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Mutations and altered expression of p16^{INK4} in human cancer

(p53 protein/tumor-suppressor gene/cyclin D1/retinoblastoma protein)

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ABSTRACT **Cell cycle arrest at the G_1 checkpoint allows completion of critical macromolecular events prior to S phase. Regulators of the G_1 checkpoint include an inhibitor of cyclin-dependent kinase, p16^{INK4}; two tumor-suppressor proteins, p53 and RB (the product of the retinoblastoma-susceptibility gene); and cyclin D1. Neither p16^{INK4} nor the RB protein was detected in 28 of 29 tumor cell lines from human lung, esophagus, liver, colon, and pancreas. The presence of p16^{INK4} protein is inversely correlated with detectable RB or cyclin D1 proteins and is not correlated with p53 mutations. Homozygous deletions of p16^{INK4} were detected in several cell lines, but intragenic mutations of this gene were unusual in either cell lines or primary tumors. Transfection of the p16^{INK4} cDNA expression vector into carcinoma cells inhibits their colony-forming efficiency and the p16^{INK4} expressing cells are selected against with continued passage *in vitro*. These results are consistent with the hypothesis that p16^{INK4} is a tumor-suppressor protein and that genetic and epigenetic abnormalities in genes controlling the G_1 checkpoint can lead to both escape from senescence and cancer formation.**

The orderly progression of cells through the cell cycle is governed by genes encoding proteins transmitting positive [e.g., activated cyclin and cyclin-dependent kinases (Cdks)] and negative (e.g., inhibitors of Cdk) signals (1–3). Dysregulation of these genes can lead to premature entry into the next phase of the cell cycle prior to completion of critical macromolecular events, including repair of DNA damage, and generate genomic instability and neoplastic transformation (4). Negative regulation of the cell cycle occurs at G_1 and G_2 checkpoints (4, 5). Phosphorylation of the RB protein by Cdk and the release of RB-associated proteins—e.g., the transcription factor E2F—is correlated with the transition across the G_1 checkpoint (6–10). The free E2F is then available to transcriptionally activate genes encoding proteins critical for S-phase function, including deoxynucleotide biosynthesis (11).

Three inhibitors of activated cyclin–Cdk complexes controlling the G_1 checkpoint of mammalian cells have recently been identified. A gene encoding a 21-kDa inhibitor (p21, WAF1, Cip1, Sdi1) of multiple cyclin–Cdk complexes is one of the downstream effectors of p53-mediated G_1 arrest and apoptosis in response to DNA damage (6, 12–15). p27^{Kip1}, an inhibitor of cyclin D2–Cdk4, has been linked to G_1 arrest of cells either exposed to transforming growth factor β1 or undergoing contact inhibition *in vitro* (16). A third inhibitor, p16^{INK4}, complexes with cyclin D1–Cdk4 or cyclin D2–Cdk4 and may act in a regulatory feedback circuit with Cdk4, D-type cyclins, and RB and RB-related proteins (17). Since genes encoding these and other inhibitors of activated cyclin–Cdk complexes are candidate tumor-suppressor genes, our investigation of this class of putative tumor-suppressor genes was initiated by examining the genomic structure and expression of p16^{INK4} in human cell lines and primary tumors.

MATERIALS AND METHODS

Cell Lines. WI-38 (human diploid cell line from normal embryonic lung tissue); A549, A427, A2182, 866MT, HuT292DM, DM592, SW1271, SK-Lu-1, Calu-1, Calu-6, NCI-H526, NCI-N417, NCI-H1155, NCI-H358, NCI-H157, NCI-H322, NCI-H596, and NCI-H446 (lung cancer cell lines); Hep 3B, Hep G2, SK-Hep-1, HuH-4, HuH-7, Ha22T/VGH, HB611, and 2.2.15 (liver cancer cell lines); CAPAN-2 and ASPC-1 (pancreatic cancer cell lines); SK-OV-3 (ovarian cancer cell line); DLD-1, COLO320, SW480, SW620, HT29, WIDR, LS174T, HCT-116, SK-CO1, SW948, SW48, SW403, and HCT-15 (colon cancer cell lines); H9, CCL119, and CCL120 (leukemia cell lines); MCF7, HLB100, HTB126, and ZRB75 (breast cancer cell lines); and U-118 (glioma cell line) were obtained from the American Type Culture Collection. M9K, M24K, M14M, and M19 were provided by K. Linnainmaa (Finnish Institute of Occupational Health, Helsinki). tc4N was provided by M. Noguchi (National Cancer Center Research Institute, Tokyo). BEAS2B, HET-1A, HCE-3, HCE-4, HCE-7, and THLE5B were established in our laboratory (National Cancer Institute).

p16^{INK4} Genomic Clones and Fluorescence *in Situ* Hybridization (FISH). Genomic clones of the p16^{INK4} gene were isolated by high-stringency screening of a λFIXII human genomic library (Stratagene) with cDNA probes. A 13-kb p16^{INK4} genomic insert was subcloned into pBluescript SK(−) (Stratagene) and labeled for FISH analysis. FISH was performed by established methods. The stained slides were counterstained with propidium iodide (for an R banding pattern) or with 4′,6-diamidino-2-phenylindole (DAPI) and actinomycin D (for a DA-DAPI banding pattern). Alignment of monochrome digital images was made by direct visualization through a triple-bandpass (DAPI/fluorescein/Texas Red) filter. Between 50 and 100 mitoses were examined. Chromosome localization was confirmed by PCR analysis from a commercially available somatic cell hybrid panel (Bios, New Haven, CT) and by dual labeling with a commercially available B-satellite probe for chromosome 9 (Oncor).

Southern and Northern Analysis. *Hin*dIII-digested DNAs were hybridized with human [α-^{32}P]dCTP-labeled p16^{INK4} cDNA and ERCC3 probes. Northern blot filters were hybridized with [α-^{32}P]dCTP-labeled human p16^{INK4} cDNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes.

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Abbreviations: Cdk, cyclin-dependent kinase; DAPI, 4′,6-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization; SSCP, single-strand conformation polymorphism.

Immunoprecipitation and Western Blot Analysis. Protein lysates were prepared as described (17). Three hundred micrograms of either ^{35}S-labeled or unlabeled protein was immunoprecipitated with anti-p16^{INK4} (17), anti-cyclin D1 (Upstate Biotechnology, Lake Placid, NY), or anti-RB (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. For detection of radiolabeled proteins, gels were fixed and exposed to x-ray film. For Western blot detection, proteins were electrophorectically transferred to poly(vinylidene difluoride) membranes (Millipore), probed with antibodies, and detected by chemiluminescence (DuPont).

PCR and Single-Strand Conformation Polymorphism (SSCP) Analysis. PCR intronic primers were identified from the p16^{INK4} genomic sequence. Primers for exon 1 were (5′–3′) 1A, CGGAGAGGGGGAGAGCAG (sense), and 1B, TCCCCTTTTTCCGGAGAATCG (antisense). PCR conditions for amplification of exon 1 consisted of a 5-min denaturation at 94°C, followed by 40 cycles of 40 sec at 94°C, 40 sec at 55°C, and 90 sec at 72°C. Primers for exon 2 were (5′–3′) 2A, CTCTACACAAGCTTCCTTTCC (sense), and 2B, GGGCTGAACTTTCTGTGCTGG (antisense). PCR conditions consisted of a 5-min denaturation at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C. For SSCP analysis, ^{32}P-labeled PCR products were heat denatured and applied to a neutral 6% polyacrylamide gel containing 2, 5, or 10% (vol/vol) glycerol. For exon 2, PCR product was digested with *Sma* I before loading.

Nucleotide Sequence Determination. PCR product was purified and sequenced by the dideoxy chain-termination method with a Sequenase kit (United States Biochemical).

Transfection. The 960-bp *Eco*RI fragment of the p16^{INK4} cDNA (17) was removed from pBluescript vector and ligated into the *Xba* I site of the vector pRc/CMV (Invitrogen). Both orientations were used to create sense and antisense constructs. Cells were transfected with 10 μg of either pCMV-p16 sense or pCMV-p16 antisense by use of a Lipofectin reagent (GIBCO/BRL). Forty-eight hours after transfection, Calu-6 and SK-OV-3 cells were selected with G418 (GIBCO/BRL) at 225 and 525 μg/ml, respectively.

Colony-Forming Efficiency Experiments. Forty-eight hours after transfection, HCE-4 cells were replated at a density of 3.3×10^5 cells per dish and M9K cells at 1.0×10^5 cells per dish. Resistant cell populations were then selected with 199 medium (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (Biofluids) and G418 at 275 μg/ml (HCE-4) or LHC-MM medium (Biofluids) with G418 at 600 μg/ml (M9K). Colonies were counted by means of an Autocount image analyzer (Dynatech).

Immunocytochemistry. Slides were seeded, fixed, and stained by conventional methods. p16^{INK4} protein was examined with a 1:50 dilution of an immunoaffinity-purified rabbit antiserum raised against glutathione *S*-transferase (GST)–p16^{INK4} fusion protein (17). Residual antibodies to GST were absorbed by incubating the affinity-purified antiserum with a 50-fold molar excess of GST at 37°C for 1–3 hr. Specificity of the affinity-purified antiserum was demonstrated by suppressing all staining by incubation with a 50-fold molar excess of GST–p16^{INK4}.

RESULTS AND DISCUSSION

The p16^{INK4} gene was localized by FISH to chromosome 9p21–22 (Fig. 1). Previous cytogenetic and allelic deletion analyses have demonstrated frequent deletions and rearrangements of chromosome 9p in carcinoma of the human bladder, lung, head and neck, and esophagus (18–21), brain glioma (22, 23), leukemia (24), mesothelioma (25), and melanoma (26). Therefore, we examined the genomic status and steady-state levels of p16^{INK4} mRNA and protein in cell lines selected from the above and other tumor types (Fig. 2; Table 1). Homozygous deletions of p16^{INK4} were found in tumor cell lines by amplifying DNA with the PCR using specific intronic primers and were confirmed by Southern blot analysis in 5 of 5 (100%) pleural mesothelioma, 7 of 18 (39%) lung carcinoma, 1 of 3 (33%) esophageal carcinoma, 1 of 8 (13%) liver carcinoma, 2 of 3 (67%) acute lymphocytic leukemia, 2 of 4 (50%) breast carcinoma, 1 of 1 (100%) glioma and 0 of 13 (0%) colon carcinoma cell lines (Table 1; Fig. 2*A*). Expression of the p16^{INK4} gene was analyzed in 29 of the above tumor cell lines (Table 1; Fig. 2*B*). p16^{INK4} mRNA and protein were detected in normal WI-38 human fibroblasts and the nontumorigenic, simian virus 40 T-antigen-immortalized human cell lines HET-1A, BEAS2B, and THLE5B. In contrast, p16^{INK4} mRNA and protein were not detected in 12 of 28 (43%) and 23 of 29 (79%) tumor cell lines, respectively (Table 1). The tumor cell lines devoid of detectable p16^{INK4} mRNA and protein often have homozygous deletions of the gene (Table 1; Fig. 2*A*). These data are consistent with the cytogenetic and allelic analyses of human cancers and tumor cell lines showing homozygous deletions and the recently described mutations of the p16^{INK4} gene, which has been termed the multiple tumor suppressor 1 (MTS1) (28) or Cdk4 inhibitor gene (29) in human cancers.

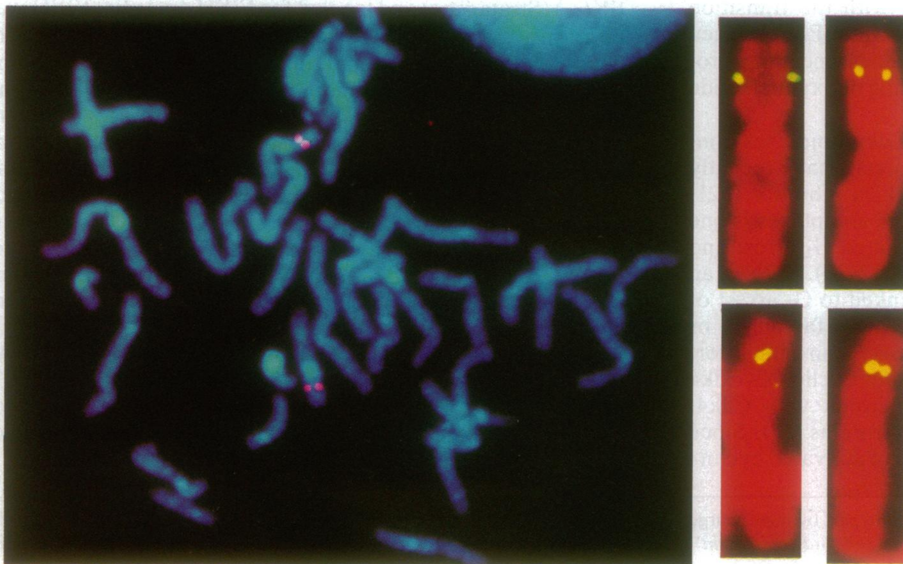

FIG. 1. Localization of the p16^{INK4} gene to chromosome 9p21–22 by FISH. (*Left*) DA-DAPI banding pattern. Dual chromatid Cy3 (indocarbocyanine) labeling shows the localization of the genomic p16^{INK4} probe to both copies of chromosome 9p. (*Right*) Higher-magnification views of propidium iodide-stained chromosomes demonstrate localization of the fluorescein-labeled probe to 9p21–22. Propidium iodide-stained chromosome pairs are from the same mitotic spread.

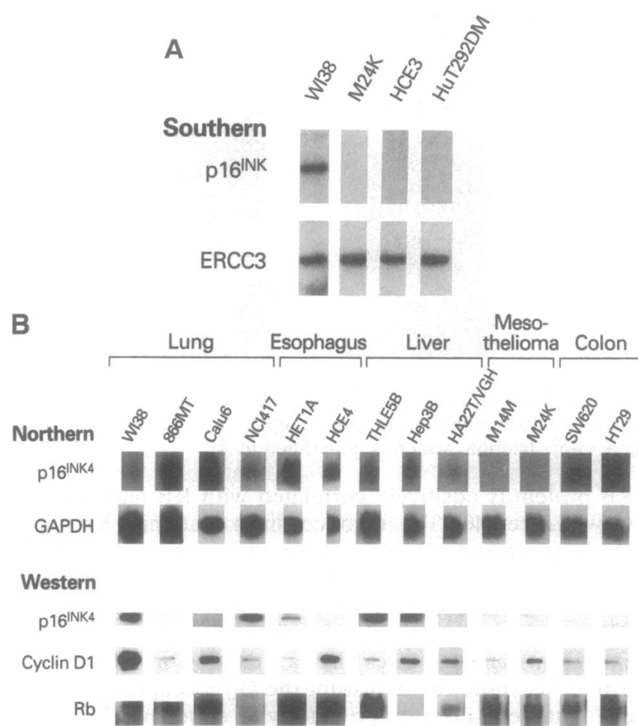

FIG. 2. (*A*) Homozygous deletions in cancer cell lines (mesothelioma, M24K; esophageal carcinoma, HCE-3; lung carcinoma, HuT292DM) compared with normal human fibroblasts (WI-38). (*B*) Steady-state levels of p16^{INK4} and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs and p16^{INK4}, RB, and cyclin D1 proteins in representative cell lines.

Since tumor cell lines expressing p16^{INK4} mRNA, but not its translated protein, may harbor nonsense and frameshift mutations in the p16^{INK4} gene, DNA from such cell lines was amplified by PCR with specific primers and then analyzed by SSCP and DNA sequencing. A lung (866MT) and a liver (Ha22T/VGH) carcinoma cell line had abnormal SSCP patterns, and DNA sequencing revealed deletions in exon 1 of the p16^{INK4} gene (Fig. 3; Table 2). When compared with the predominantly nuclear localization of p16^{INK4} protein in normal cells (WI-38 and HET-1A), p16^{INK4} was not detected by immunocytochemistry in 866MT cells, which contain a p16^{INK4} mutation (Fig. 4). Several other tumor cell lines had abnormal SSCP patterns in exon 1 (DLD-1, HCT-116, HCT-15, and CAPAN-2) or exon 2 (NCI-H157), and some of them were confirmed by DNA sequencing (Table 2). These deletions and insertions are consistent with the DNA polymerase slippage model of endogenous mutagenesis (27) and may have occurred either *in vitro* or *in vivo*. The remaining tumor cell lines have normal SSCP patterns, suggesting either defects of p16^{INK4} mRNA at the level of processing and translation or enhanced degradation of the p16^{INK4} protein.

We also initiated a survey of primary tumors analyzed by SSCP and DNA sequencing. Tumor-specific SSCP patterns were not observed in 12 esophageal carcinomas, 7 lung carcinomas, and 6 liver carcinomas. One germline mutation was found at codon 127, GCA (Ala) to TCA (Ser). Further studies are needed to determine whether this alteration is related to cancer-proneness or is a polymorphism unrelated to cancer risk. These results and others (30, 31) indicate that the frequency of intragenic p16^{INK4} gene mutations in some types of primary tumors (i.e., lung, bladder, kidney, head and neck, brain, colon, and ovary) is quite low. Nevertheless, Mori *et al.* (32) recently reported that mutations of the p16^{INK4} gene were detected in 52% of Japanese esophageal carcinomas. Although we examined microdissected Chinese

Table 1. Expression of p16^{INK4}, cyclin D1, and RB genes and genomic status of p16^{INK4} and p53 genes in normal and neoplastic human cells

	p16^{INK4}						
	DNA (SSCP)						
Cell type	Exon 1	Exon 2	mRNA	Protein	p53 gene*	RB	Cyclin D1
Fibroblast							
WI-38†	P(W)	P(W)	+	+	W	+	+
Esophagus							
HET-1A‡	P(W)	P(W)	+	+	W	+	+
HCE-4	P(W)	P(W)	+	−	M	+	+
Lung							
BEAS2B‡	P(W)	P(W)	+	+	W	+	+
866MT	A(M)	P(W)	+	−	M	+	+
Calu-1	P(W)	P(W)	+	−	M	+	+
Calu-6	P(W)	P(W)	+	−	M	+	+
NCI-N417	P(W)	P(W)	+	+	M	−	+
NCI-H1155	P(W)	P(W)	+	+	M	−	−
NCI-H358	P(W)	P(W)	+	−	M	+	+
NCI-H157	P(W)	P(M)	+	−	M	+	+
NCI-H322	D	D	−	−	M	+	+
NCI-H596	P(W)	P(W)	ND	+	M	−	+
Liver							
THLE5B‡	P(W)	P(W)	+	+	W	+	+
Hep 3B	P(W)	P(W)	+	+	M	−	+
Hep G2	P(W)	P(W)	+	+	W	+	+
HuH4	P(W)	P(W)	+	−	M	+	+
Ha22T/VGH	A(M)	P(W)	+	−	M	+	+
HB611	P(W)	P(W)	+	+	W	+	+
Pancreas							
ASPC-1	P(W)	ND	+	−	M	+	+
Mesothelioma							
M9K	D	D	−	−	W	+	+
M24K	D	D	−	−	W	+	+
M14M	D	D	−	−	W	+	+
M19	D	D	−	−	W	+	+
M33K	D	D	−	−	W	+	+
Colon							
DLD-1	P(M)	P(W)	−	−	M	+	+
SW620	P(W)	P(W)	+	−	M	+	+
HT29	P(W)	P(ND)	+	−	M	+	+
LS174T	P(W)	P(W)	−	−	W	+	+
HCT-116	P(M)	ND	−	−	M	+	+
SW948	P(W)	P(ND)	−	−	ND	+	−
SW403	P(W)	P(W)	−	−	ND	+	−
HCT-15	P(M)	P(W)	−	−	ND	+	+

See *Materials and Methods* for sources of cell lines. Glyceraldehyde-3-phosphate dehydrogenase mRNA was present in all cell lines. +, Expression; −, not detected; ND, not done; A, abnormal; P, present; D, deleted; W, wild type; M, mutant.
*Data from ref. 27.
†Human diploid cell line from normal embryonic lung tissue.
‡Human epithelial cell line immortalized by simian virus 40 T antigen.

esophageal carcinoma samples, we did not find any mobility shifts by PCR–SSCP analysis in exons 1 and 2. One possibility is that there are some false negative cases by PCR–SSCP analysis. However, we used several conditions for PCR–SSCP analysis and PCR products of <300 bp were analyzed by SSCP, which generally has a false negative rate of <10% (33). Another possibility is that there are different genetic and environmental factors for esophageal carcinogenesis between the Japanese and the Chinese.

The functional significance of the loss of p16^{INK4} was investigated by transfection of a vector containing the p16^{INK4} cDNA driven by a cytomegalovirus promoter into a human esophageal carcinoma cell line (HCE-4). The colony-

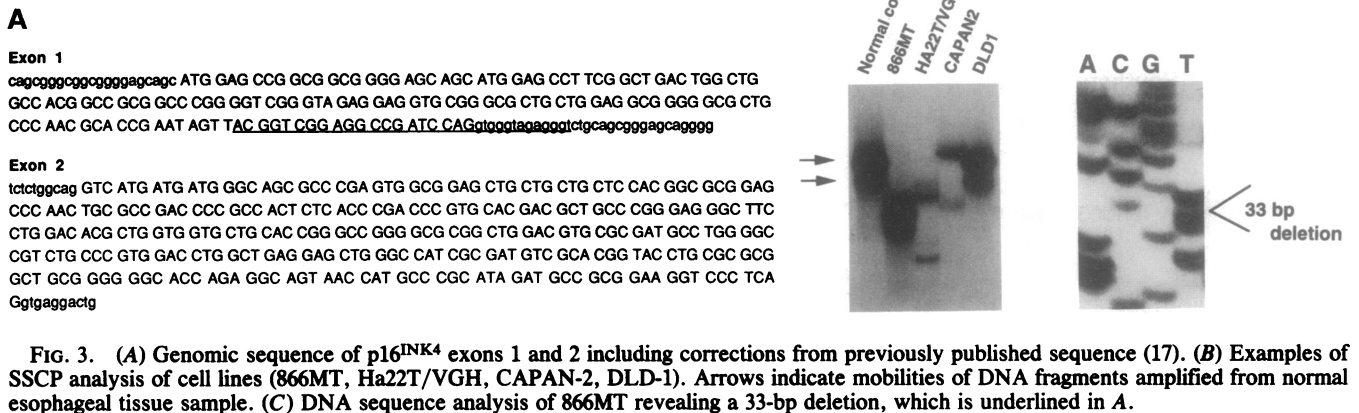

FIG. 3. (*A*) Genomic sequence of p16^{INK4} exons 1 and 2 including corrections from previously published sequence (17). (*B*) Examples of SSCP analysis of cell lines (866MT, Ha22T/VGH, CAPAN-2, DLD-1). Arrows indicate mobilities of DNA fragments amplified from normal esophageal tissue sample. (*C*) DNA sequence analysis of 866MT revealing a 33-bp deletion, which is underlined in *A*.

forming efficiency of the p16^{INK4}-transfected cells was 112 ± 8 (mean ± SD) compared with 192 ± 3 in the cells transfected with antisense p16^{INK4} (68%). A similar degree of inhibition was found using a human mesothelioma cell line (M9K) which had homozygous deletion of p16^{INK4} (53%), whereas the RB-deficient cell line Hep 3B was inhibited to a lesser extent (83%). We transfected the p16^{INK4} expression vector into Calu-6 and SK-OV-3, which lack p16^{INK4} expression, and isolated clones were expanded. SK-OV-3 showed rearrangement of the p16^{INK4} gene. Although p16^{INK4} mRNA was transiently expressed at 48 hr, p16^{INK4} protein was not detected by Western analysis in the expanded cell populations. Expression of transfected wild-type p53 also reduces colony-forming efficiency of human cancer cells to varying degrees and the cells expressing wild-type p53 are selected against during clonal expansion (34, 35). Therefore, expression of transfected p16^{INK4} inhibits growth and is selected against in these cells.

The finding of homozygous deletions and nonsense point mutations in cancer cell lines and inhibition of their growth by expression of a transfected p16^{INK4} gene is genetic and functional evidence consistent with the hypothesis that p16^{INK4} is a recessive tumor-suppressor gene (Fig. 2*A*; Table 1; refs. 2, 27 and 28). However, additional studies are required to search for germline mutations that segregate with cancer in families and to determine the frequency of p16^{INK4} mutations in a wide spectrum of primary tumors. The spectrum of somatic p16^{INK4} mutations differs from that of the p53 gene. p53 mutations are primarily missense (reviewed in refs. 36 and 37), whereas p16^{INK4} mutations are primarily deletion and nonsense mutations (Table 2; refs. 28 and 29). This indicates a loss of function of the p16^{INK4} gene product with inhibition of activated cyclin D–Cdk4 phosphorylation of cell cycle-related substrates, including the RB protein, whereas missense mutations of p53 can produce both loss of suppressor function and gain of oncogenic activity (38).

The G_1 checkpoint can be abrogated by genetic and epigenetic mechanisms. Mutations in the *RB* gene can inhibit RB protein neutralization of the transcriptional activity of E2F (reviewed in ref. 39). Therefore, we examined the status of RB protein in the cell lines (Table 1; Fig. 2*B*), since loss of RB protein is positively correlated with RB mutations (reviewed in ref. 40). The tumor cell lines containing RB protein rarely expressed p16^{INK4} protein, and conversely, p16^{INK4}-expressing tumor cell lines rarely had detectable RB protein. This inverse relationship between expression of p16^{INK4} and RB protein is statistically significant ($P < 0.0006$, Fisher exact test) and indicates that a mutation in either RB or p16^{INK4} is sufficient to disrupt the G_1 checkpoint pathway. p53 mutations do not correlate with either p16^{INK4} or RB expression in these tumor cell lines ($P = 0.38$ and 0.13, respectively), indicating that p53 participates in an independent pathway such as regulation of the G_1 checkpoint in response to DNA damage.

Since cyclin D1 overexpression accelerates entry of cells into S phase (41, 42), negates the G_1 arrest induced by RB introduced into *RB*-null cell lines (43, 44), and neoplastically transforms rat embryo fibroblasts alone or in cooperation with the E1A or Ha-*ras* oncogene (45–47), the steady-state levels of cyclin D1 were analyzed (Fig. 2*B*; Table 1). Although the expression of p16^{INK4} and cyclin D1 is not correlated, the positive correlation between RB and cyclin D1 proteins ($P = 0.05$) is consistent with the recent observation of RB protein regulating the expression of cyclin D1 (48).

Continuous, long-term culture may select for cells with loss of senescence genes. p16^{INK4} is a candidate for the cellular senescence gene mapped to chromosome 9 by cell–cell hybrid analysis of immortal cells (49). The G_1-arrest state of senescent cells is associated with persistent hypophosphorylated RB protein and inactive cyclin–Cdk complexes and, thus, has much in common with quiescent mortal cells (50). Functional defects of senescence genes, including p21 (15) and perhaps p16^{INK4}, would allow cells to escape programmed senescence and enhance the probability of their neoplastic transformation.

The overexpression and gene amplification of G_1 cyclins; the mutations in the p16^{INK4}, p53, and *RB* genes; and the inactivation of the p53 and *RB* genes by oncoproteins of certain DNA viruses such as human papillomavirus implicate dysregulated control of the G_1 checkpoint in carcinogenesis and tumor progression. The low frequency of p16^{INK4} muta-

Table 2. Mutations of p16^{INK4} in cell lines and primary tumors

Sample	Mutation	Coding effect	Location*
866MT (lung)	33-base deletion	Splice alteration	Codon 44 base 2 to intron 1 base 13
Ha22T/VGH (liver)	23-base deletion	Stop codon in exon 1	Codon 21 base 1 to codon 28 base 2
CAPAN-2 (pancreas)	6-base insertion	ACG-GCC (Thr-Ala)	Between codons 19 and 20
HCT-116 (colon)	1-base insertion	Stop codon in exon 1	Codon 23
Lung primary†	GCA → TCA	Ala → Ser	Codon 127

*Codon numbers are based on numbering 1 at the start codon ATG (Fig. 3*A*).
†This mutation was detected both in the tumor and in the corresponding normal tissue.

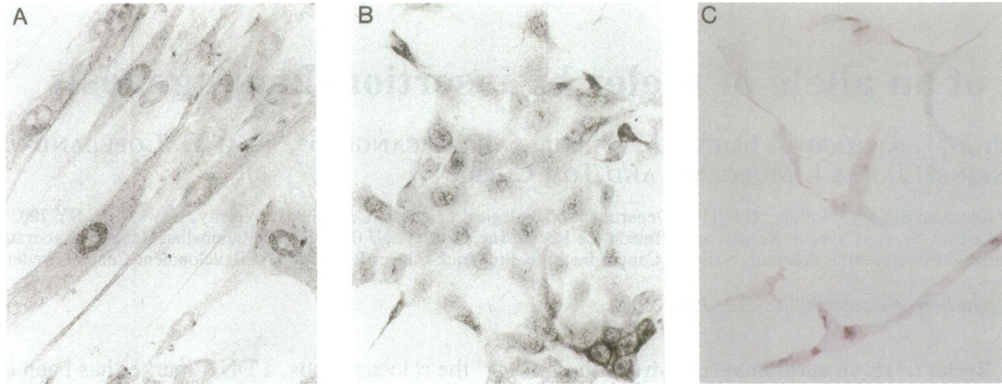

FIG. 4. Immunocytochemical staining of p16^{INK4} protein. (*A*) Normal human fibroblasts (WI-38, no counterstain) showed cytoplasmic and nuclear staining which varied among cells. (*B*) A nontumorigenic, simian virus 40 T-antigen-immortalized human esophageal epithelial cell line (HET-1A, no counterstain) showed less intense staining. (*C*) A lung cancer cell line (866MT, eosin counterstain) was negative.

tions in primary tumors when compared with the high frequency in tumor cell lines indicates that one should be cautious in extrapolating data from cell lines to primary tumors. Since p16^{INK4} may be inactivated in the late stages of tumor progression, a comparison of primary tumors and their metastases is warranted.

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