

Alterations of $p14^{ARF}$, $p53$, and $p73$ Genes Involved in the E2F-1-mediated Apoptotic Pathways in Non-Small Cell Lung Carcinoma

Siobhan A. Nicholson,¹ Nader T. Okby,¹ Mohammed A. Khan, Judith A. Welsh, Mary G. McMenamin, William D. Travis, James R. Jett, Henry D. Tazelaar, Victor Trastek, Peter C. Pairolero, Paul G. Corn, James G. Herman, Lance A. Liotta, Neil E. Caporaso, and Curtis C. Harris²

Laboratory of Human Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20892 [S. A. N., M. A. K., J. A. W., M. G. M., L. A. L., N. E. C., C. C. H.]; Orange Pathology Associates, Middleton, New York 10940 [N. T. O.]; Armed Forces Institute of Pathology, Washington, DC 20306 [S. A. N., W. D. T.]; Mayo Clinic, Rochester, Minnesota 55905 [J. R. J., H. D. T., V. T., P. C. P.]; and The Johns Hopkins Oncology Center, Baltimore, Maryland 21231 [P. G. C., J. G. H.]

ABSTRACT

Overexpression of *E2F-1* induces apoptosis by both a $p14^{ARF}$ - $p53$ - and a $p73$ -mediated pathway. $p14^{ARF}$ is the alternate tumor suppressor product of the *INK4a/ARF* locus that is inactivated frequently in lung carcinogenesis. Because $p14^{ARF}$ stabilizes $p53$, it has been proposed that the loss of $p14^{ARF}$ is functionally equivalent to a $p53$ mutation. We have tested this hypothesis by examining the genomic status of the unique exon 1 β of $p14^{ARF}$ in 53 human cell lines and 86 primary non-small cell lung carcinomas and correlated this with previously characterized alterations of $p53$. Homozygous deletions of $p14^{ARF}$ were detected in 12 of 53 (23%) cell lines and 16 of 86 (19%) primary tumors. A single cell line, but no primary tumors, harbored an intragenic mutation. The deletion of $p14^{ARF}$ was inversely correlated with the loss of $p53$ in the majority of cell lines ($P = 0.02$), but this relationship was not maintained among primary tumors ($P = 0.5$). *E2F-1* can also induce $p73$ via a $p53$ -independent apoptotic pathway. Although we did not observe inactivation of $p73$ by either mutation or DNA methylation, haploinsufficiency of $p73$ correlated positively with either $p14^{ARF}$ or $p53$ mutation or both ($P = 0.01$) in primary non-small cell lung carcinomas. These data are consistent with the current model of $p14^{ARF}$ and $p53$ interaction as a complex network rather than a simple linear pathway and indicate a possible role for an *E2F-1*-mediated failsafe, $p53$ -independent, apoptotic pathway involving $p73$ in human lung carcinogenesis.

INTRODUCTION

The Rb^3 and $p53$ -mediated checkpoints are central to the control of cell cycle progression (1, 2). As linchpins of tumor suppression, the disruption of their function is a hallmark of carcinogenesis. These pathways are linked, and their activities are influenced, in turn, by a single genetic locus, *INK4a*, located on chromosome 9p21 (3, 4). This locus encodes two tumor suppressors in a manner unique to eukaryotic cells. $p16^{INK4a}$ is encoded by exons 1 α , 2, and 3, and inhibits the cyclin-dependent kinases 4 and 6. This then inhibits the phosphorylation of Rb, resulting in the sequestration of E2F transcription factors and prevents the transition from G₁ to S-phase of the cell cycle (5). $p16^{INK4a}$ was identified as a tumor suppressor as a result of its association with germ-line mutations in familial melanomas (6–8) and with frequent deletion, mutation, and promoter methylation in sporadic human tumors and cell lines (9–12).

The recently discovered murine $p19^{ARF}$ and its human homologue $p14^{ARF}$ are the alternate transcripts of this locus (13). $p14^{ARF}$ is

encoded by a separate exon 1 β that lies ~20 kb upstream of exon 1 α and shares exons 2 and 3 as read in an ARF, giving rise to a protein completely unrelated to $p16^{INK4a}$ (14). Despite its unrelated structure, $p14^{ARF}$ also is capable of causing cell cycle arrest in G₁ and G₂. $p14^{ARF}$ binds to and antagonizes the actions of MDM2, a negative regulator of $p53$ (15). Thus, it interferes with the ability of MDM2 to block transcription, to ubiquitinate, and to transport $p53$ to the cytoplasm for degradation (16–22). In response to DNA damage, the accumulation of $p53$ results in cell cycle arrest or apoptosis. The loss of $p14^{ARF}$ increases $p53$ degradation, thereby diminishing the $p53$ response to genotoxic stress. The role of $p14^{ARF}$ in carcinogenesis was first demonstrated in mice by showing that ARF-null mice are highly tumor prone (23) and develop sarcomas, lymphomas, carcinomas, and gliomas and thus, die early in life (24). Mice that are heterozygous for ARF also develop tumors but die later in life. Mouse embryonic fibroblasts that lack ARF do not undergo replicative senescence in culture and can be transformed by oncogenic *ras* alone (23). On the basis of this evidence, it was hypothesized that $p14^{ARF}$ functions as a tumor suppressor *in vivo*.

The modulation of the $p53$ response to genotoxic stress by $p14^{ARF}$ also raises the possibility that patients, whose tumors show a loss of $p14^{ARF}$, have a poorer prognosis. This protective role has been demonstrated in mice, where ARF protects cells against Myc-induced tumors, and on an ARF-null background, E μ -Myc transgenic mice die of aggressive lympholeukemias at an early age (25). Although alterations of $p16^{INK4a}$ have been demonstrated in ~50% of NSCLCs (26–31), the status of $p14^{ARF}$ has not been as well characterized. Although $p14^{ARF}$ deletions are well described, intragenic mutations have not been identified within the unique exon 1 β .

There may be additional associations between $p14^{ARF}$ and other cell cycle regulators, oncoproteins, and tumor suppressors. $p14^{ARF}$ is not activated directly by DNA damage, but rather by a subset of abnormal hyperproliferative signals including oncoproteins *myc*, *ras*, and *v-abl* (32) and *E2F-1* (33). As one of the targets of phosphorylated Rb, the induction of $p14^{ARF}$ by *E2F-1* establishes cross-talk between the *Rb* and $p53$ pathways (33). The activities of the E2F family of transcription factors influence both cell cycle progression and apoptosis, and the loss of regulation is seen in many human cancers (34). In addition to $p14^{ARF}$, $p73$, an evolutionary relative of $p53$ and putative tumor suppressor, also is a downstream target of *E2F-1*. The activation of $p73$ has been shown recently to provide a means for *E2F-1* to induce cell death in the absence of $p53$ (35). Furthermore, the disruption of $p73$ function inhibits *E2F-1*-induced apoptosis in $p53$ -null cells (36). Because *E2F-1*-induced apoptosis uses both a $p53$ -independent pathway (mediated by $p73$) and a $p53$ -dependent pathway (mediated by $p14^{ARF}$; Ref. 37), the loss of this apoptotic signal through the disruption of both pathways may enhance tumor development, raising the selection pressure for these genetic alterations in tumors. We tested these hypotheses by studying alterations of exon 1 β of $p14^{ARF}$ and $p73$ in a series of NSCLCs and sought correlations with other clinical, genetic, pathological, and epidemiological markers examined previ-

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¹ These authors contributed equally to this work.

² To whom requests for reprints should be addressed, at Laboratory of Human Carcinogenesis, National Cancer Institute, NIH, Building 37, Room 2C05, 37 Convent Drive, Bethesda, MD 20892-4255. Phone: (301) 496-2048; Fax: (301) 496-0497; E-mail: Curtis_Harris@nih.gov.

³ The abbreviations used are: Rb, retinoblastoma; ARF, alternative reading frame; MDM, murine double minute; SCLC, small cell lung carcinoma; NSCLC, non-SCLC; LOH, loss of heterozygosity; SSCP, single strand conformation polymorphism; WT, wild type.

ously by our group including p53 status. We assessed the prognostic significance of the loss of p14^{ARF} alone or in combination with p53 and p73 in primary-resected tumors. Furthermore, because the p53 and Rb pathways are cross-linked intimately through p14^{ARF}, we also studied p14^{ARF} in a series of human cell lines in conjunction with data acquired previously on the other members of these two pathways including p73, p16^{INK4a}, p53, Rb, and cyclin D1.

MATERIALS AND METHODS

Primary Tumor Samples. DNA extracted from archival NSCLCs and nontumor tissue of 106 patients surgically treated at the Mayo Clinic between 1991 and 1992 formed one arm of this study. An epidemiological profile was available for each patient including demographics, medical, smoking, family, and occupational history, in addition to survival data that now include 9 years of follow-up. The series was composed of 63 males and 43 females, and tumor histology included adenocarcinoma ($n = 55$), squamous cell carcinoma ($n = 29$), and large cell carcinoma ($n = 22$). As part of an ongoing study of these NSCLCs, data were available from previous analyses including p53 mutations and the expression of *c-erbB-2* (38), p21^{waf1}, transforming growth factor- β 1 (39), bcl-2 (40), cyclooxygenase-2, NOS2 (41), LOH in the putative tumor suppressor gene *FHIT* (42), and genetic polymorphisms of *CYP1A1*, *CYP2E1*, and *GSTM1* (43).

DNA Extraction from Tumor and Nontumor Tissue. After microdissection of tumor tissue, DNA was isolated by proteinase K and chloroform phenol extraction according to standard protocols (44).

Cell Lines. The 53 cell lines analyzed were derived from 11 NSCLCs, 6 SCLCs, 2 mesotheliomas, 11 colon carcinomas, 5 breast carcinomas, 3 pancreatic carcinomas, 5 hepatocellular carcinomas, 2 hepatoblastomas, 1 SV40-immortalized liver cell line, 1 cervical carcinoma line, 1 oral squamous cell carcinoma, 1 lymphoblastic leukemia, 1 lymphoma, 1 glioblastoma, and 1 ovarian carcinoma. We used DNA extracted from stocks of cells frozen at the time of the previous studies (Table 1).

PCR and Sequence Analysis of p14^{ARF}. Oligonucleotide primers for exon 1 β of p14^{ARF} were synthesized (Bioserve Biotechnologies Ltd., Laurel, MD) according to published sequences. The sequences were 5'-TCCAGTCTG-CAGTTAAGG-3' and 5'-GTCTAAGTCGTTGTAACCCG-3'. The expected 439-bp product includes the 267-bp exon 1 β of p14^{ARF} and 172 bp of noncoding intronic material. Three μ l of a DNA template were amplified with AmpliTaq DNA polymerase (Perkin-Elmer) in a total reaction volume of 100 μ l that included 10 μ l 10 \times PCR buffer (Perkin-Elmer), 10 μ l of DMSO, 2 μ l of deoxynucleotide triphosphates, and 20 pmol (2 μ l) of each primer. Conditions adapted from Sanchez-Cespedes *et al.* (45) were 94°C for 5 min and 35 cycles of 94°C for 1 min; 58°C for 1 min; and 72°C for 1 min, followed by a 4-min extension at 72°C on a GeneAmp PCR System 9700 (PE Applied Biosystems). The presence and integrity of DNA was checked by amplifying exons 6 and 7 of p53, which yielded a 150- and 486-bp fragment, respectively. Normal human placental DNA (Sigma Chemical Co., St. Louis, MO) and water included in each reaction served as positive and negative controls, respectively. The amplified 439-bp product was electrophoresed on a 3% agarose gel, visualized by staining with ethidium bromide and UV illumination, and documented by a computer-linked camera. Persistent absence of a band after repeated PCRs with increasing volumes of DNA template, in the presence of a positive result with p53 primers, was interpreted as a homozygous deletion of p14^{ARF} exon 1 β . Seven cases that also failed to amplify the 486-bp product of exon 7 of p53, when known to be present from previous analysis, were excluded from the final series of 86 cases.

The PCR products were column purified (QIAquick PCR Purification kit; Qiagen, Inc., Valencia, CA) and sequenced directly using an ABI PRISM BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems). The same set of primers was used for sequencing and for PCR. The 20- μ l reaction volume consisted of 5 μ l of PCR product, 2 μ l of DMSO, 20 pmol of forward or reverse primer, 3 μ l of water, and 8 μ l of terminator mix. Reaction conditions were: 25 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The samples were electrophoresed on an ABI prism 377 DNA Sequencer (PE Applied Biosystems) after a second purification step. The sequence obtained was compared with the known sequence of exon 1 β of p14^{ARF} downloaded from GenBank.

Allelic Deletion Analysis of p73. Oligonucleotide primers to the chromosome 1p36 gene locus, *DIS2893*, were retrieved from the Internet site, www.gdb.org, and synthesized. The sequences were 5'-AAAACATCAA-CTCTCCCCTG-3' and 3'-CTCAAACCCCAATAAGCCTT-5'. Five μ l of a DNA template were amplified with AmpliTaq DNA Polymerase (Perkin-Elmer) in a total reaction volume of 100 μ l as described above. The conditions were 35 cycles of 94°C for 1 min, 50°C for 1 min, followed by 72°C for 30 s. The 215-bp product was confirmed on a 3% agarose gel. LOH was determined by running each tumor and nontumor amplicon in adjacent lanes on a 5% MetaPhor gel to resolve allelic bands and to identify informative cases. Informative cases with either complete absence or at least 50% attenuation of intensity of allelic bands in tumor samples relative to nontumor samples were considered to have LOH.

SSCP. The confirmation of LOH was performed by SSCP. Genomic DNA from the LOH pairs was reamplified with [³³P]dATP using identical PCR conditions. Sample pairs were heat denatured and run on a 6% polyacrylamide, 5% glycerol gel for 3–5 h at 700 V. The gels were fixed in a 10% acetic acid, 10% methanol solution, and dried and exposed to photographic film for band identification. Cases confirmed by SSCP were subjected to mutational analysis.

PCR and Sequence Analysis of Exons 1–13 of p73. Samples were analyzed using previously published primers and methods (46). A modified protocol was used using the Advantage-GC cDNA PCR kit from Clontech. The PCR products were column purified (Concert Rapid PCR Purification System kit; Life Technologies, Inc.). Purified PCR products were sequenced as before using the ABI PRISM BigDye Terminator Cycle Sequencing Reaction kit.

DNA Methylation of p73 Promoter. The 10 cases that demonstrated LOH of p73 were further analyzed for methylation of the p73 promoter, using a human bacterial artificial chromosome clone containing exon 1 and the 5' region of p73 and methylation-specific PCR. The method has been described previously (47).

Statistical Analysis. p14^{ARF} and p73 status within the NSCLC series was correlated with clinicopathological, epidemiological, and survival data acquired previously by our group using a commercially available statistical package, SPSSv10. Associations between the status of p14^{ARF} and p73, and other clinicopathological and genetic markers, were examined using χ^2 and Fisher exact tests. Associations were considered significant if two-tailed P s were <0.05 . Kaplan-Meier curves plotted cumulative survival against time, and differences in patient survival were analyzed using the log-rank test. Associations between the p14^{ARF} and p53 status and other cell cycle pathway proteins within cell lines also were examined using χ^2 tests.

RESULTS

p14^{ARF} Deletions in Cell Lines. Exon 1 β of p14^{ARF} was deleted from 12 of 53 (23%), mutated in 1 of 53 (2%), and contained WT sequence in the remaining 40 of 53 (75%) cell lines examined (Table 1). Deletions were present in 4 of 11 (36%) NSCLCs (A2182, A-427, A549, and NCI-H292), 2 of 2 mesothelioma (M24 and M9K), 2 of 3 (67%) pancreatic carcinoma (ASPC-1 and MIA PaCa-2), 1 of 5 (20%) hepatocellular carcinoma (SK-HEP-1), 1 of 1 lymphoma (H9), 1 of 1 glioma (U-118-MG), and 1 of 1 ovarian carcinoma-derived cell line (SK-OV-3). Five of 5 breast carcinomas and 10 of 11 (91%) colon carcinoma-derived cell lines were WT at the genomic level (Fig. 1). In the colon carcinoma cell line HCT 116, a missense mutation was identified at nucleotide 171 in codon 56, where a G-C bp deletion was found (Fig. 2).

In 13 cell lines with deleted or mutated p14^{ARF}, the p53 status was WT in 9 of 13 (69%) and mutated in 4 of 13 (31%). In 30 of 40 (75%) cell lines WT for p14^{ARF}, p53 abnormalities consisted of mutations in 26, deletions in 4, and p53 was WT in the remaining 10 (25%). Thus, an inverse association was established between p14^{ARF} and p53 in 74% of the cell lines studied ($P = 0.002$). The correlation was not perfect, however, and p14^{ARF} and p53 were found to be deleted or mutated in 4 cell lines and WT in 10 cell lines (Table 2).

The deletion of p14^{ARF} was associated with the deletion of p16 exon 1 α or the loss of p16 protein expression. Although p16 status

Table 1 Status of p14^{ARF}, p16^{INK4a}, p53, p73, Rb, and cyclin D1 in cell lines

Cell line	p14 ^{ARF} exon 1β	p53 ^a	p16 ^{INK4a} exon 1α ^b	p16 ^{INK4a} exon 2 ^b	p16 ^{INK4a} mRNA ^b	p16 ^{INK4a} protein ^b	Rb ^b	Cyclin D1 ^b	p73 ^c
NSCLC									
866MT	WT ^d	MUT	MUT	WT	+	-	+	+	WT
A2182	DEL	WT	DEL	N/A	-		+	N/A	WT
A-427	DEL	WT	DEL	N/A	-		+	N/A	12 bp DEL
A549	DEL	WT	DEL	N/A	-		+	N/A	WT
Calu-1	WT	DEL	WT	WT	+	-	+	+	WT
Calu-6	WT	MUT	WT	WT	+	-	+	+	WT
NCI-H1155	WT	MUT	WT	WT	+	+	-	-	790 G → T
NCI-H157	WT	WT	WT	MUT	+	-	+	+	WT
NCI-H292	DEL	WT	N/A	N/A	N/A	N/A	N/A	N/A	Silent
NCI-H358	WT	DEL	WT	WT	+	-	+	+	WT
NCI-H596	WT	MUT	WT	WT	N/A	+	-	+	WT
SCLC									
DMS92	WT	MUT	WT	N/A	+	+	+	N/A	2 and 4 bp DEL
NCI-H146	WT	WT	N/A	N/A	N/A	N/A	N/A	N/A	WT
NCI-H446	WT	WT	WT	N/A	+	+	-	N/A	WT
NCI-H526	WT	MUT	WT	N/A	+	+	-	N/A	WT
NCI-H82	WT	MUT	WT	N/A	+	N/A	+	N/A	WT
NCI-N417	WT	MUT	WT	WT	+	+	-	+	WT
Mesothelioma									
M9K	DEL	WT	DEL	DEL	-	-	+	N/A	WT
M24	DEL	WT	DEL	DEL	-	-	+	+	Silent
Colon carcinoma									
COLO 320DM	WT	MUT	N/A	N/A	N/A	N/A	N/A	N/A	Silent
DLD-1	WT	MUT	MUT	WT	-	-	+	+	WT
HCT 116	MUT	WT	MUT	N/A	-	-	+	+	WT
HT-29	WT	MUT	WT	N/A	+	-	+	+	WT
LS 174T	WT	WT	WT	WT	-	-	+	+	Silent
RKO	WT	WT ^e	N/A	N/A	N/A	N/A	+ ^e	N/A	WT
SW403	WT	MUT	WT	WT	-	-	+	+	WT
SW48	WT	MUT	N/A	N/A	N/A	N/A	N/A	N/A	WT
SW480	WT	MUT	N/A	N/A	N/A	N/A	N/A	N/A	WT
SW620	WT	MUT	WT	WT	+	-	+	+	WT
WiDr	WT	MUT	N/A	N/A	N/A	N/A	N/A	N/A	WT
Breast carcinoma									
HS 578T	WT	N/A	N/A	N/A	N/A	N/A	N/A	N/A	WT
MCF7	WT	WT	DEL ^f	DEL ^f			+ ^{f,g}	+ ^{h,i}	WT
MDA-MB-468	WT	MUT	N/A	N/A	N/A	N/A	N/A	N/A	Silent
T-47D	WT	MUT	N/A	N/A	N/A	N/A	N/A	+ ⁱ	WT
ZR-75-1	WT	MUT	N/A	N/A	N/A	N/A	N/A	+ ^{ij}	WT
Pancreatic carcinoma									
ASPC-1	DEL	MUT	WT	N/A	+	-	+	+	WT
Capan-2	WT	MUT	N/A	N/A	N/A	N/A	N/A	N/A	WT
MIA PaCa-2	DEL	MUT	N/A	N/A	N/A	N/A	N/A	N/A	Silent
HCC									
HA22T/VGH	WT	DEL	MUT	WT	+	-	+	+	WT
HEP 3B	WT	DEL	WT	WT	+	+	-	+	Silent
HUH4	WT	MUT	WT	WT	+	-	+	+	WT
HUH7	WT	MUT	N/A	N/A	N/A	N/A	N/A	N/A	Silent
SK-HEP-1	DEL	WT	N/A	N/A	N/A	+	+ ^k	N/A	Silent
Hepatoblastoma									
HB611	WT	WT	WT	WT	+	+	+	+	WT
Hep G2	WT	WT	WT	WT	+	+	+	+	WT
Liver									
THLE-5B	WT	WT	WT	WT	+	+	+	+	WT
Cervical carcinoma									
Ca Ski	WT	WT	N/A	N/A	N/A	N/A	N/A	N/A	WT
Oral squamous cell carcinoma									
SCC-4	WT	MUT	N/A	N/A	N/A	N/A	N/A	N/A	WT
Ovarian Ca									
SK-OV-3	DEL	WT	N/A	N/A	N/A	- ^l	+ ^l	N/A	WT
Lymphoma									
H9	DEL	MUT	N/A	N/A	N/A	N/A	N/A	N/A	WT
Leukemia									
CCRF-CEM	WT	MUT	N/A	N/A	N/A	N/A	N/A	N/A	WT
Glioblastoma									
U-118-MG	DEL	MUT	N/A	N/A	N/A	N/A	N/A	N/A	WT

^a Data from Greenblatt *et al.* (73).

^b Data from Okamoto *et al.* (12, 74).

^c Data from Yoshikawa *et al.* (46).

^d WT, wild-type; DEL, deleted; MUT, mutated; N/A, not available; +, present; -, absent; HCC, hepatocellular carcinoma.

^e Data from van Bree *et al.* (75).

^f Data from Craig *et al.* (76).

^g Data from Lee *et al.* (77).

^h Data from Russell *et al.* (61).

ⁱ Data from Russell *et al.* (78).

^j Data from Hosokawa and Arnold (79).

^k Data from Puisieux *et al.* (80).

^l Data from Todd *et al.* (81).

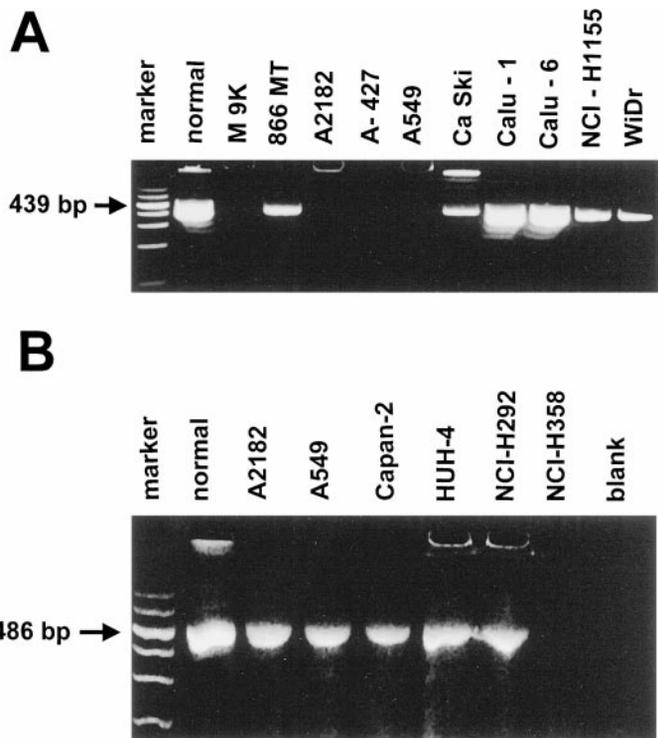


Fig. 1. PCR demonstration of *p14^{ARF}* in cell line DNA. A, the 439-bp product is deleted from M9K, A2182, A-427, and A549, but is amplified in 866 MT, Ca Ski, Calu-1, Calu-6, NCI-H1155, and WiDr. B, exon 7 of *p53*, a 486-bp fragment was amplified as an internal control. The NCI-H358 cell line contains a known deletion of *p53*. Normal human placental DNA served as a positive control in each reaction.

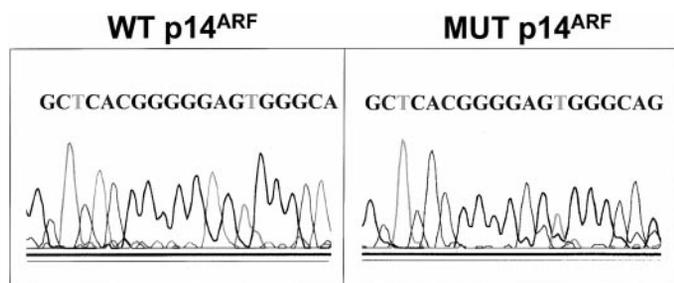


Fig. 2. Sequencing analysis of *p14^{ARF}*. Nucleotides 160–180 of exon 1 β are illustrated. HCT 116 on the right demonstrates a G-C bp deletion at base 171 compared with a WT sequence on the left.

Table 2 Comparison of *p14^{ARF}* deletion and *p53* status in cell lines

	<i>p53</i> WT ^a	<i>p53</i> MUT	Total
<i>p14^{ARF}</i> WT	10	30	40
<i>p14^{ARF}</i> DEL ^b	9	4	13
Total	19	34	53

^a WT, wild-type; DEL, deleted; MUT, mutated.

^b Correlates with *p53* WT status in cell lines ($P = 0.002$).

was not known for every cell line analyzed for *p14^{ARF}*, in 9 of 13 where *p14^{ARF}* was deleted or mutated, there also was a deletion or mutation of exon 1 α or a loss of p16 protein expression. One of 13 retained p16 expression, and information was not available for the remaining 3. No association was found with *Rb* or *cyclin D1* status.

Molecular Analysis of Primary NSCLC. Of the 106 patient total, 86 cases yielded suitable DNA for *p14^{ARF}* analysis, whereas 87 cases were available for *p73* analysis. In 68 cases, data were available for both, but data were only available for either *p14^{ARF}* or *p73* in 18 and 16 cases, respectively.

***p14^{ARF}* Status in Primary NSCLC.** *p14^{ARF}* was homozygously deleted from 16 of 86 (19%) NSCLCs and was WT in 70 of 86 (81%; Fig. 3). No intragenic mutations were found. A statistically significant inverse correlation between *p14^{ARF}* and *p53* status was not observed ($P = 0.6$). In 7 cases, *p14^{ARF}* was deleted, and *p53* was mutated. *p14^{ARF}* deletions were almost equally distributed among 7 *p53* mutated and 7 WT *p53* tumors. The *p53* status in the remaining 2 NSCLCs with *p14^{ARF}* deleted was not available because of technical difficulties (Table 3). *p14^{ARF}* deletions were associated with an over-expression of *p21^{waf1}* ($P = 0.04$) and negative staining for *bcl-2* ($P = 0.05$; data not shown). In an examination of prognostic significance, the loss of *p14^{ARF}* was not associated with a statistically significant shortened 5-year survival for the group as a whole ($P = 0.8$) nor within the group of WT *p53* tumors ($P = 0.3$).

***p73* Status in Primary NSCLC.** There were 48 of 86 (56%) informative cases that demonstrated 2 allelic bands present in nontumor DNA. Ten of 48 (21%) of these informative cases demonstrated LOH in tumor DNA (Fig. 4). No correlation between *p73* and *p53*

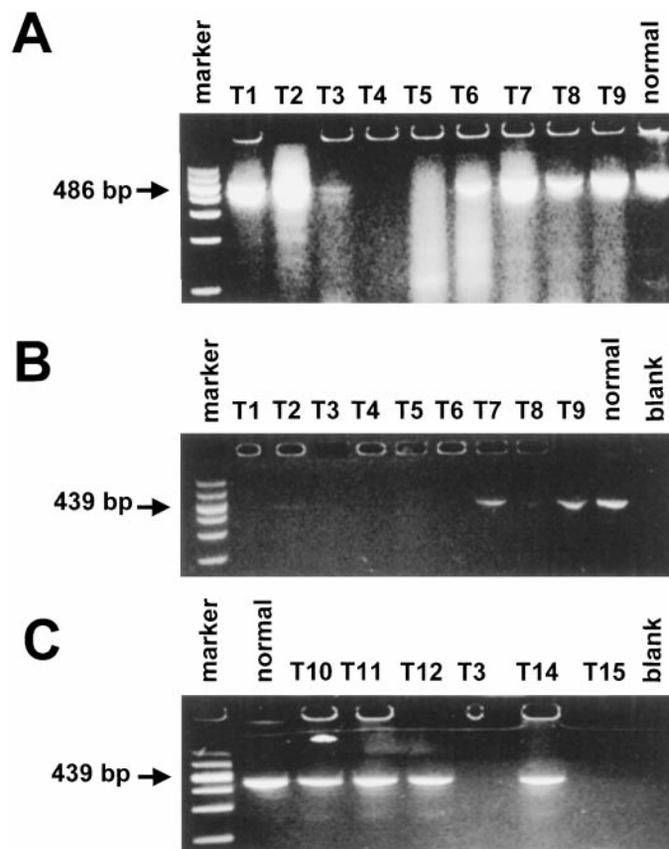


Fig. 3. PCR demonstration of *p14^{ARF}* in primary NSCLC. A, a similarly sized fragment, exon 7 of *p53*, was amplified as a control of DNA quality and integrity. B, detection of *p14^{ARF}* deletion. *p14^{ARF}* is deleted apparently from Lanes T1–T6; however, Lanes T4 and T5 are excluded from the final set because they fail to amplify exon 7 of *p53*. Lanes T1–T3 are interpreted as true homozygous deletions. The 439-bp product is detected in Lanes T7–T9, albeit faintly in Lane T8. C, *p14^{ARF}* is detected in Lanes T10–T12 and T14, but is deleted from Lanes T13 and T15. Normal human placental DNA served as a positive control in each reaction.

Table 3 Comparison of *p14^{ARF}* deletion and *p53* status in primary NSCLCs

	<i>p53</i> WT ^a	<i>p53</i> MUT	Total
<i>p14^{ARF}</i> WT	38	27	65
<i>p14^{ARF}</i> DEL ^b	7	7	14
Total	45	34	79

^a WT, wild-type; DEL, deleted; MUT, mutated.

^b Does not correlate with *p53* WT status in primary NSCLC ($P = 0.6$).

status was observed ($P = 0.08$). None of these 10 cases with LOH showed evidence of DNA methylation of the promoter region of *p73*. Sequencing of exons 1–13 revealed WT sequences with no mutations. Five LOH tumor samples (50%) showed a single guanine (G) insertion at nucleotide 778 in the 3' intron of exon 7. The resultant intron did not cause a frameshift mutation or affect the coding region of the gene. Five corresponding normal samples revealed the same insertion, indicating a polymorphism. A single heterozygote A:T to G:C transition was identified at nucleotide 1243 of exon 8. This change was not in the coding region and did not affect the amino acid sequence.

Neither the deletion of *p14^{ARF}* nor *p73* LOH correlated with patients' age, gender, family, smoking, occupational history, tumor histology, size, stage, or grade. We did not detect a statistically significant relationship between either a *p14^{ARF}* deletion or *p73* LOH and other markers studied previously in this series (*FHIT* LOH; immunohistochemical expression of *c-erbB-2*, cyclooxygenase-2, NOS2, or genetic polymorphisms of *CYP1A1*, *CYP2E1*, and *GSTM1*). However, we identified a statistically significant association between *p73* LOH and *p14^{ARF}* deletion ($P = 0.02$; Table 4). Furthermore, in a three-part analysis of the status of *p53*, *p73*, and *p14^{ARF}*, we noted a statistically significant correlation between *p53* mutation, *p14^{ARF}* deletion, and *p73* LOH ($P = 0.02$). Viewed from another perspective, we demonstrated that *p73* LOH occurred only in cases where either *p53* or *p14^{ARF}*, or both together, were mutated or deleted, but not in cases where both were normal ($P = 0.01$; Table 5).

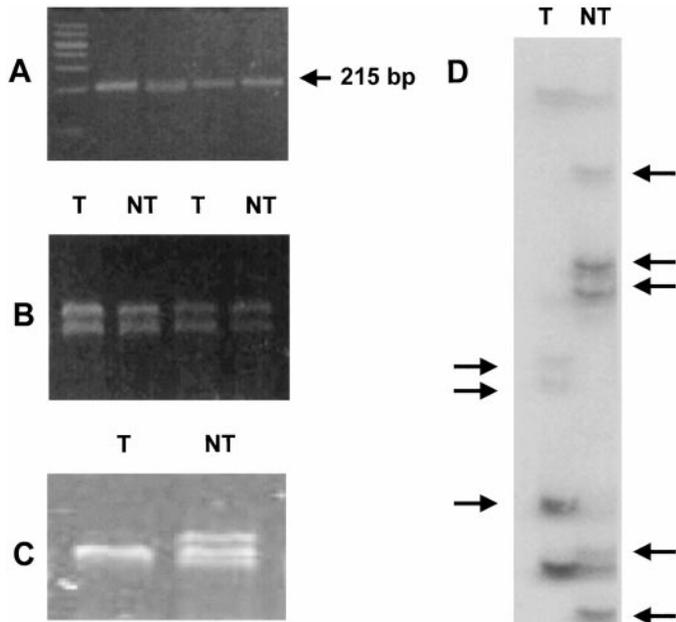


Fig. 4. Demonstration of *p73* status by PCR and SSCP. A, 215-bp PCR product of the chromosome 1p36 locus. B, *p73* informative cases. Two allelic bands, present in tumor (T) and nontumor (NT), without LOH. C, *p73* informative case with LOH. Note 2 allelic NT bands but only one T band. D, SSCP. Note the altered band patterns in the T versus NT sample.

Table 4 Comparison of *p14^{ARF}* and *p73* status in primary NSCLCs

	<i>p73</i>	<i>p73</i> LOH ^a	Total
<i>p14^{ARF}</i> WT	28	1	29
<i>p14^{ARF}</i> DEL	4	3	7
Total	32	4	36

^a Correlates with *p14^{ARF}* deletion in primary NSCLC ($P = 0.02$), Fisher's exact test. DEL, deleted.

Table 5 Comparison of *p73* status with abnormalities of *p14^{ARF}* and *p53* combined

	<i>p73</i>	<i>p73</i> LOH ^a
<i>p14^{ARF}/p53</i> both WT	14	0
<i>p14^{ARF}/p53</i> either or both abnormal	15	9

^a Correlates with abnormalities of either *p14^{ARF}* or *p53*, or both ($P = 0.01$), Fisher's exact test.

DISCUSSION

We have identified *p14^{ARF}* deletions in a significant number of human cell lines 12 of 53 (25%). Deletions were frequent in NSCLC cell lines (7 of 11), and deletions also were identified in a significant percentage of resected primary NSCLC tumors (19%). Although this figure is <37% cited in an earlier study of resected NSCLCs (45), these results add support to the premise that *p14^{ARF}* is a tumor suppressor in lung carcinogenesis. The loss of *p14^{ARF}* may be more significant in the pathogenesis of NSCLC than SCLC, given that *p14^{ARF}* deletions in lung cancer cell lines were confined to NSCLC, whereas all 6 SCLC cell lines tested were WT at the genomic level. The high frequency of *p53* mutation in SCLC (48) may lessen the selection pressure for *p14^{ARF}* deletion. Other cell lines with *p14^{ARF}* deletions in this study were mesothelioma, glioblastoma, and lymphoma derived, which are consistent with previous studies of these tumor types in which alterations of the *INK4a/ARF* locus have been recognized as frequent events (49–57). *p14^{ARF}* deletions were not identified in colon or breast carcinoma cell lines and in only 1 of 5 (20%) hepatocellular carcinoma lines studied.

A missense mutation identified in exon 1β of *p14^{ARF}* of the colon carcinoma cell line HCT 116 demonstrates that an intragenic mutation, not described previously, may occur in exon 1β of *p14^{ARF}*, although none were identified in the primary lung tumors studied. HCT 116 is known to harbor a separate mutation in exon 1α of *p16^{INK4a}* that results in a stop codon (12). Thus, in this cell line, both *p16^{INK4a}* and *p14^{ARF}* are altered by discreet point mutations. This suggests that although the deletion of *p14^{ARF}* was associated largely with the deletion of *p16^{INK4a}* within the group of cell lines studied, abrogation of *p16^{INK4a}* and *p14^{ARF}* function may not be governed exclusively by overlapping mutational events.

The subset of cell lines that harbored deletions or mutations of both *p53* and *p14^{ARF}* consisted of a glioblastoma, 2 pancreatic carcinomas, and 1 lymphoblastic lymphoma-derived cell line. These tumor types are noted for their aggressive behavior, suggesting that the loss of both *p53* and *p14^{ARF}* may have additive and cooperative effects in tumorigenesis. It has been shown recently that *p14^{ARF}* has molecular targets other than *p53* (58), and therefore, the inactivation of *p53* may contribute to tumor progression. A report of mice lacking *ARF*, *MDM2*, and *p53* showed a wider range of tumor types than animals lacking either gene alone. This also was noted in a study of non-Hodgkin's lymphoma where concomitant alteration of the *p53* and the *INK4a/ARF* locus was associated with a shorter survival (56).

Nine cell lines and 40 primary tumors were WT for both *p53* and *p14^{ARF}* at the genomic level. Nevertheless, the majority of cell lines in this subset are targeted by mechanisms that act epistatically to disrupt the function of the *Rb* or *p53* pathway. These include the inactivation of *p53* by the HPV E6/E7 oncoproteins (Ca Ski; Ref. 59) or the T- antigen of SV40 (THLE 5B; Ref. 60), the loss of p16 protein expression (LS174T; Ref. 12), the deletion of *p16^{INK4a}* and accumulation of cyclin D1 (MCF7; Ref. 61), the loss of *Rb* (NCI-H146 and NCI-H446), and the methylation of the *ARF* promoter (RKO; Ref. 62). The disruption at this functional level highlights the fundamental significance of the *Rb* and *p53* pathways to carcinogenesis.

Because of the downstream location of *p53*, the loss of ARF was predicted to be equivalent to *p53* inactivation, with the corollary that

in any given tumor system the loss of both is functionally redundant and rare (23, 63). However, emerging evidence suggests a more complex interrelationship and points to *p53*-independent functions of *p14^{ARF}* involving targets other than MDM2 (58). We found that the correlation of *p14^{ARF}* with *p53* status differed when cell lines, as opposed to primary lung tumors, were examined. An inverse correlation between *p53* and *p14^{ARF}* status was observed among cell lines, which conforms to the earlier model of linear interrelationships between the 2 tumor suppressors. This relationship was not maintained among the primary tumors studied, which echoes the contradictory results reported by others in studies of lung cancer and other tumor types. The evidence that supports the premise of an inverse correlation between *p14^{ARF}* and *p53* is derived from a small number of studies. A statistically significant negative correlation between *p14^{ARF}* deletions and *p53* mutations was established in an analysis of 29 gliomas using a differential PCR method (50). Acknowledging that this differed from some lung cancer studies, the authors hypothesized that selection pressures for *p53* pathway mutagenesis may vary in different tissues. In a study of 97 non-Hodgkin's lymphomas, ARF deletion was found in 8 cases (9%), mainly in tumors with WT *p53*, as measured by a multiplex PCR method (56). In a study of 49 NSCLCs (29), the loss of expression of ARF protein, as measured immunohistochemically, correlated inversely with *p53* overexpression. Normal lung tissue that exhibited nuclear and cytoplasmic staining served as a positive control. Furthermore, the exon 1 β transcript was identified by reverse transcription-PCR in all of a subset of tumors examined. Normal lung also was used as a positive control in a further study of ARF expression in a large group of NSCLCs and high-grade neuroendocrine carcinomas (64). No relationship was established between *p14^{ARF}* and *p53* status. Reports of immunohistochemical detection of the ARF protein must be interpreted with caution, however, given that the ARF protein is localized primarily in the nucleolus, and extremely low normal levels in adult tissue are detectable only by reverse transcription-PCR (65). Hence, it is not entirely clear whether the staining pattern observed with rabbit polyclonal antibodies should be interpreted as specific for *p14^{ARF}*, and whether absence of staining should be regarded as representative of the loss of expression.

In a recent study of 38 NSCLCs, the status of *p14^{ARF}* was extrapolated from measurements of the LOH by microsatellite analysis. Homozygous deletions were reported in 37% of the cases, and *p14^{ARF}* inactivation was not inversely correlated with *p53* mutation (45). In a study of colon carcinoma, methylation and inactivation of the ARF promoter was identified in 31 of 110 cases by a methylation-specific PCR. Methylation was overrepresented slightly, but was not restricted to tumors with WT *p53* when compared with tumors harboring *p53* mutations (62). We did not address the question of methylation of the ARF promoter in the current study, but it has been demonstrated in 1 of 20 (5%) primary lung cancers.⁴ We did not establish a negative correlation between *p53* and *p14^{ARF}* in NSCLC at the genomic level, and recognizing the complexity of the cell cycle control network *in vivo*, we do not consider *p14^{ARF}* inactivation equivalent to the disruption of the *p53* pathway by *p53* mutation. This is supported by a recent study of NSCLC that demonstrated multiple impairments of 3 to 4 molecular pathways in 43% of tumors (28).

We found LOH of *p73* in 21% of informative cases, but did not identify intragenic mutations in the expressed allele, which is consistent with the findings of other investigators (66–70). However, we did observe a statistically significant positive correlation between *p73* LOH and the deletion of *p14^{ARF}*, which suggests that the reduction of *p73* to hemizyosity has functional significance in the dysregulation

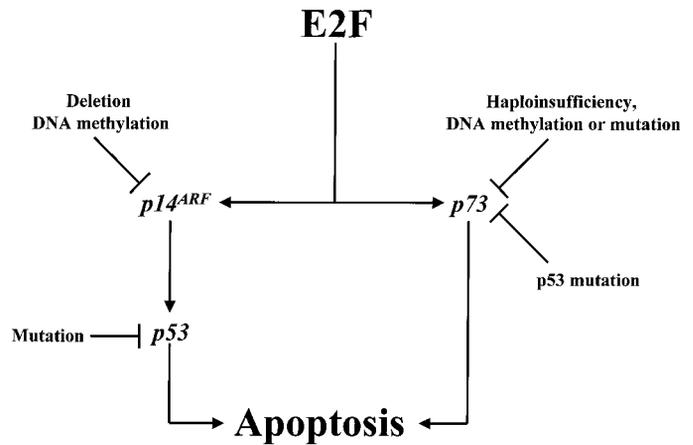


Fig. 5. A model of dysregulation of E2F-1-induced apoptosis. If the apoptotic signals, mediated by *p14^{ARF}* and *p53*, are abrogated by deletion, mutation, or DNA methylation, then the *p73* safeguard assumes more significance in tumor suppression. However, if *p73* function is attenuated by haploinsufficiency, DNA methylation, mutation, or binding to certain mutants of *p53*, then both apoptotic pathways are inhibited and proliferation is favored.

of E2F-1-induced apoptosis. Because *E2F-1* directly activates the transcription of *p73*, which leads to the activation of *p53*-responsive target genes that result in apoptosis, and the inhibition of *p73* function partially rescues cells from *E2F-1*-induced apoptosis, thus *p73* might constitute a *p53*-independent, safeguard apoptotic mechanism (37). It has been shown recently that tumor-derived *p53* mutants can bind to and inactivate *p73* (71). Others also have suggested that haploinsufficiency of *p73* may lead to dysfunction (72). Our demonstration of a correlation between *p73* LOH and *p14^{ARF}* deletion in a subset of primary NSCLC is supportive of this hypothesis. Moreover, we propose a model whereby, in the setting of *p14^{ARF}* loss and/or *p53* mutation resulting in a diminution of *p53* levels, the “failsafe” *p53*-independent pathway of apoptosis, mediated by *p73*, may assume greater significance (Fig. 5). Should the *p73* response be attenuated by mutant *p53*, haploinsufficiency, or less commonly, by DNA methylation or mutation (46), then the cell proliferative effects of *E2F-1* are favored, tipping the balance toward tumor development. In the current data set, we also have shown that LOH of *p73* occurred only where either *p14^{ARF}* or *p53*, or both, were abnormal, which suggests that this model of *E2F-1* dysregulation is indeed a plausible paradigm of tumor development.

In summary, we have demonstrated that the deletion of exon 1 β of *p14^{ARF}* occurs in a significant percentage of human cell lines and primary NSCLCs. We also identified LOH of *p73* in a similar percentage of lung tumors and a statistically significant correlation with *p14^{ARF}* deletion and/or *p53* mutation that is consistent with the hypothesis about selection pressures for the dysregulation of *E2F-1*-induced apoptosis in lung carcinogenesis. Furthermore, a statistically significant trend toward inverse correlation between *p14^{ARF}* and *p53* status was seen within the group of cell lines studied, but not within primary tumors. This is consistent with the current model that shows the relationship of *p14^{ARF}* to *p53* as part of a complex network of interactions, rather than a simple linear pathway.

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⁴ J. G. Herman, unpublished data.

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