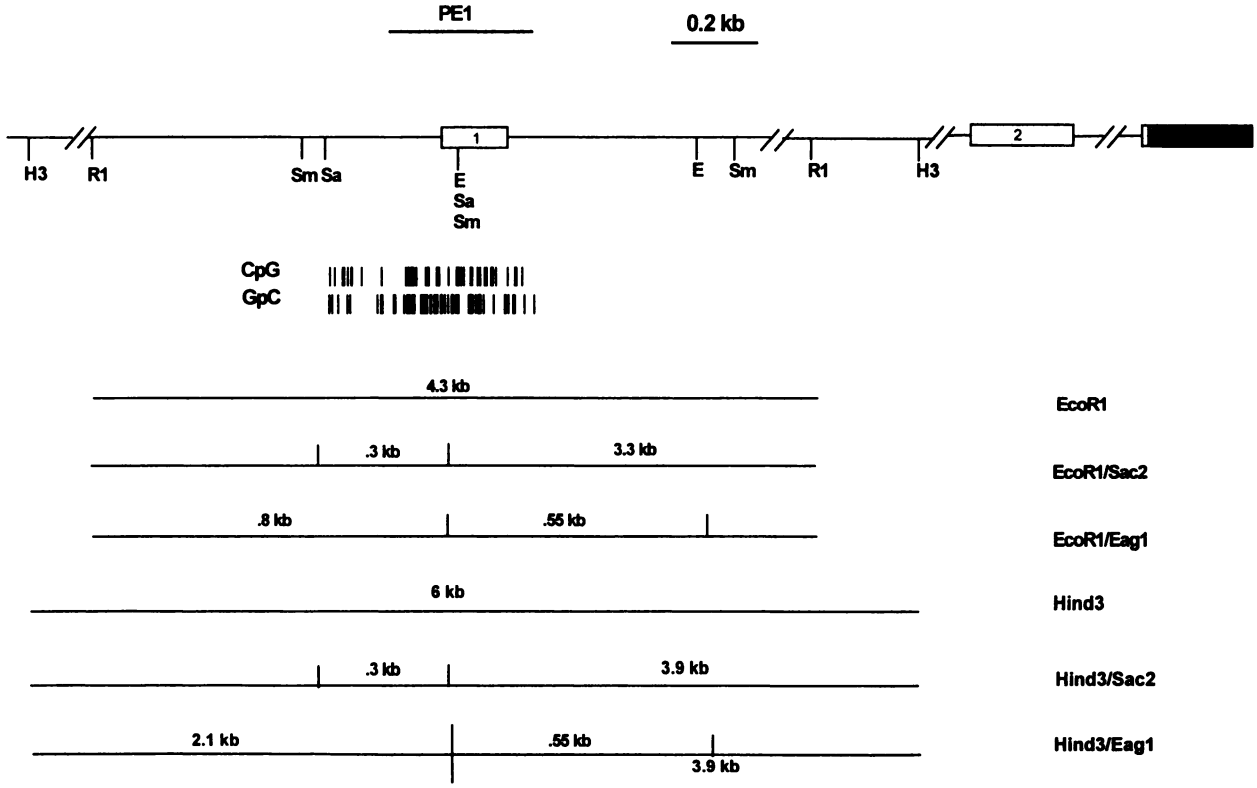


Fig. 1. Representation of the *p16* gene. PE1 is the probe used for Southern analysis. *Boxes* in the *second line* are exons 1–3, with coding regions in *white* and the 3′ untranslated region *shaded gray*. A restriction map including the flanking enzymes *Hin*dIII (*H3*) and *Eco*RI (*R1*) and the position of the methylation-sensitive restriction enzyme sites used to determine the methylation status of this CpG island, including *Eag*I (*E*), *Sac*II (*Sa*), and *Sma*I (*Sm*), is also shown. The density of CpG and GpC dinucleotides are shown below the gene, and the predicted restriction fragments for the enzymes in the study are depicted at *bottom*.

the methylation-sensitive restriction enzymes *Eag*I or *Sac*II produced a pattern of complete (T47D) or predominant (ZR-75–1) methylation (Fig. 2*A*), while one breast cancer cell line (MDA-MB-468) was unmethylated. We also examined renal carcinomas analyzed previously for mutations and hypermethylation of *VHL* (10). Of 26 cell lines examined, 13 had homozygous deletion, consistent with rates of homozygous deletion reported previously (1). However, 6 of the remaining 13 cell lines displayed abnormally hypermethylated *p16* (Table 1).

Primary tumors also displayed aberrant methylation of *p16*, with a renal cell carcinoma providing a vivid example. DNA from this renal cell carcinoma was hypermethylated at *p16* in cell lines obtained from both the primary and metastatic tumor from the same patient. In addition, DNA from the original primary tumor and this metastasis contained the same hypermethylated alleles of *p16* (Fig. 3*A*). These data indicate that the aberrant methylation was present *in vivo* and occurred prior to metastatic spread. DNA from this patient's normal adjacent kidney, as well as DNA from the normal kidneys of four other patients with renal cancer and two patients without cancer, did not contain these hypermethylated alleles. In primary breast cancer, 5 of 16 tumors (31%) also had hypermethylated *p16* alleles. In these breast tumors, this *p16* methylation was still detectable (Fig. 3*B*; and was confirmed on repeat analysis), despite a considerable amount of contaminating normal cells certain to prevent accurate assessment of homozygous deletion.

These findings confirmed that *p16* methylation occurs frequently in common tumors in which homozygous deletions are also a common mechanism of inactivation. However, we have also found the methylation events in common cancer cell lines where homozygous deletions of *p16* have never (colon) or rarely (prostate) been described (1, 16). In 3 of 5 prostate cancer cell lines (PC3, TSUPr1, and DuPro-1) and 12 of 13 colon cancer cell lines, the *p16* CpG island of exon 1 was completely methylated, while only two prostate cancer cell lines (LNCaP and DU145) and one colon cancer cell line were unmethylated (SW1417; Fig. 2). None of these 18 cell lines contained homozygous deletions of the gene, consistent with prior observations. In 20 primary colorectal carcinomas, we again found no evidence of homozygous deletion in this tumor type, but 8 of the 20 tumors (40%) had hypermethylation of the 5′ CpG island of *p16* (Fig. 3*C*). In contrast, *p16* was unmethylated in 22 normal adjacent colons and normal colons from individuals without cancer. We also examined six adenomatous polyps, the preinvasive lesion from which colorectal carcinoma arises, for evidence of hypermethylation at *p16*. One large 40-mm adenoma contained aberrant methylation of *p16*, whereas the remaining five smaller polyps displayed the normal methylation pattern, suggesting that inactivation of *p16* by methylation occurs during progression from this early lesion to the carcinomatous lesion. These overall results in colon and prostate cancer were surprising and demonstrate that *p16* is much more frequently inactivated in human tumors than realized previously.

Our findings of *p16* hypermethylation in colon cancer were further striking, not only because homozygous deletions were absent, but also since LOH at 9p21 is rare in this tumor type (16). Therefore, we examined these cell lines for evidence of LOH. Four of 13 colon cancer cell lines contained just 1 allele at all 5 microsatelite markers surrounding this region, suggesting monosomy of chromosome 9. Of the remaining cell lines, seven remained heterozygous at all markers (data not shown) and included the single colon cancer cell line (SW1417) with an unmethylated *p16*. Two other cell lines were considered noninformative, since they were heterozygous for only some of the five markers. Retention of both alleles in six of the cell

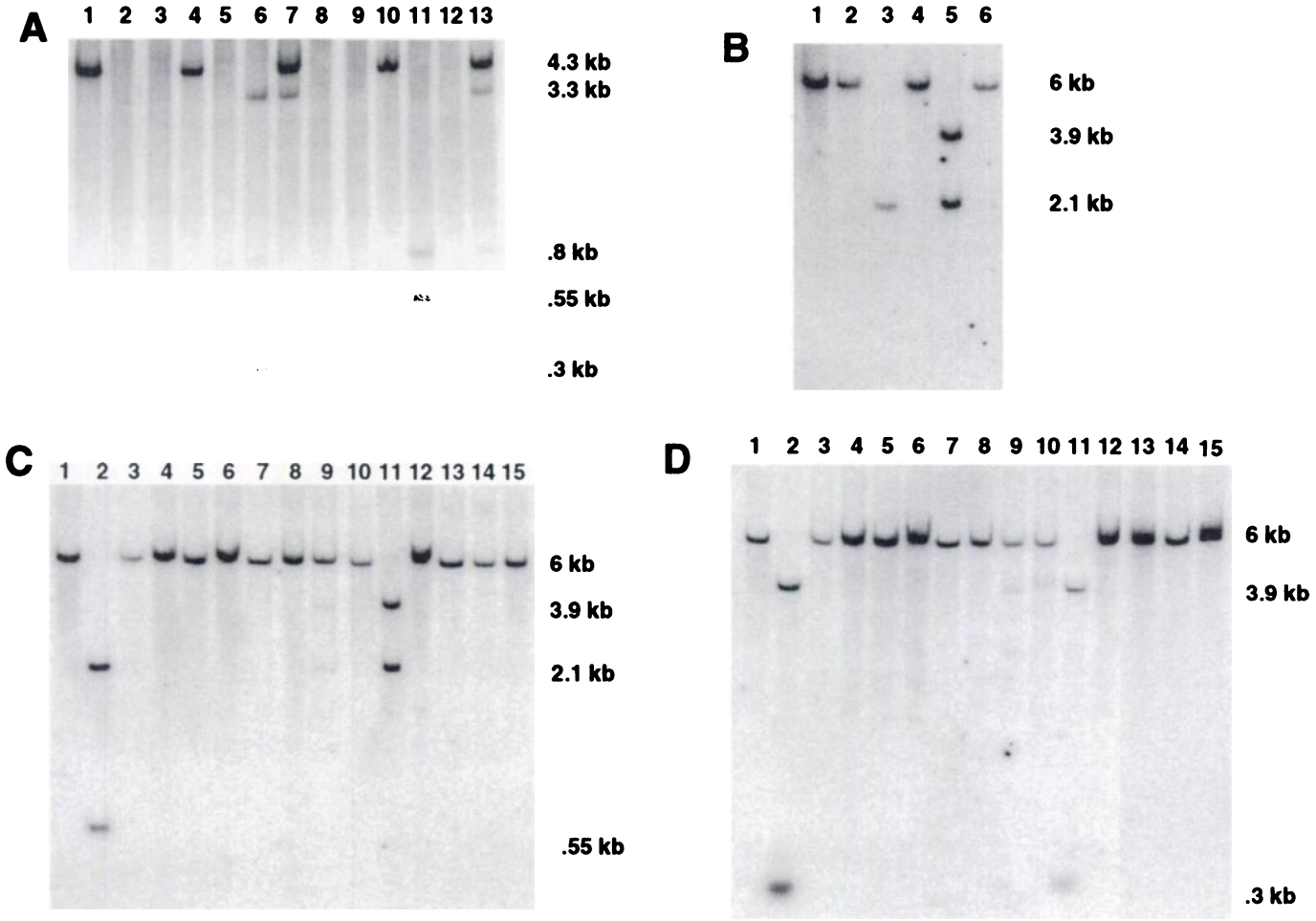


Fig. 2. Methylation and homozygous deletion of *p16* DNA in cancer cell lines. In each panel, the highest molecular weight band (4.3 kb for *A* and 6.0 kb for *B-D*) reflects full methylation of the methylation-sensitive site (*Eag*I or *Sac*II). *A*, breast cancer cell lines. *Lane 1* is DNA restricted with *Eco*RI alone for reference, while DNA in *Lanes 2–7* is restricted with *Eco*RI and *Sac*II and in *Lanes 8–13* with *Eco*RI and *Eag*I. Cell lines shown are Hs578t (*Lanes 2* and *8*), MCF-7 (*Lanes 3* and *9*), T47D (*Lanes 4* and *10*), MDA-MB-231 (*Lanes 5* and *12*), MDA-MB468 (*Lanes 6* and *11*), and ZR-75–1 (*Lanes 7* and *13*). *B*, DNA from prostate cancer cell lines restricted with *Hin*dIII and *Eag*I. *Lane 1*, *Hin*dIII-restricted DNA for reference; *Lane 2*, DuPro; *Lane 3*, DU145; *Lane 4*, PC3; *Lane 5*, LNCaP; *Lane 6*, TSU-Pr1. *C*, colon cancer cell lines. *Lanes 1* and *2* are DNA, for reference, for an unmethylated lung cancer cell line restricted with *Hin*dIII (*Lane 1*) or *Hin*dIII and *Eag*I (*Lane 2*). *Lanes 3–15* are all restricted with *Hin*dIII and *Eag*I, and represent, in order, Colo320, WIDR, SW48, HT29, SW837, SW1463, SW948, SW1116, SW1417, Colo205, RKO, $CaCO_2$, and SW480. *D*, DNA from the same colon cancer cell lines, restricted with *Hin*dIII and *Sac*II, and shown in identical order to *C*.

Table 1 *Inactivation of* p16 *in cell lines and primary tumors*

	Homozygously deleted	Methylated *p16*	Inactivated *p16*	Intact[a] *p16*
Cell lines (*n*)				
Breast cancer (6)	3 (50%)	2 (33%)	5 (83%)	1 (17%)
Prostate cancer (5)	0	3 (60%)	3 (60%)	2 (40%)
Colon cancer (13)	0	12 (92%)	12 (92%)	1 (8%)
Renal cancer (26)	13 (50%)	6 (23%)	19 (73%)	7 (27%)
Primary tumors (*n*)				
Breast cancer (16)	0[b]	5 (31%)	5 (31%)	11 (69%)
Colon adenoma (6)	0[b]	1 (16%)	1 (16%)	5 (84%)
Colon cancer (20)	0[b]	8 (40%)	8 (40%)	12 (60%)

[a] Tumors were not sequenced to exclude point mutations, which are infrequent in these tumor types.
[b] Failure to detect homozygous deletion may result from residual normal tissue.

lines with aberrant methylation of *p16* suggests that both alleles are inactivated by hypermethylation without loss of the other allele.

We demonstrated previously that hypermethylation in this 5′ promoter region was associated with lack of transcription of the normal mRNA. Therefore, we examined cell lines of breast, prostate, and colon cancer for *p16* expression by RT-PCR (Fig. 4). No methylated cell line (T47D, DuPro-1, PC-3, TSU-PR1, HT29, $CaCO_2$, SW480, or RKO) expressed the expected *p16* product (minimal expression was seen in T47D). In contrast, normal female breast tissue and three of four tested normal colon samples had detectable *p16* message (Fig. 4). We also found that the unmethylated cell lines (MDA-MB-468, Du145, and LNCaP) of breast and prostate cancer also expressed the gene. The breast cancer cell line ZR-75–1, which contained both methylated and unmethylated *p16* alleles, expressed *p16* message. To confirm that this DNA methylation blocked transcription, we treated the colon cancer cell lines RKO and SW480 with the demethylating agent 5-deoxyazacytidine (1 μM for 3 days). As in our studies of lung and head and neck tumors (11), both colon cancer cell lines had detectable *p16* mRNA after treatment with 5-deoxyazacytidine (Fig. 4), suggesting that the aberrant DNA methylation is essential for maintaining transcriptional silencing.

Aberrant methylation of *p16* may be analogous to homozygous deletion, leading to lack of *p16* expression and a selective growth advantage to tumor cells. The inverse relation of both aberrant methylation and homozygous deletion to genetic alterations of *Rb* lends further evidence to the concept that both genes act in the same pathway of cell cycle control (4, 11, 17). Our current data amply validate this concept. The only breast cancer cell line with an intact *CDKN2/p16* gene, MDA-MB468, was reported previously to contain

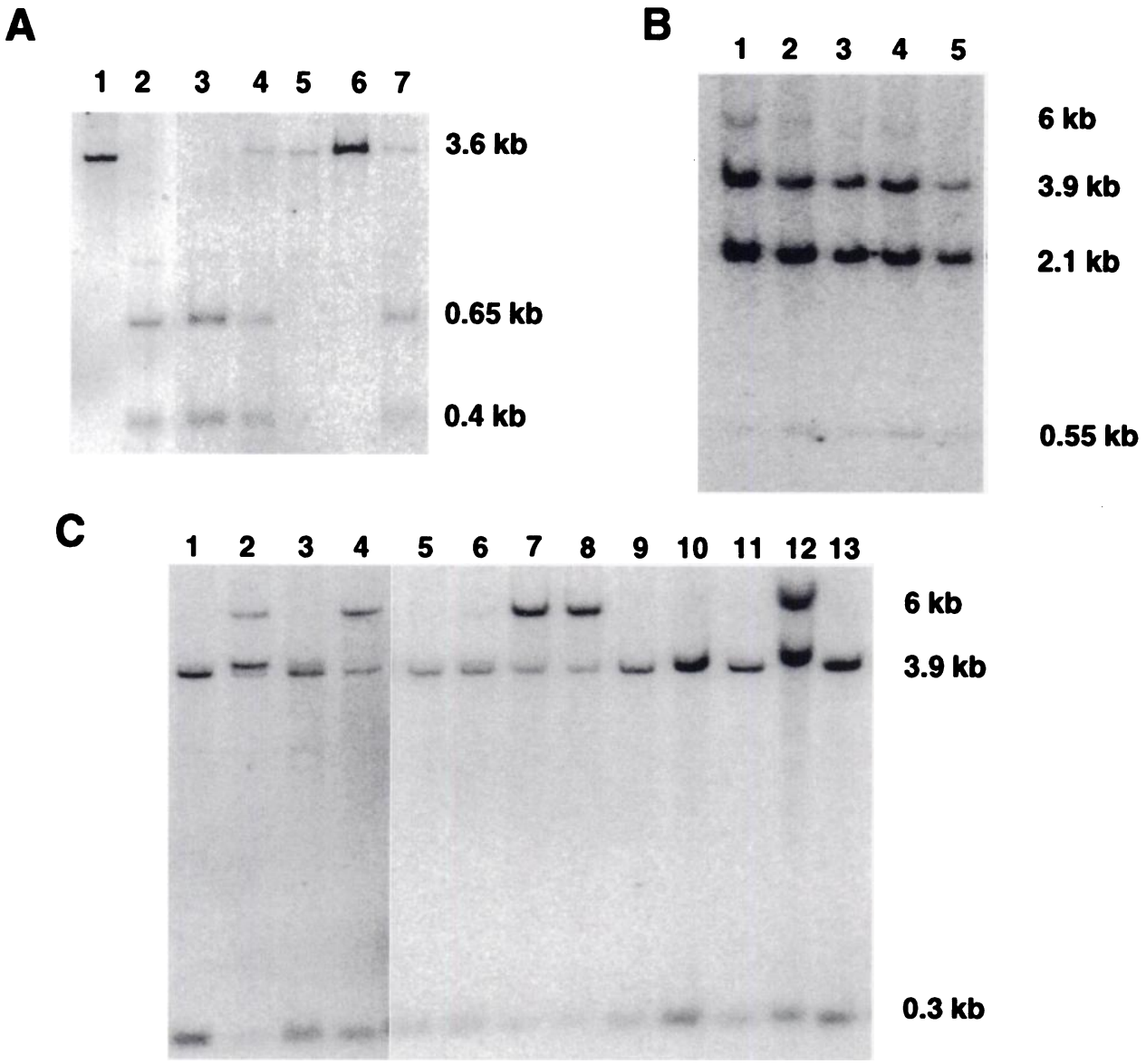
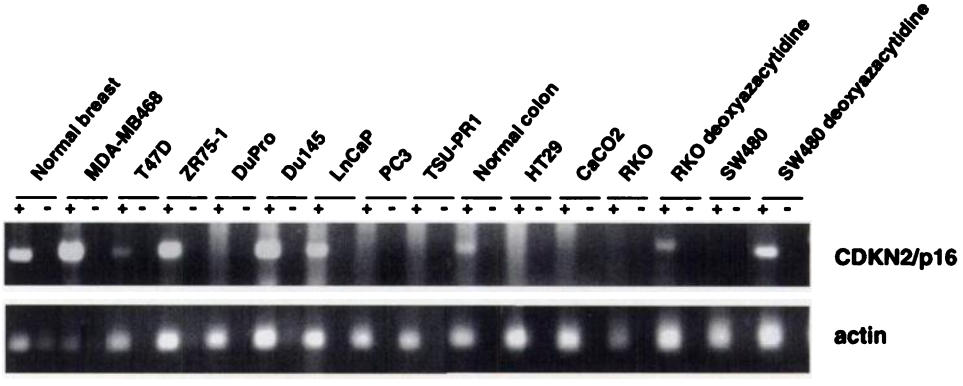


Fig. 3. Methylation of primary neoplasms. As in Fig. 2, the highest molecular weight fragment (3.6 kb in *A* or 6.0 kb in *B–C*) represent complete methylation of the methylation-sensitive restriction sites (*Sma*I or *Sac*II). *A*, renal cancer. DNA from normal kidney DNA restricted with *Xba*I (*Lane 1*) or *Xba*I and *Sma*I (*Lane 2*) are shown for reference. *Lanes 3–7*, DNA from a single patient restricted with both *Xba*I and *Sma*I, including normal kidney (*Lane 3*), primary renal carcinoma (*Lane 4*) and the cell line derived from this primary (*Lane 5*), metastatic tumor (*Lane 7*) and the cell line derived from this metastatic tumor (*Lane 6*). In primary tumors, normal unmethylated alleles present likely represent contaminating normal tissue. *B*, breast tumor DNA restricted with *Hin*dIII and *Eag*I showing examples having hypermethylated alleles (*Lanes 1, 2,* and *4*) and those without methylated alleles (*Lanes 3* and *5*). *C*, DNA from normal colonic mucosa (*Lanes 1, 3,* and *5*) and colon cancer (all other lanes) are shown following restriction with *Hin*dIII and *Sac*II. Some primary colon cancers had hypermethylated *p16* alleles (*Lanes 2, 4, 6–8,* and *12*), while others were unmethylated (*Lanes 9–11* and *13*).

Fig. 4. RT-PCR analysis for expression of *p16*. Cancer cell lines and corresponding normal tissue are shown. Analyses included samples with RT (+) and without RT (−). *Right*, the expected 428-bp p16 product and 400-bp actin products. *p16* gene mRNA was seen in the colon cancer cell lines RKO and SW480 only after treatment with 5-deoxyazacytidine.



mutant *Rb*, while the other cell lines retain wild-type *Rb* (MCF-7, MDA-MB-231, Hs578t, and T47D; Ref. 18). Moreover, colon cancers frequently inactivate *p16* by aberrant methylation and rarely, if ever, inactivate Rb (19), including many of the cell lines examined in this study. The impact of these cell cycle regulatory genes on proliferation rates is further reflected in prostate cancer cell lines, where the four lines with *p16* (DuPro-1, PC3, and TSU-PR1) or Rb (DU 145) inactivation (20) have a mean *in vitro* doubling time of 30 h (range, 22–36 h; Refs. 21–24), while the only cell line with both genes intact, LNCaP, has a cell doubling time of 60 h (25).

A consistent observation in this study and others is that cell lines have a higher rate of *p16* abnormalities than do primary tumors (3–8, 11). For homozygous deletions of the gene, this may in part represent the technical problems of residual *p16* signal in tumors contaminated by normal cells. Detection of aberrant DNA methylation of the *p16* gene is not so limited by this problem, since gain of an abnormal band on Southern blots is more readily detectable, even if it represents contribution from only a percentage of cells. In either case, the incidence of inactivation of *p16* (either by homozygous deletions or aberrant methylation) may be an underestimation in noncultured samples of many tumor types.

Alternatively, abnormalities of *p16* may be present in subpopulations of cells that are expanded during tumor progression and may be selected for in cell culture. In other studies, higher rates of mutation in cell lines than primary tumors have been reported for *p53* and *Rb* in lung and breast cancer (18, 26). These findings may reflect selection of subclones of cells within an individual tumor that have a growth advantage that facilitates the establishment of an immortal cell line. However, the importance of *p16 in vivo* is still supported by its consistent inactivation in the primary tumors of lung, glioma, colon, and breast cancer. In brain tumors (27) and lung carcinomas (28), later stage tumors were reported to have higher rates of homozygous deletion of p16, suggesting that *p16* abnormalities may be late progression events for some tumors as well or that later or metastatic tumors contain fewer contaminating nonneoplastic cells.

In summary, our current results and those reported previously by others show that *p16* is inactivated in a high percentage of many tumors, including the most common forms of adult cancers. However, this inactivation is somewhat unconventional in that, with the exception of a few tumor types such as pancreatic carcinoma (29) and familial melanoma (1, 30), inactivation does not frequently involve point mutation of one allele and loss of the other allele, as found in many other inactivated tumor suppressor genes. In contrast to other tumor suppressor genes, the two most common mechanisms for loss of *p16* function are homozygous deletion and loss of transcription associated with hypermethylation of the 5′ CpG island region. To date, *p16* appears to be unique among tumor suppressor genes for the high incidence of inactivation by these processes. In fact, in colon cancer, the methylation changes can be the sole inactivating mechanism and, in this setting, frequently function as a homozygous deletion by inactivating both retained alleles of *p16*. Taking into account all of our data in the present and our previous study (11) and those of others (1, 2, 16, 31), inactivation of *p16* by either homozygous deletions or aberrant methylation appears to be one of the most frequent genetic events in human neoplasia.

In conclusion, we have not observed hypermethylation of *p16* in adjacent normal tissues from patients with lung, renal, or colon cancer or from any other normal tissue. However, after submission of this paper, we became aware of the findings of Gonzalez-Zulueta *et al.* (31), described in an accompanying paper, concerning the presence of *p16* hypermethylation in normal colon mucosa. We have since obtained some of the same samples studied by this group, which were enriched for colonic mucosa, and they were found to be methylated by a PCR-based assay (31). DNA from these normal mucosa samples does contain, by our Southern analysis, a small (<10%) percentage of alleles hypermethylated at both the *Sac*II and *Eag*I sites shown in Fig. 1. However, by our RT-PCR assay using a higher number of PCR cycles, some expression was found in these samples. We cannot determine whether this expression was from the normal epithelial cells or from any remaining cells of different origin. These data suggest that subpopulations of cells may be present in the normal colonic epithelium that may already harbor abnormalities of *p16* methylation. Further studies may provide insight into this and whether the presence of such cells may explain the high frequency of hypermethylation and the lack of homozygous deletion observed in colon cancer cell lines and primary colon cancers.

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