Deletion of *p16*^{INK4A}/*CDKN2* and *p15*^{INK4B} in Human Somatic Cell Hybrids and Hybrid-derived Tumors[1]

Steven J. Kuerbitz,[2] Jennifer Malandro, Nicole Compitello, Stephen B. Baylin, and Jeremy R. Graff[3]

Departments of Pediatrics [S. J. K., J. M., N. C.] and Genetics [S. J. K.] and The Ireland Cancer Center [S. J. K.], Case Western Reserve University School of Medicine, Cleveland, Ohio 44106; and The Oncology Center, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21231 [S. B. B., J. R. G.]

Abstract

Deletion or epigenetic inactivation of the tumor suppressor gene *p16*^{INK4A}/*CDKN2* (*p16*) has been observed in multiple human tumors. We assayed hybrid cell lines between human diploid fibroblasts and fibrosarcoma cells for *p16* allelic status and expression and found that *p16* was expressed in the parental diploid fibroblast cell lines used, whereas the parental fibrosarcoma cell line HT1080.6TG exhibited homozygous deletion of *p16*. Most immortalized hybrid cell lines derived from these parent cell lines, whether tumorigenic or nontumorigenic, exhibited loss of fibroblast-derived *p16* alleles. All *p16*-negative hybrid cell lines also exhibited deletion of *p15*^{INK4B} (p15). Hybrid cell lines yielded tumors upon s.c. injection into athymic *nude* mice regardless of *p16*/*p15* status. Tumors derived from six *p16*/*p15*-positive hybrid cells, however, revealed deletions of both *p16* and *p15*. When human diploid fibroblasts were fused with A388.6TG squamous cell carcinoma cells, which exhibit aberrant methylation of *p16*, the resulting hybrids again exhibited deletion of the unmethylated fibroblast-derived *p16* alleles. Transfection of both HT1080.6TG and A388.6TG cells with wild-type *p16* expression vector resulted in decreased clonogenicity in culture. Although the determinants directing genetic *versus* epigenetic inactivation of *p16* and *p15* remain unclear, these results demonstrate that *p16*-mediated growth suppression could be abrogated by either mechanism in somatic cell hybrids.

Received 8/19/98; revised 11/8/98; accepted 11/16/98.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported in part by National Cancer Institute Grants K11 CA01620 (to S. J. K.) and R01 CA43318 (to S. B. B.) and funding provided to S. J. K. by the Department of Pediatrics, Case Western Reserve University.
[2] To whom requests for reprints should be addressed, at the Department of Genetics, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, OH 44106-4955.
[3] Present address: Eli Lilly and Co., Indianapolis, IN 46285.

Introduction

Inactivation of the cyclin-dependent kinase 4 inhibitor-encoding genes *p16*^{INK4A}/*CDKN2* (*p16*) and *p15*^{INK4B} (*p15*) in many tumors strongly suggests a role for these genes in the regulatory programs disturbed during cellular transformation. Although homozygous deletions involving chromosomal segment 9p21 in melanoma cell lines led to the initial identification of these genes (1, 2), deletions involving one or both loci have been described in multiple tumors, including cancers of the head and neck (3, 4), prostate, bladder, and lung (3); tumors of the central nervous system (5); and acute leukemias (6). Interestingly, although germ-line mutations of *p16* were identified in families exhibiting a predisposition to melanoma (7), point mutations in the *p16* gene in sporadic tumors appear to be relatively uncommon (8–10). More recently, epigenetic transcriptional repression, characterized by aberrant methylation of the CpG-rich promoter, has been shown to inactivate both *p16* (11–15) and *p15* (16) in a variety of tumors. Similar aberrant epigenetic silencing has also been shown to inactivate the retinoblastoma (17), von Hippel-Lindau (18), and human *mutL* homologue (19) tumor suppressor genes in nonfamilial tumors.

Somatic cell hybrids constructed by fusing neoplastic and nonneoplastic cells have proven to be important tools for testing the relevance of putative tumor-inducing inactivating mutations observed in primary tumors and cell lines. The recapitulation in the hybrid cells of these deletions and mutations on alleles derived from the nonneoplastic parental cell line, associated with the acquisition of neoplastic phenotypes, provides functional evidence for tumor suppressor activity encoded by the gene or within the chromosomal region in question (20, 21). We assessed the *p16* and *p15* status of somatic cell hybrids constructed between nonimmortalized human fibroblasts and either human fibrosarcoma cells, in which *p16* and *p15* are deleted, or human squamous cell carcinoma cells, in which *p16* exhibits epigenetic inactivation. The results support the genetic data, indicating a growth suppressor role for *p16* and *p15*. The data underscore the applicability of somatic hybridization experiments to the study of epigenetic mechanisms in tumorigenesis and demonstrate the potential role of epigenetic processes in gene inactivation events associated with neoplastic progression in somatic cell hybrids.

Results

Deletion of *p16* and *p15* in HT1080.6TG Fibrosarcoma Cells and in Human Diploid Fibroblast × Fibrosarcoma Hybrid Cells. To assess the status of *p16* in parental and hybrid cell lines, we used multiplex PCR assays in which a *p16* exon 2 fragment was coamplified from genomic DNA with a c-*myc* fragment to control for integrity of the DNA sample. Analyses of the parental cell lines GM02291 and

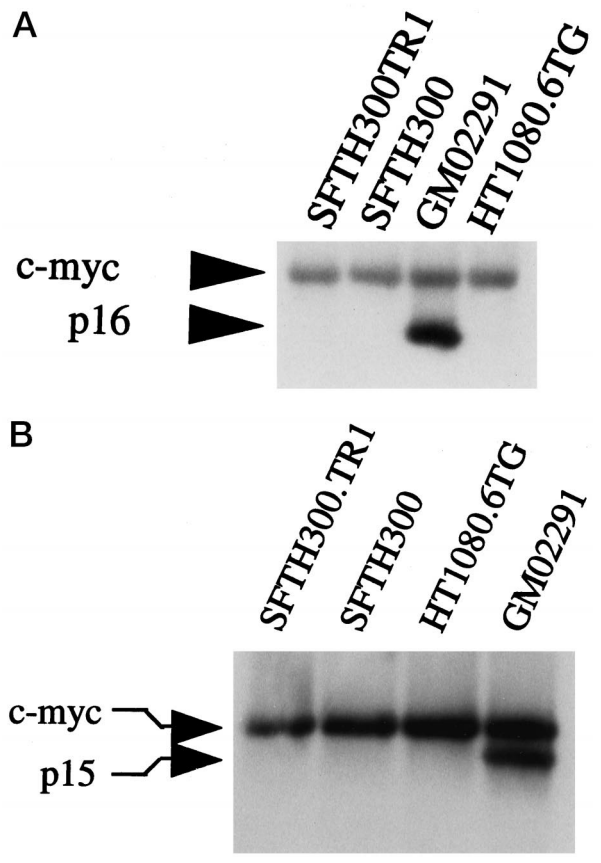

Fig. 1. Deletion of *p16* and *p15* in HT1080.6TG fibrosarcoma cells and hybrid cell lines. Genomic DNA was amplified with primers specific for c-*myc* and *p16* [exon 2 (*A*)] or c-*myc* and *p15* [exon 1 (*B*)].

HT1080.6TG as well as the hybrid cell lines SFTH300 and SFTH300TR1 (22) are shown in Fig. 1*A*. A band corresponding to the c-*myc* amplification product was detected in PCRs from all four cell lines. A band corresponding to the *p16* amplification products was also observed in the GM02291 fibroblast reaction. No *p16*-specific product was apparent, however, in PCRs using genomic DNA from HT1080.6TG fibrosarcoma cells or from either the nontumorigenic SFTH300 or the tumorigenic SFTH300TR1 hybrid cell lines (Fig. 1*A*). The status of *p15* was also assessed using primers generating a fragment encompassing *p15* exon 1. Again, a *p15*-specific amplification product was observed only in the GM02291 reaction (Fig. 1*B*). Thus, the parental HT1080.6TG fibrosarcoma cells showed homozygous deletion of *p16* and *p15*. In addition, the deletion of fibroblast-derived alleles of *p16* and *p15* in both the nontumorigenic and tumorigenic hybrid cell lines demonstrates that inactivation of these genes accompanied events leading to immortalization and preceded tumorigenic transformation in this system.

Deletion of *p16* in K894 Hybrid Cell Lines and in K894-derived Tumors. To test the hypothesis that deletion/inactivation of *p16* is a step in neoplastic transformation of fibroblast × fibrosarcoma hybrids, we analyzed a second hybrid series comprising the parental cell lines BBT and 1080NR.4 as well as 50 BBT × 1080NR.4 hybrid cell lines designated K894 (Table 1). Multiplex PCR for *p16*/c-*myc* of BBT fibroblast-derived genomic DNA revealed the expected *p16*-specific product (Fig. 2*A*). Analysis of the K894 hybrid cell lines at passage 2, however, revealed the *p16* band in only 19 of 50 cell lines (data not shown). Thus, as was observed with the SFTH hybrid cells, the majority of K894 hybrid cell lines exhibited early deletion of fibroblast-derived *p16* alleles.

To determine whether tumorigenicity correlated with *p16*/*p15* status in the hybrid cell lines, cells from six *p16*-positive and six *p16*-negative K894 cell lines were injected s.c. into the flank tissues of athymic *nude* mice. Tumors developed in all evaluable animals injected with parental 1080NR.4 fibrosarcoma cells or any of the 12 hybrid cell lines, whereas no tumors developed in mice injected with parental BBT fibroblasts (Table 2). Although tumors derived from hybrid line K894.19 developed more slowly than others, no general difference in tumor latency was noted between *p16*-positive and *p16*-negative cell lines. Tumors derived from the six *p16*-positive hybrid cell lines (Fig. 2*A*) were then assayed for *p16* status. Of 12 independent tumors tested, *p16* was undetectable by PCR analysis of 11 samples (Fig. 2*B*). Although a faint *p16*-specific band was detectable in PCR products from genomic DNA of K894.12T1 tumor cells (Fig. 2*B*), no *p16*-specific band was detectable by Southern blot analysis of genomic DNA from these cells (data not shown). The total or near total loss of *p16* in all tumors derived from *p16*-positive hybrid cell lines confirmed that growth of these cell lines, either in culture or as tumor xenografts, entailed a selection against *p16* expression.

Deletion of *p15* in K894 Hybrids and Hybrid-derived Tumors. Because HT1080.6TG cells and the hybrid cell lines SFTH300 and SFTH300TR1 exhibited loss of both *p16* and *p15*, we asked whether *p15* was also deleted in K894 hybrids and hybrid-derived tumors. PCR analysis of six *p16*-positive hybrid cell lines revealed a *p15*-specific band (Fig. 3), whereas analysis of multiple *p16*-negative hybrid lines revealed concordant loss of *p15* (data not shown). When tumors derived from *p16*/*p15*-positive hybrid cells were analyzed for *p15* status, all exhibited loss of *p15* (Fig. 3), suggesting that growth of the hybrid cell lines was also facilitated by the deletion of *p15*.

Expression of *p16* and *p15* mRNAs in Fibroblasts and in *p16*-/*p15*-positive Hybrid Cell Lines. To document expression of *p16* or *p15* mRNA in fibroblasts and in hybrid cell lines that were *p16*-/*p15*-positive by genomic PCR, we used multiplex RT-PCR[4] assays. Such documentation was of particular significance with respect to *p16* expression because hybrid cell lines positive for *p16* exon 2 by genomic PCR could still harbor a deletion involving exon 1 and/or its adjacent promoter (23, 24). Therefore, primers specific for *p16* exon 1 and exon 3 sequences were combined with primers specific for a portion of the *GAPDH* transcript to control for RNA integrity in a multiplex RT-PCR assay. BBT parental

[4] The abbreviations used are: RT-PCR, reverse transcription-PCR; *GAPDH*, *glyceraldehyde 3-phosphate dehydrogenase*; EMEM, Eagle's MEM; FBS, fetal bovine serum.

Table 1 Cell lines

Parental cell lines	Hybrid cell lines	Properties
GM02291		Human fibroblast, nonimmortalized
HT1080.6TG		Human fibrosarcoma, immortalized, tumorigenic
	SFTH300	Immortalized, nontumorigenic
	SFTH300TR1	Immortalized, tumorigenic
BBT		Human fibroblast, nonimmortalized
1080NR.4		Neomycin-resistant subclone of HT1080.6TG
	K894	Immortalized
GM01429		Human fibroblast, nonimmortalized, *p16* alleles unmethylated
388NR.4		Human squamous cell carcinoma, immortalized, tumorigenic, *p16* alleles methylated
	Hy397	Immortalized

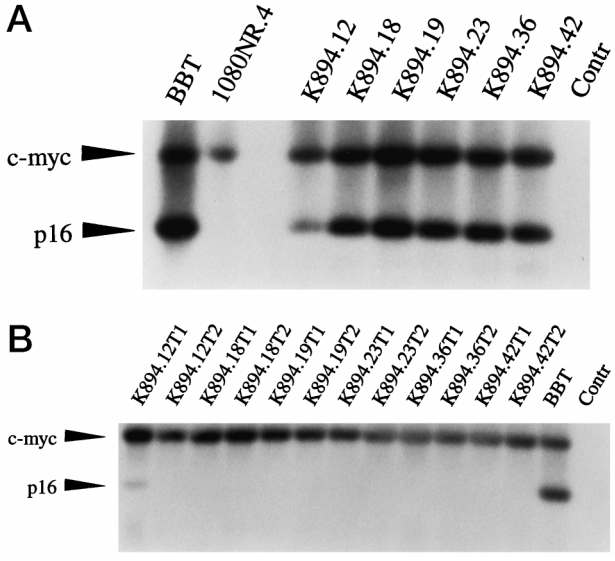

Fig. 2. Deletion of *p16* in tumors derived from *p16*-positive hybrid cell lines. *A*, genomic DNA from the parental fibroblast and fibrosarcoma cell lines as well as six K894 hybrid cell lines was amplified with *p16* (exon 2) and c-*myc* primers. No DNA template was added to the control reaction. *B*, *p16* and c-*myc* genomic PCR products amplified from DNA of two independent tumors derived from each hybrid cell line are shown.

Table 2 Tumorigenicity of K894 hybrid cell lines

Cell line	*p16* status	No. of tumors/ no. of mice injected	Latency (weeks)[a]
Parental cell lines			
BBT	+	0/2	NA[b]
1080NR.4	−	5/5	2
Hybrid cell lines			
K894.11	−	4/4	2
K894.12	+	2/4[c]	4
K894.16	−	4/4	3
K894.18	+	4/4	3
K894.19	+	4/4	6
K894.22	−	4/4	3
K894.23	+	4/4	3
K894.32	−	4/4	3
K894.36	+	4/4	3
K894.39	−	4/4	3
K894.40	−	4/4	3
K894.42	+	4/4	3

[a] Average time until tumors measured 0.5 cm in largest dimension.
[b] NA, not applicable.
[c] Two mice died before tumors were identifiable.

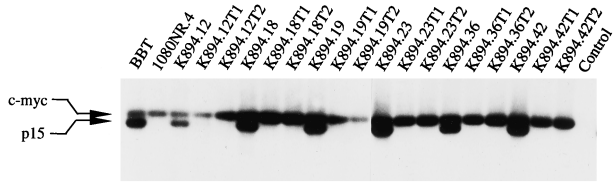

Fig. 3. Deletion of *p15* in tumors derived from *p15*-positive K894 hybrid cell lines. Genomic DNA from the parental fibroblast and fibrosarcoma cell lines as well as six K894 hybrid cell lines and two independent tumors (designated T1 and T2) derived from each hybrid cell line was amplified with primers for for *p15* (exon 1) and c-*myc*.

fibroblasts, 1080NR.4 parental fibrosarcoma cells, and three K894 hybrid cell lines that were *p16* positive by genomic PCR were analyzed. A *p16*-specific RT-PCR product was detected in all cell line samples except 1080NR.4 (Fig. 4*A*). Similarly, the assay for *p15* expression used primers spanning the *p15* coding sequence in combination with *GAPDH*-specific primers. Analysis once again revealed *p15* expression only in the fibroblasts and the *p15* genomic PCR-positive hybrid cell lines (Fig. 4*B*). Therefore, these assays confirmed *p16* and *p15* expression in those cell lines that were positive by *p16* or *p15* genomic PCR.

Chromosome 9p Deletions in HT1080.6TG Cells and K894 Hybrid-derived Tumors. To determine the extent of the chromosome 9p deletion in HT1080.6TG cells and hybrid-derived tumors, PCR analysis using microsatellite markers was performed. As summarized in Table 3, the homozygous 9p deletion in HT1080.6TG cells was bounded proximally by the marker D9S171 and distally by D9S157. Seven tumors derived from *p16*/*p15*-positive K894 hybrid cell lines were then analyzed to map deletions of parental fibroblast-derived chromosome 9 alleles. Because the parental HT1080.6TG cells and fibroblasts shared one common allele at D9S197 and D9S269, we could not establish unambiguous deletion of both fibroblast-derived alleles at these loci in the hybrid cell lines. Nevertheless retention of unique fibroblast-derived alleles of these and other loci in two of the seven hybrid cell lines confined the largest region of deletion to that region bounded by D9S259 proximally and by

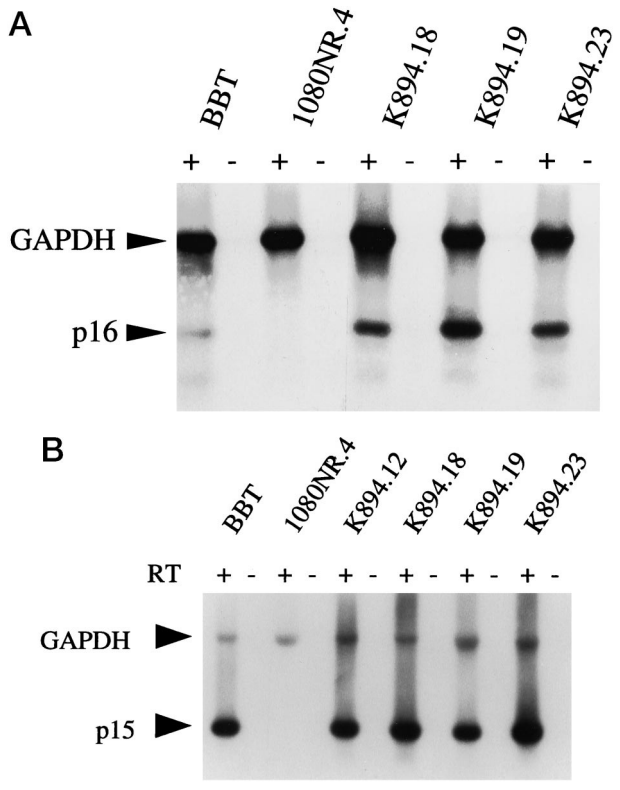

Fig. 4. Expression of *p16* and *p15* in genomic PCR-positive cell lines. RNA from parental and hybrid cell lines was reverse transcribed, and the resultant cDNA was amplified using primers for *GAPDH* and (*A*) *p16* (exons 1α and 3) or (*B*) *p15* (exons 1 and 2).

D9S157 distally (indicated by the dashed box in Table 3). This deletion was similar in extent to the deletion in HT1080.6TG cells. No other regions of fibroblast-derived chromosome 9 were consistently deleted in the tumor cells.

Deletion of p16 in Human Squamous Cell Carcinoma × Human Fibroblast Hybrids. Epigenetic transcriptional repression associated with aberrant promoter methylation and chromatin condensation is an alternative mechanism of tumor suppressor gene inactivation. Because deletion of *p16* was observed exclusively in K894 hybrid cells, we extended our analysis to somatic cell hybrids constructed from a parental cell line in which *p16* was inactivated by epigenetic repression rather than deletion. We made hybrids between the human fibroblast cell line GM01429, which has normal, unmethylated *p16* alleles, and 388HR.4, a squamouscell carcinoma cell line that exhibits aberrant methylation of the *p16* promoter (Table 1). Five hybrid cell lines, termed Hy397, were derived from two cell fusion experiments, selecting for retention of the GM01429-derived t(Xq;9p) chromosome by culturing the hybrids in hypoxanthine, aminopterin, and thymidine medium. An informative *Msp*I polymorphism in *p16* exon 3 (10) permitted discrimination between parental *p16* alleles in these hybrid cells. Although analysis of all Hy397 hybrid cell lines at passage 2 revealed both GM01429-derived and 388HR.4-derived *p16* alleles (data not shown), repeat analysis at passage 20 (~50 population doublings) revealed only 388HR.4-derived alleles (Fig. 5*A*). To verify further that the hybrids originally contained at least one GM01429-derived chromosome 9, we assessed the Hy397 hybrids for retention of some element of the fibroblast-derived chromosome. Microsatellite analysis for chromosome 9 loci confirmed the retention of one GM01429-derived allele at locus D9S273 (9p21-q21) in all Hy397 hybrid cell lines at passage 20 (Fig. 5*B*). These data demonstrate that passage in cell culture selected for the deletion but not the *de novo* methylation of unmethylated GM01429-derived *p16* alleles in Hy397 hybrid cells, whereas methylated 388NR.4-derived alleles were retained.

Suppressed Clonogenicity in p16-transfected HT-1080.6TG Cells and A388.6TG Cells. Because the genetic and epigenetic changes demonstrated in these hybrid cell lines and hybrid-derived tumors could potentially inactivate genes other than *p16* and *p15*, we asked directly whether *p16* expression conferred growth suppression in the parent tumor cell lines. HT1080.6TG cells and A388.6TG cells were transfected with pCDKN2AWT, an expression vector encoding the wild-type *p16* cDNA driven by a cytomegalovirus promoter. For both tumor cell lines, transfection with the wild-type *p16*-containing plasmid resulted in substantially reduced clonogenicity in cell culture compared to transfection to the vector alone (Table 4). The magnitude of this suppression, although less than total, was comparable to that observed in previous studies (9, 25).

Discussion

The hybrid cell lines SFTH300 and SFTH300TR1 have been used previously to analyze genetic and epigenetic events in relationship to cellular immortalization, tumorigenicity in athymic *nude* mice, and other neoplastic phenotypes (22, 26, 27). We recently demonstrated the deletion in immortalized, tumorigenic SFTH300TR1 cells of chromosome 17 loci that were retained in immortalized but nontumorigenic SFTH300 cells, suggesting a possible role for these genetic events in tumorigenic transformation rather than immortalization (26). Here, in contrast, *p16* and *p15*, deleted in the parental HT1080.6TG cells, were also deleted in nontumorigenic SFTH300 cells, demonstrating that *p16/p15* deletion was not sufficient to establish tumorigenicity in these hybrid cells and suggesting that the process of immortalization in culture selected against cells expressing these genes.

Data from a second series of hybrid cell lines supported these results. Early deletion (passage 2) of fibroblast-derived *p16* and *p15* was observed in the majority of K894 hybrid cell lines. In tumorigenicity assays, tumors developed rapidly in *nude* mice injected with K894 hybrid cell lines, regardless of *p16/15* status. Importantly, however, tumors derived from *p16/p15*-positive hybrids exhibited total or near total loss of fibroblast-derived *p16* and *p15*. As assessed by the retention or loss chromosome 9 loci in the tumors (Table 3), the gene(s) accounting for the tumor suppressive effect inferred by the deletion of fibroblast-derived alleles map to the region 9p21-p22, and *p16* and *p15* remain the best candidates.

Although the data from the K894 hybrids do not demonstrate an exclusive association of *p16/p15* inactivation with immortalization *versus* tumorigenicity, the early deletion of

Table 3 Chromosome 9p deletions in HT1080.6TG and hybrid-derived tumor cells

Tumor or cell line	D9S162	D9S157	*p16*	*p15*	D9S171	D9S259	D9S169
HT1080.6TG	+[a]	+	−	−	+	+	+
K894.12T2	ND	D/U	−	−	U	D	D/U
K894.18T1	ND	R	−	−	U	R	R
K894.18T2	ND	R	−	−	U	R	R
K894.19T1	ND	D/U	−	−	U	D	D/U
K894.19T2	ND	D/U	−	−	U	D	D/U
K894.23T1	ND	D/U	−	−	U	D	D/U
K893.23T2	ND	D/U	−	−	U	D	D/U

[a] The allelic status of loci is depicted as follows: +, alleles detected; −, no alleles detected; R, fibroblast-derived allele(s) retained; D, no fibroblast-derived alleles retained; U, uninformative; D/U, one fibroblast-derived allele deleted, other allele uninformative; ND, not done.

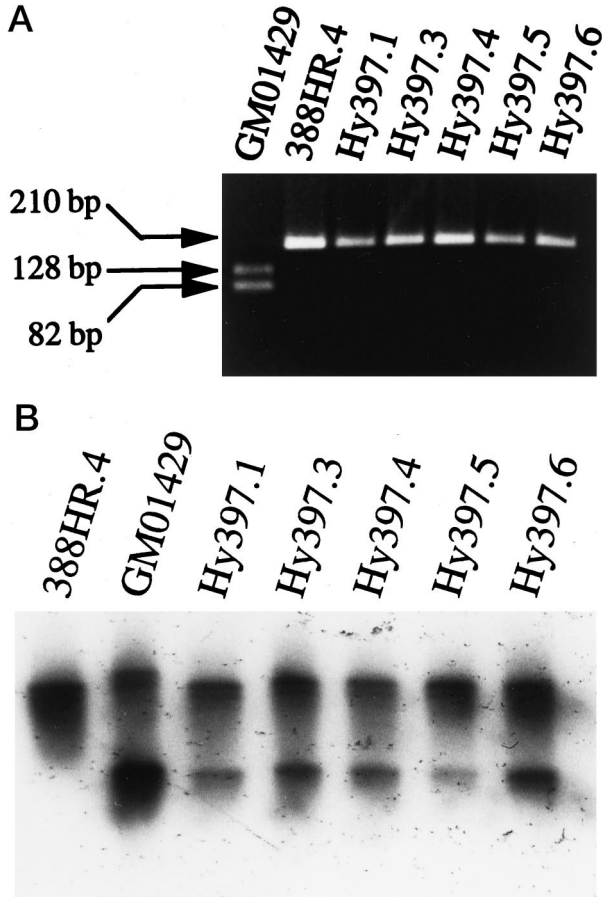

Fig. 5. Deletion of fibroblast-derived *p16* alleles in Hy397 hybrid cells containing fibroblast-derived chromosome 9 markers. *A*, the *p16* exon 3 region containing an *Msp*I polymorphism was amplified from genomic DNA of parental fibroblast and squamous cell carcinoma cell lines as well as five Hy397 hybrid cell lines. PCR products were digested with *Msp*I. Note that GM01429 alleles are completely digested, whereas 388HR.4 alleles are fully resistant to digestion. *B*, genomic DNA from the parental and hybrid cell lines was amplified using primers specific for the chromosome 9 microsatellite locus D9S273. Note that the smaller, GM01429-specific allele is present in each of the hybrid cell lines.

Table 4 *p16*-induced suppression of clonogenicity in parental tumor cell lines

Cell line	No. of transfectant colonies (±SD)		Clonogenicity fraction[a]
	pCDKN2AWT	pCDNA3	
HT1080.6TG	224 (13)	502 (18)	0.45
A388.6TG	28 (4)	97 (5)	0.29

[a] No. of pCDKN2AWT clonies/no. of pCDNA3 colonies.

p16 and *p15* in more than half of the cell lines indicatesthat expression of these genes probably suppressed growth of the hybrid cells in culture. The deletion of fibroblast-derived *p16* alleles in Hy397 hybrids following extended passage in culture suggests further that loss of *p16*-facilitated growth in cell culture. Because the assay for *p16* and *p15* status was PCR based, the deletion of *p16* and *p15* observed in tumors derived from hybrid cell lines positive for both genes may reflect heterogeneity of the original hybrid cell population. Indeed, the short latency periods associated with tumor formation suggest selective expansion of a preexisting *p16*-/*p15*-negative cell population. The reduced clonogenicity observed in the *p16*-transfected parental HT1080.6TG and A388.6TG tumor cell lines confirms the growth-suppressive effect of *p16* inferred by the deletion of the gene during growth of hybrid cells. Our results support previous data for *p16*- (9, 25, 28) or *p15*- (29) mediated growth suppression.

Serrano, *et al.* (30) suggested a role for *p16* activity in senescence of fibroblasts based on the enhanced clonogenic potential and lack of senescent growth delay exhibited by *p16* null (−/−) mouse embryo fibroblasts. Furthermore, they noted an increased frequency of fibrosarcoma development, both spontaneously and following UV radiation or chemical mutagenesis, in *p16* nullizygous mice compared to controls. The specific role of *p16* inactivation in the evolution of these transformation events has been called into question by recent experiments using mice that were nullizygous for $p19^{ARF}$, the product of an alternate reading frame encoded at the *INK4A* locus, but expressed *p16*. Embryo fibroblasts from these mice also exhibit increased immortalization frequency, and the mice show an increased susceptibility to spontaneous and carcinogen-induced tumors, including fibrosarcomas (31). Our results support a role for *p16* and/or *p15* inactivation in fibroblastic tumors or tumors of primitive mesenchymal origin, in agreement with recent data (32). Because the deletions we observed encompassed both the *INK4A* and *INK4B* loci, however, our data do not permit discrimination between the potential roles of *p15*, *p16*, and *p19* inactivation in these events.

Aberrant methylation of the *p16* and *p15* gene promoters has been associated with transcriptional repression in pre-

vious reports (11–16). We used the somatic cell hybrid systems to ask whether epigenetic inactivation is the functional equivalent of genetic inactivation of tumor suppressor alleles and, potentially, to gain insight into the determinants that direct epigenetic *versus* genetic inactivation. We assessed the status of the fibroblast-derived *p16* alleles in hybrid cell lines between human fibroblasts and 388HR.4 cells, a squamous cell carcinoma cell line that has inactivated *p16* by methylation-associated epigenetic repression. The deletion of fibroblast-derived *p16* alleles and concomitant retention of methylated 388NR.4-derived alleles observed after 20 passages in tissue culture suggests that epigenetic inactivation and genetic deletion of the parental tumor cell *p16* alleles were functionally equivalent in these hybrid cell systems.

The molecular processes resulting in aberrant methylation-associated transcriptional repression of *p16* and *p15* are not well defined. The deletion, rather than methylation, of fibroblast-derived *p16* alleles observed in all five Hy397 hybrid cell lines suggests that *p16* methylation in this system is probably not attributable to a 388HR.4-derived factor capable of acting in *trans* to silence the *p16* locus. We have shown previously that parental cell methylation patterns were retained at alleles of most CpG island loci K894 hybrid cells (26). These data further suggest that the determinants of aberrant *p16* repression act in *cis* with respect to the inactivated locus. Further investigation will be necessary to identify these putative *cis*-acting signals.

One approach to the identification of tumor suppressor genes is to map deletions of nonneoplastic parental cell-derived alleles in transformed somatic cell hybrids and then to identify inactivating mutations of neoplastic parental cell-derived alleles of candidate genes. Our results demonstrate that deletions of nonneoplastic cell-derived chromosomes in somatic cell hybrids can mark neoplastic cell-derived loci inactivated by epigenetic as well as genetic mechanisms. This observation suggests that a rigorous analysis of candidate suppressor loci identified in cell hybridization and, perhaps, chromosome transfer experiments should include methylation or chromatin structural analysis as well as mutation and deletion analyses.

Materials and Methods

Cell Lines. The cell lines used in this study are listed and described in Table 1. BBT human diploid fibroblasts were the generous gift of Dr. Darwin J. Prockop (Thomas Jefferson Medical College, Philadelphia, PA). GM02291 and GM01429 human fibroblasts were purchased from the Coriell Cell Repositories (Camden, NJ). Hypoxanthine phosphoribosyltransferase-deficient HT1080.6TG human fibrosarcoma cells, SFTH300 and SFTH300TR1 hybrid cells, and A388.6TG squamous cell carcinoma cells were the gift of Dr. Bernard Weissman (University of North Carolina, Chapel Hill, NC). All cell lines were maintained in EMEM supplemented with 10% FBS and incubated at 37°C at 5% CO_2. The generation of K894 hybrid cell lines has been described (26). To generate Hy397 hybrids, we transfected A388.6TG cells with a plasmid conferring hygromycin resistance. These cells, termed 388HR.4, were then fused as described previously (26) with GM01429 fibroblasts, which carry an (Xq;9p) translocation, and hybrids were selected in medium supplemented with hygromycin (150 μg/ml) and 1× hypoxanthine, aminopterin, and thymidine (Sigma Chemical Co., St. Louis, MO). Individual clones were isolated after 3–5 weeks.

Multiplex Genomic PCR for *p16* and *p15*. PCRs were carried out in 10 mM Tris (pH 8.6), 50 mM KCl, 100 μg/ml gelatin, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM each primer, 50 ng of genomic DNA, and 1 unit of Taq polymerase (Life Technologies, Inc.) per 50-μl reaction. Oligonucleotide primers for *p16*/c-*myc* PCR were: *p16* (exon 2), forward, 5′-CCACCCTGGCTCTGACCATTCTGT, and reverse, 5′-5′-CATCAGTCCTCACCTGAGCCT; and c-*myc*, forward, 5′-AAGTGCGTCTCCGAGATAGCAGGG, and reverse, 5′-CGTCCGGGTCGCAGATGAAACTCT. Reactions were supplemented with 4% DMSO. Cycling parameters were as follows: 94°C for 5 min; then 35 cycles of 94°C for 40 s, 62° for 45 s, and 72° for 40 s; and final extension at 72°C for 5 min. Coamplification of *p15* and c-*myc* was performed using *p15* primers flanking exon 1 (forward, 5′-TTTCCCAGAAGCAATCCAGGCGCG, and reverse, 5′-CGCTCTAGGTTCCAGCCCCGATCC) in reactions supplemented with 4% DMSO. Cycling parameters were: 94°C for 5 min; then 35 cycles of 94°C for 40 s, 65°C for 35 s, 72°C for 40 s; and final extension at 72°C for 5 min. Ten μl of the reaction products were fractionated on a 2% agarose gel. DNA was then transferred to nylon membranes and hybridized to random hexamer-labeled probes generated from the cloned PCR fragments.

Multiplex RT-PCR for *p16* and *p15* mRNA Expression. Total cellular RNA (2.5 μg; Ref. 33) was reverse-transcribed in 30-μl reactions containing 150 ng of random hexamers (Life Technologies, Inc., Gaithersburg, MD), 0.5 μM dNTPs, 0.3 μl of RNAsin (Promega, Madison, WI), buffer, DTT as supplied by the manufacturer (Life Technologies, Inc.), and 1.5 μl (300 units) murine Moloney leukemia virus reverse transcriptase (Life Technologies, Inc.) or water. Reactions were incubated 1 h at 37°C, heat-inactivated for 5 min at 95°C, and then held on ice. Five μl of the RT reactions were used as template for PCRs with *p16* primers (exon 1α, forward, 5′-ATGGAGCCTTCGGCTGACTGGCTG; and reverse, 5′-CGAGGTTTCTCAGAGCCTCTCTGG) or *p15* primers (forward, 5′-ATGCGCGAGGAGAACAAGGGCATG; and reverse, 5′-AAGTCGTTGTGGGCGGCTGGGGAA) and *GAPDH* primers (*GAPDH*, forward, 5′-TCTTCTTTTGCGTCGCCAGCCGAG; and reverse, 5′-AATGCCAGCCCCAGCGTCAAAGGA) in reactions supplemented with 4% DMSO. Cycling parameters for both *p16/GAPDH* and *p15/GAPDH* PCRs were: 94°C for 5 min; then 35 cycles of 94°C for 40 s, 67°C for 30 s, and 72°C for 40 s; and final extension at 72°C for 5 min. Ten μl of the reaction products were separated on agarose gels, transferred, and hybridized as described above.

PCR Analysis of Microsatellite Markers. Oligonucleotide primers specific for loci D9S157, D9S162, D9S169, D9S171, D9S259, and D9S273 were purchased from Research Genetics (Huntsville, AL). PCR amplification was performed using 25 ng of genomic DNA in 25-μl reactions. Cycling parameters were as follows: 94°C for 4 min; then 30–35 cycles of 94°C for 40 s, 55°C for 30 s, 72°C for 1 min; and final extension at 72°C for 4 min. Reaction products were separated in 3% low melting point agarose gels and visualized by UV fluorescence after staining in ethidium bromide. Alternatively, one oligonucleotide primer per reaction series was end-labeled with [γ-^{32}P]ATP and Klenow fragment. PCR products were then resolved on 5% acrylamide/8 M urea sequencing gels and visualized after autoradiographic exposure of the dried gels.

***p16 Msp*I Polymorphism Analysis.** The region containing a RFLP identified by *Msp*I in exon 3 of the *p16* gene (10) was amplified with primers P16E3.1 (5′-TAGGGACGGCAAGAGAGGAGGGC) and P16E3.2 (5′-TGAAAACTACGAAAGCGGGGTGGG) in 25-μl reactions containing 25 ng of genomic DNA and 4% DMSO. Cycling parameters were 94°C for 5 min; then 35 cycles of 94°C for 40 s, 64°C for 45 s, 72°C for 30 s; and final extension at 72°C for 5 min. PCR products (10 μl of each 25-μl reaction) were then digested for 2 h with 10 units of *Msp*I (New England Biolabs, Beverly, MA) in the buffer supplied by the manufacturer in a reaction volume of 20 μl. The digestion products were then separated on a 2% agarose gel and visualized by UV fluorescence of the ethidium bromide-stained gel.

Tumorigenicity Assays. Cells (2×10^6–5×10^6) were injected s.c. into the flanks of athymic *nude* mice (one injection per mouse; four mice per cell line). Individual tumors were excised and genomic DNA was prepared by cell lysis and digestion SDS-proteinase K followed by phenol-chloroform extraction.

Transfection Assays. Cells were grown to ~75% confluence in 100-mm tissue culture dishes. Cells were transfected with 10 μg of *Bgl*II-linearized DNA from the plasmids pCDNA3 (Invitrogen) or pCDKN2AWT (the generous gift of Dr. H-J. Su Huang, Ludwig Institute for Cancer Research, La Jolla, CA) or mock-transfected with TE buffer. Transfection was performed with Lipofectamine (Life Technologies, Inc.) in EMEM without FBS for 5 h, after which transfection medium was removed and cells were incubated overnight in EMEM supplemented with 10% FBS. On the following day, cell monolayers were trypsinized and equivalent numbers of cells were plated in 100-mm dishes in EMEM with 10%

FBS containing G418 (Life Technologies, Inc.) at 300 μg/ml (A388.6TG) or 500 μg/ml (HT1080.6TG). Macroscopic colonies were counted after 10 days by staining with methylene blue.

Acknowledgments

We thank Drs. Bernard Weissman and Darwin J. Prockop for providing cell lines, Dr. James G. Herman for providing a *p16* exon 1 probe, and Dr. H-J. Su Huang for providing the plasmid pCDKN2AWT. We also thank Dr. Huntington F. Willard for critical review of the manuscript.

References

1. Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S., III, Johnson, B. E., and Skolnick, M. H. A cell cycle regulator potentially involved in the genesis of many tumor types. Science (Washington DC), *264:* 436–440, 1994.

2. Weaver-Feldhaus, J., Gruis, N. A., Neuhausen, S., Le Paslier, D., Stockert, E., Skolnick, M. H., and Kamb, A. Localization of a putative tumor suppressor gene by using homologous deletions in melanomas. Proc. Natl. Acad. Sci. USA, *91:* 7563–7567, 1994.

3. Reed, A., Califano, J., Cairns, P., Westra, W. H., Jones, R., Koch, W., Bartek, J., and Sidransky, D. High frequency of p16 (CDKN2/MTS1/INK4A) inactivation in head and neck squamous cell carcinoma. Cancer Res., *56:* 3630–3633, 1996.

4. Cairns, P., Polascik, T. J., Eby, Y., Tokino, K., Califano, J., Merlo, A., Mao, L., Herath, J., Jenkins, R., Westra, W., Rutter, J. L., 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.

5. Jen, J., Harper, W., Bigner, S., Bigner, D., Papadopoulos, N., Markowitz, S., Willson, J. K. V., Kinzler, K., and Vogelstein, B. Deletion of *p16* and *p15* genes in brain tumors. Cancer Res., *54:* 6353–6358, 1994.

6. Okuda, T., Shurtleff, S. A., Valentine, M. B., Raimondi, S. C., Head, D. R., Behm, F., Curcio-Brint, A. M., Liu, Q., Pui, C-H., Sherr, C. J., Beach, D., Look, A. T., and Downing, J. R. Frequent deletion of $p16^{INK4A}$/*MTS1* and $p15^{INK4B}$/*MTS2* in pediatric acute lymphoblastic leukemia. Blood, *85:* 2321–2330, 1995.

7. Hussussian, C. J., Struewing, J. P., Goldstein, A. M., Higgins, P. A. T., Ally, D. S., Sheahan, M. D., Clark, W. H., Jr., Tucker, M. A., and Dracopoli, N. C. Germline p16 mutations in familial melanoma. Nat. Genet., *8:* 15–21, 1994.

8. Cairns, P., Mao, L., Merlo, A., Lee, D. J., Schwab, D., Eby, Y., Tokino, K., van der Riet, P., Blaugrund, J. E., and Sidransky, D. Rates of *p16 (MTS1)* mutations in primary tumors with 9p loss. Science (Washington DC), *265:* 415–417.

9. Okamoto, A., Demetrick, D. J., Spillare, E. A., Koichi, H., Hussain, S. P., Bennett, W. P., Forrester, K., Gerwin, B., Serrano, M., Beach, D., and Harris, C. Mutations and altered expression of $p16^{INK4}$ in human cancer. Proc. Natl. Acad. Sci. USA, *91:* 11045–11049, 1994.

10. Ueki, K., Rubio, M-P., Ramesh, V., Correa, K. M., Rutter, J. L., von Deimling, A., Buckler, A. J., Gusella, J. F., and Louis, D. N. *MTS1/CDKN2* gene mutations are rare in primary human astrocytomas with allelic loss of chromosome 9p. Hum. Mol. Genet., *3:* 1841–1845.

11. 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 CDKN2/p16 in human cancers. Nat. Med., *7:* 686–692, 1995.

12. Gonzales-Zulueta, M., Bender, C. M., Yang, A. S., Nguyen, T., Beat, 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 tissues correlates with gene silencing. Cancer Res., *55:* 4531–4535, 1995.

13. Costello, J. F., Berger, M. S., Huang, H-J. S., and Cavanee, W. K. Silencing of p16/CDKN2 in human gliomas by methylation and chromatin condensation. Cancer Res., *56:* 2405–2410, 1996.

14. Lo, K-W., Cheung, S-T., Leung, S-F., van Hasselt, A., Tsang, Y-S., Mak, K-F., Chung, Y-F., Woo, J. K. S., Lee, J. C. K., and Huang, D. P. Hypermethylation of the *p16* gene in nasopharyngeal carcinoma. Cancer Res., *56:* 2721–2725, 1996.

15. Herman, J. G., Civin, C. I., Issa, J-P. J., Collector, M. I., Sharkis, S. J., and Baylin, S. B. Distinct patterns of inactivation of $p15^{INK4B}$ and $p16^{INK4A}$ characterize the major types of hematological malignancies. Cancer Res., *57:* 837–841, 1997.

16. Herman, J. G., Jen, J., Merlo, A., and Baylin, S. B. Hypermethylation-associated inactivation indicates a tumor suppressor role for $p15^{INK4B}$. Cancer Res., *56:* 722–727, 1996.

17. Sakai, T., Toguchida, J., Ohtani, N., Yandell, D. W., Rapaport, J. M., and Dryja, T. P. Allele-specific methylation of the retinoblastoma tumor suppressor gene. Am. J. Hum. Genet., *48:* 880–888, 1991.

18. Herman, J. G., Latif, F., Weng, Y., Lerman, M. J., Zbar, B., Lin, S., Samid, D., Duan, D-S. R., Gnarra, J. R., Linehan, M., and Baylin, S. B. Silencing of the VHL tumor suppressor gene by DNA methylation in renal carcinoma. Proc. Natl. Acad. Sci. USA, *91:* 9700–9704, 1994.

19. Kane, M. F., Massimo, L., Gaida, G. M., Lipman, J., Mishra, R., Goldman, H., Jessup, J. M., and Kolodner, R. Methylation of the *hMLH1* promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Res., *57:* 808–811, 1997.

20. Chen, P., Ellmore, N., and Weissman, B. E. Functional evidence for a second tumor suppressor gene on human chromosome 17. Mol. Cell. Biol., *14:* 534–542, 1994.

21. Klingelhutz, A. J., Wu, S-Q., Huang, J., and Reznikoff, C. A. Loss of 3p13–p21.2 in tumorigenic reversion of a hybrid between isogeneic non-tumorigenic and tumorigenic human uroepithelial cells. Cancer Res., *52:* 1631–1634, 1992.

22. Benedict, W. F., Weissman, B. E., and Stanbridge, E. J. Tumorigenicity of HT1080 fibrosarcoma × normal fibroblast hybrids: chromosome dosage dependency. Cancer Res., *44:* 3471–3479, 1984.

23. Mao, L., Merlo, A., Shapiro, G. I., Edwards, C. D., Rollins, B. J., and Sidransky, D. A novel $p16^{INK4A}$ transcript. Cancer Res., *55:* 2995–2997, 1995.

24. Stone, S. Jiang, P., Dayananth, P., Tavtigian, S., Katcher, H., Parry, D., Peters, G., and Kamb, A. Complex structure and regulation of the *p16* (*MTS1*) locus. Cancer Res., *55:* 2988–2994, 1995.

25. Liggett, W. H., Jr., Sewell, D. A., Rocco, J., Ahrendt, S. A., Koch, W., and Sidransky, D. *p16* and *p15* are potent growth suppressors of head and neck squamous carcinoma cells *in vitro*. Cancer Res., *56:* 4119–4123, 1996.

26. Kuerbitz, S. J., and Baylin, S. B. Retention of unmethylated CpG island alleles in human diploid fibroblast × fibrosarcoma cell lines expressing high levels of DNA methyltransferase. Cell Growth Differ., *7:* 847–853, 1996.

27. Tlsty, T. D., White, A., and Sanchez, J. Suppression of gene amplification in human cell hybrids. Science (Washington DC), *255:* 1425–1427, 1992.

28. Arap, W., Nishikawa, R., Furnari, F. B., Cavanee, W. K., and Huang, H. J. Replacement of the *p16/CDKN2* gene suppresses human glioma cell growth. Cancer Res., *55:* 1351–1354, 1995.

29. Stone, S., Dayananth, P., Jiang, P., Weaver-Feldhaus, J. M., Tavtigian, S. V., Cannon-Albright, L., and Kamb, A. Genomic structure, expression and mutational analysis of the *p15* (*MTS2*) gene. Oncogene, *11:* 987–991, 1995.

30. Serrano, M., Lee, W-H., Chin, L., Cordon-Cardo, C., Beach, D., and DePinho, R. A. Role of the *INK4a* locus in tumor suppression and cell mortality. Cell, *85:* 27–37, 1996.

31. Kamijo, T, Zindy, F, Roussel, M. F., Quella, D. E., Downing, J. R., Ashmun, R. A., Grosveld, G., and Sherr, C. J. Tumor suppression at the mouse *INK4a* locus mediated by the alternative reading frame product. p19ARF. Cell, *91:* 649–659, 1997.

32. Nielsen, G. P., Burns, K. L., Rosenberg, A. E., and Louis, D. N. *CDKN2A* gene deletions and loss of p16 expression occur in osteosarcomas that lack RB alterations. Am J. Pathol., *153:* 159–163, 1998.

33. Chomczynski, P., and Sacchi, N. Single step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *162:* 156–159, 1987.