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Mutational Analysis of the *p63/p73L/p51/p40/CUSP/KET* Gene in Human Cancer Cell Lines Using Intronic Primers

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Abstract

After the identification of *p73*, a second homologue of the human *p53* tumor suppressor gene has been reported and named *p63/p73L/p51/p40/CUSP/KET*. We have investigated the hypotheses that: (*a*) *p63* is mutated in diverse types of human cancers; and (*b*) *p63* functions in the same pathway as *p53* and *p73* in the process of carcinogenesis; therefore, mutations in these three genes would be mutually exclusive. We have analyzed the genomic structure of the *p63* gene and have performed mutational analyses on 54 human cell lines using intronic primers flanking each exon. We have confirmed that the human *p63* open reading frame encodes the same length of protein as murine *p63* that was initially reported to be 39 amino acids longer than human *p63*. By mutational analysis, we have shown that DLD1 and SKOV3 cells have either heterozygous mutations or polymorphisms in the putative DNA binding domain of *p63*. In these cell lines, *p63* is biallelically expressed. We conclude that mutations in the *p63* gene are rare in human cell lines. The fact that DLD1 is abnormal for both *p63* and *p53* genes suggests that they may not be involved in the same tumor suppressor pathway.

Introduction

The existence of a *p53* homologue had long been considered unlikely. However, emerging evidence indicates that there is a *p53* gene family (1). After the identification of the *p73* gene (2), the second homologue of *p53* was reported from several investigators under the names *p73L* (3), *p51* (4), *p40* (5), *CUSP*,[2] *KET* (6), and *p63* (7). Although the amino acid sequences and the molecular weights were reported to be different, they have proven to be isotypes derived from a single gene with two promoters, two 3′-end exons, and at least three alternative splicing patterns (summarized in Table 1). In this report, the name *p63* will be used hereafter. *p53*, *p63*, and *p73* have much sequence homology throughout their length and share domain structures that include a transactivation domain, a DNA binding domain, and an oligomerization domain from the NH_2 terminus to the COOH terminus. Because *p63* and *p73* share more homology than *p53*, they are considered to be evolutionarily more closely related. A prominent feature of *p63* is its lack of the transactivation domain (ΔNp63s: shown in Table 1) in some isotypes transcribed from the second promoter (7). *p63* isotypes having the transcriptional transactivation domain transactivate *p53* target sequences such as the $p21^{WAF-1}$ promoter (4) and the minimal *p53* binding sequence, PG-13 (7). Overexpression of *p63* induces apoptosis, although this ability differs among isotypes (4, 7). The expression of *p63* is tissue specific, and many tissues have dominantly expressed isotypes (3–5, 7). By immunohistochemical analysis, high *p63* expression was observed in the basal cells of various epithelial tissues (7). Mice lacking *p63* show developmental defects in organs of ectodermal origin (8, 9); mice lacking *p53* have exencephaly and other developmental defects in about 25% of the embryos (10–12). This suggests that *p63* and *p53* activate different target genes. Because *p63* has a high homology with *p53* and *p73*, we tested two hypotheses: (*a*) that *p63* is mutated in diverse types of human cancers; and (*b*) that p63 is functioning in the same pathway as *p53* and *p73* in the process of carcinogenesis so that mutations would be exclusive among the three genes. Detailed mutational analysis is necessary to investigate these hypotheses. In addition to the six isotypes shown in Table 1, *p63* has even more minor isotypes produced by additional alternative splicing patterns (4, 8, 13). This makes mutation detection by RT-PCR-SSCP[3] analysis difficult because the differently spliced mRNAs exhibit additional bands on the gel (4). In this report, we studied the *p63* genomic sequence, designed PCR primers in the introns flanking each exon, and then performed the mutation screening using genomic DNA. This enables accurate *p63* mutational analysis without being affected by the presence of multiple isotypic mRNAs produced by alternative splicing.

Materials and Methods

Isolation of Human *p63* Genomic Clones. To obtain human genomic clones that contain the entire human *p63* gene, human genomic libraries made in YAC (CEPH-Généthon, distributed by Research Genetics), PAC (Genome Systems), and BAC (Research Genetics) were screened by PCR using primer pairs CF1 (5′-GTCCCAGAGCACACAGACAAATG-3′) and CB1 (5′- TTTGTCGCACCATCTTCTGATG-3′) or CF51 (5′-AGATTGCCCCTCCTAGTCATTTG-3′) and CB68 (5′-CGGTTCATCCCTCCAACACAAC-3′), arbitrarily designed from the *p63* coding sequence. The CF1-CB1 pair and the CF51-CB68 pair were later found to amplify exon 1-intron 1-exon 2 and exon 6-intron 6-exon 7 fragments, respectively.

Determination of the Intronic Sequences Flanking Each Exon of the Human *p63* Gene. The intronic sequences that flank each exon of the human *p63* gene were determined by the "Long Distance Sequencer" method (14–16) from YAC clone yhCEPH913D2. In brief, YAC DNA was digested by *Hinc*II, *Rsa*I, *Pvu*II, NlaIV, *Hae*III, or *Cac*8I (New England Biolabs) and ligated with a vectorette unit. PCR was then performed using a gene-specific primer and the 224 M13 primer (14). A total of 69 gene-specific primers were designed from the reported cDNA sequences (3–6, 7) to determine all of the exon/intron boundaries. Two to four amplified fragments were selected and directly sequenced on the fluorescent DNA sequencer 370A (Perkin-Elmer) using the −21 M13 primer (Amersham) and the ThermoSequenase dye terminator sequencing kit (Amersham). Our genomic sequences were compared with the available cDNA sequences (3–6, 7), and the exon/intron boundaries were assigned by the GT/AG rule (17). The nucleotide sequences of the 5′ untranslated regions of mRNAs with transactivation domains (TAp63s in Table 1) or without transactivation domains (ΔNp63s in Table 1) had been reported in GenBank (accession numbers AB016072 and AF091627, respectively). We determined the genomic sequences corresponding to these untranslated regions; then the first translation start codons (ATG) of the open reading frames

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[2] Genbank accession number AF091627.

[3] The abbreviations used are: RT-PCR, reverse transcriptase-PCR; SSCP, single strand conformation polymorphism; YAC, yeast artificial chromosome; PAC, P1 artificial chromosome; BAC, bacterial artificial chromosome.

Table 1 *p63 isotypes*

Six major splicing forms of the *p63* gene, their synonyms, and the exons used for each isotype are summarized from the literature. TA isotypes start translation from exon 1, whereas the ΔN isotypes start translation from exon 3′. Reference numbers are shown in parentheses.

p63 isotypes (8)	Synonyms	Exons used (8)
TAp63α	*p51B* (4), *KET* (7)	1–14
TAp63β		1–12, 14
TAp63γ	*p51A* (4)	1–10, 15
ΔNp63α	*CUSP* (6), *p73L* (3)	3′, 4–14
ΔNp63β		3′, 4–12, 14
ΔNp63γ	*p40* (5)	3′, 4–10, 15

were assigned. The presence of the upstream in-frame stop codons, which defines the open reading frames of both TAp63s and ΔNp63s, was also searched.

Preparation of DNA. Fifty-four human cell lines were grown in the recommended medium. Cell lines used were as follows: 11 colon cancers (HCT116, DLD1, SW620, HT-29, SW480, COLO320DM, SW48, WiDr, LS174T, RKO, and SW403), 11 non-small cell lung cancers (866 MT, A2182, NCI-H292, Calu6, A427, Calu1, NCI-H358, NCI-H1155, NCI-H157, NCI-H596, and A549), 6 small cell lung cancers (NCI-N417, DMS92, NCI-H446, NCI-H146, NCI-H82, and NCI-H526), 5 breast cancers (MDA-MB-468, T-47D, MCF7, Hs578T, and ZR-75–1), 5 hepatocellular carcinomas (HA22T/VGH, HUH4, HEP3B, HUH7, and SK-HEP-1), 1 hepatoblastoma (HepG2), 1 hepatoblastoma transfected by hepatitis B virus (HB611), 1 SV40 immortalized liver cell line (THLE-5B), 3 pancreatic cancers (MIAPaCa-2, Capan-2, and AsPC-1), 2 oral cancers (FaDu and SSC-4), 2 mesotheliomas (M9K and M24), 1 T-cell lymphoblastic leukemia (CCRF CEM), 1 T-cell lymphoma (H9), 1 ovarian cancer (SKOV3), 1 cervical cancer (CaSki), 1 esophageal cancer (HCE7), and 1 glioblastoma (U118 MG). DNA was extracted using the Nucleon I DNA Extraction kit (Scotlab) and dissolved in 10 mM Tris-HCl (pH 8.0)/1 mM EDTA to a final concentration of 50 ng/μl.

PCR-SSCP Analysis. Sixteen primer sets were designed from the intronic sequences to amplify all coding exons and the splicing junctions of the *p63* gene. The primers and the lengths of the PCR fragments are shown in Table 2. PCR was performed in a 25-μl reaction containing 1× XL buffer II (Perkin-Elmer), 1.1 mM $Mg(OAc)_2$, 200 μM deoxynucleotide triphosphates, 300 nM of each primer, and 2 units of rTth DNA polymerase, XL (Perkin-Elmer) using 40 cycles of 94°C for 40 s, 55°C for 30 s, and 68°C for 2 min. After confirming the amplification by agarose gel electrophoresis, the PCR fragments were labeled by five additional cycles of 94°C for 40 s, 55°C for 30 s, and 68°C for 2 min in a 5-μl labeling solution containing 1× XL buffer II, 1.1 mM $Mg(OAc)_2$, 50 μM deoxynucleotide triphosphates, 300 nM of each primer, 0.2 μCi of [α-^{33}P]dATP (DuPont), and 0.1 unit of rTth DNA polymerase, XL. For fragments longer than 300 bp, the labeled fragment was then digested by an appropriate restriction enzyme (see Table 2) to divide it into two shorter fragments of <300 bp. Labeled fragments were denatured by heating at 70°C after adding an equal amount of denaturing solution containing 98% formamide, 0.025% xylene cyanol, and 0.025% bromphenol blue and were then run on a 0.5× MDE gel (FMC Bioproducts) at 20°C. The gel was dried and exposed to Kodak XAR film overnight.

Direct Sequencing of the PCR Fragments. The PCR fragments that gave abnormal bandshifts on the MDE gel were subjected to direct sequencing. The DNA from each cell line was amplified by the intronic primers that were tagged by either −21 M13 or −28 M13 reverse sequences (Amersham), purified by the Wizard PCR Prep kit (Promega), and then sequenced using either −21 M13 or −28 M13 reverse DYEnamic ET primers and ThermoSequenase (Amersham) with the fluorescent DNA sequencer 370A (Perkin-Elmer).

RT-PCR using total RNA from DLD1 and SKOV3 cells was done as follows. Total RNA was isolated using the RNA Extraction kit (Stratagene). One μg of total RNA was reverse transcribed using SuperScript II reverse transcriptase (Life Technologies, Inc.) and then amplified by rTth DNA polymerase, XL using −21 M13 or −28 M13 reverse sequence-tagged primer pairs Ex5M13 (TGTAAAACGACGGCCAGTCCATGAGCTGAGCCGTGAATTC) and Ex6rev (AGGAAACAGCTATGACCATCAGCACACTCTGTCTTCCTGTGAT) to check exon 6 and Ex12M13 (TGTAAAACGACGGCCAGTTATCCCACAGATTGCAGCATTGT) and Ex13rev (AGGAAACAGCTATGACCATATCCATGGAGTAATGCTCAATCTG) to check exon 13.

Table 2 *Intronic primers for p63*

List of the intronic primers used to amplify each exon. The sizes of each exon and of each amplified fragment are also shown. The sizes of exons 1 and 3′ are those from the start codons (ATG nucleotide sequence) and the exon/intron boundaries. The sizes of exons 14 and 15 are those from the intron/exon boundaries to the stop codons. For amplified fragments longer than 300 bp, each fragment was digested with the appropriate restriction enzyme to give two fragments smaller than 300 bp before the SSCP analysis. The names of the restriction enzymes used and the sizes of the digested fragments are given in parentheses.

Exon		Primer	Exon size (bp)	PCR fragment size (bp)
Exon 1	F	GACCCTATTGCTTTTAGCCTC	62	235
	B	CATTCATAATACACAAGGCACTTC		
Exon 2	F	CCTGCATGGTTTTATAGATTCACTTG	129	305 (EcoRI 197 + 108)
	B	GACCACCCACATTTACCTATTTAG		
Exon 3	F	CCCTTTCCATGCCTAACTCACT	133	209
	B	CCAAAGACTGAAGAGAAAGCCTG		
Exon 3′	F	GGCAAAATCCTGGAGCCAGAAG	42	140
	B	AAAGCATCTCTAAATGGAGTGC		
Exon 4	F	GGCTTCAGCGGCTAATATTGGG	255	353 (AflIII 252 + 101)
	B	GTGAAGCCCATCCTTGGACTTG		
Exon 5	F	TCTCCTTCCTTTCTCCACTGGC	187	284
	B	TGCCCACAGAATCTTGACCTTC		
Exon 6	F	GCCACCAACATCCTGTTCATGC	116	259
	B	GTCTACTCAGTCCATAGAGGTGTTG		
Exon 7	F	GAAGGAACAACGTCAGTTTAAACCC	110	245
	B	AAAGCAGCCACGATTTCACTTTGCC		
Exon 8	F	GTGGTAGATCTTCAGGGGACTTTC	137	263
	B	CCAACATCTGGAGAAGATTCAACC		
Exon 9	F	GTGTTCCCAGGATGAAACTTGC	83	153
	B	GAAGCAACCATGAACACCCAAGG		
Exon 10	F	CCACACTTCTAACAGTTCTACAGC	137	278
	B	TCATCAATCACCCTATTGCTGATCC		
Exon 11	F	CGTGCTCACCATCATTTCCATG	158	239
	B	CTAGCCTGTTCATCCTTCAGCC		
Exon 12	F	CACTGGGATGCTGGTACATGATG	145	228
	B	GGGCACATGCTGTGGACTC		
Exon 13	F	CTGTTTCCTCCTTCCTCTTCC	94	212
	B	CAGGGATTGAACTACAAGGC		
Exon 14	F	CCCTGTTTTCATTCTCCATGACAC	297	366 (EcoRI 245 + 121)
	B	GGGATAGGAAGAGCTCACATGG		
Exon 15	F	CATGAAGGTATAAGGAGTGTGTTTCTG	115	240
	B	ACACACACTTAAAATATAGAGATAGGGC		

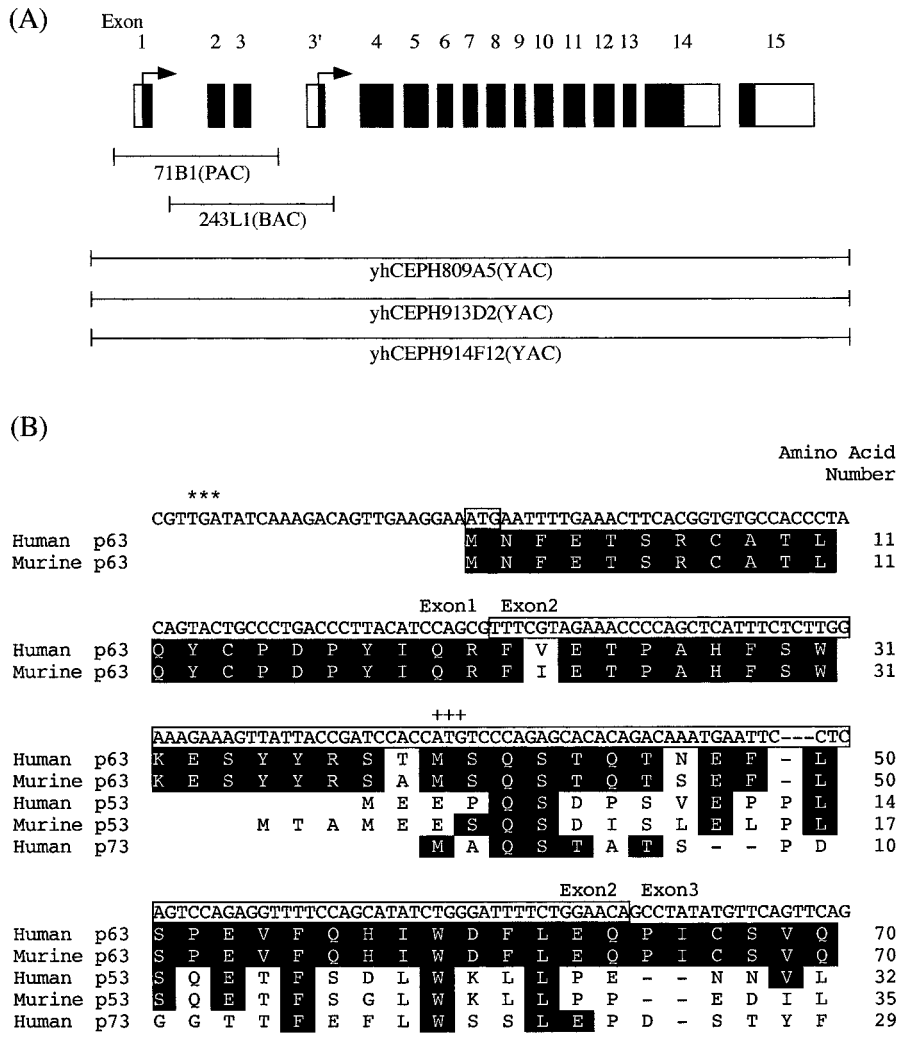

Fig. 1. Analysis of the human *p63* gene. *A*, location of the human genomic clones (*YAC, PAC,* and *BAC*). YAC yhCEPH913D2 containing the entire *p63* gene was used in this study. The extent of the insert of each clone was determined by mapping individual exons on the clone. The distances between exons have not yet been determined. *B*, comparison of the NH_2 termini of the human and murine p63, human and murine p53, and human p73 proteins, with the human nucleotide sequence shown above. p63 has a longer open reading frame than either p53 or p73 in the NH_2 terminus. The translation start codon is located in exon 1. The nucleotide sequence of exon 2 is *boxed*. *Dashes* (-) were inserted to maximize the alignment. *Left*, amino acid numbers for each protein. Amino acids common to human and murine are shown in *reverse text*, as well as any in p53 or p73 common to conserved amino acids in p63. ***, in-frame upstream stop codon. +++, "ATG" codon initially reported as the translation start codon.

Each primer was designed on different exons to span the intervening intron and thus avoid amplification from contaminating genomic DNA. The nucleotide sequence was determined as described above. The correct exon/exon boundary sequences were confirmed to guarantee the mRNA-derived sequences.

Results and Discussion

Screening of the human YAC, PAC, and BAC genomic libraries using the *p63*-specific primer pairs gave five clones shown in Fig. 1*A*. To find a clone that contained the entire *p63* gene, we first screened 270,000 PAC and BAC clones using primer pairs CF1-CB1 and CF51-CB68. Each PAC or BAC clone had an average insert size of 100 kb; thus, 270,000 clones were enough to cover the entire human genome nine times. Even so, we were not able to find a single clone that was positive for both primer pairs, suggesting that *p63* is so large that it does not fit into a single PAC or BAC clone. We then screened a human YAC library. Most of these clones have from 500-kb to 2-Mb inserts. We found clones yhCEPH809A5, yhCEPH913D2, and yhCEPH914F12 that contain the entire *p63* gene. These three clones are reported to have the STS marker *D3S1288* that had been mapped to chromosome 3q27.[4] This is consistent with the reported location of the *p63* gene at 3q27–29 (3, 4, 6, 7). The intronic sequences flanking each exon were determined from yhCEPH913D2 by the Long Distance Sequencer method (14). The sequences were deposited in GenBank under accession numbers AF124528–AF124540. PAC clone 71B1 and BAC clone 243L1, obtained using the primer pair CF1-CB1, were also mapped on the *p63* genomic structure (Fig. 1*A*). Each clone was found to contain only three exons. This suggests that the 5′ exons of the *p63* gene are widely dispersed in the genome, as is the case for the *p53* and *p73* genes (18).

For isotypes with the transactivation domain (TAp63s), human *p63* was originally reported to be 39 amino acids shorter than the murine *p63* in the NH_2 terminus (4, 7); however, the recently reported human *KET* sequence has the same length of the NH_2 terminus with murine *p63* (6). By investigating the genomic sequence, we found that the human *p63* gene does have the 39-amino acid stretch, which is highly homologous with its murine counterpart. The in-frame stop codon 24 nucleotides upstream from the translation start codon defines this open reading frame. As shown in Fig. 1*B*, the highly conserved 39-amino acid domain is specific to *p63* and not present in either *p53* or *p73*. This suggests that the domain may play an important role in distinguishing specific *p63* functions. For the isotypes without transactivation domain (ΔNp63s), we also found an in-frame stop codon upstream of the reported start codon (3, 6, 7), which defines the reported open reading frames.

We designed PCR primers in the introns to amplify all of the coding exons individually from the genomic DNA. This enables the mutational analysis to be independent of either the level of expression of each allele or of the presence of the alternative splicing patterns. We

[4] Internet address: http://kiwi.imgen.bcm.tmc.edu:8088/bio/access_yac.html.

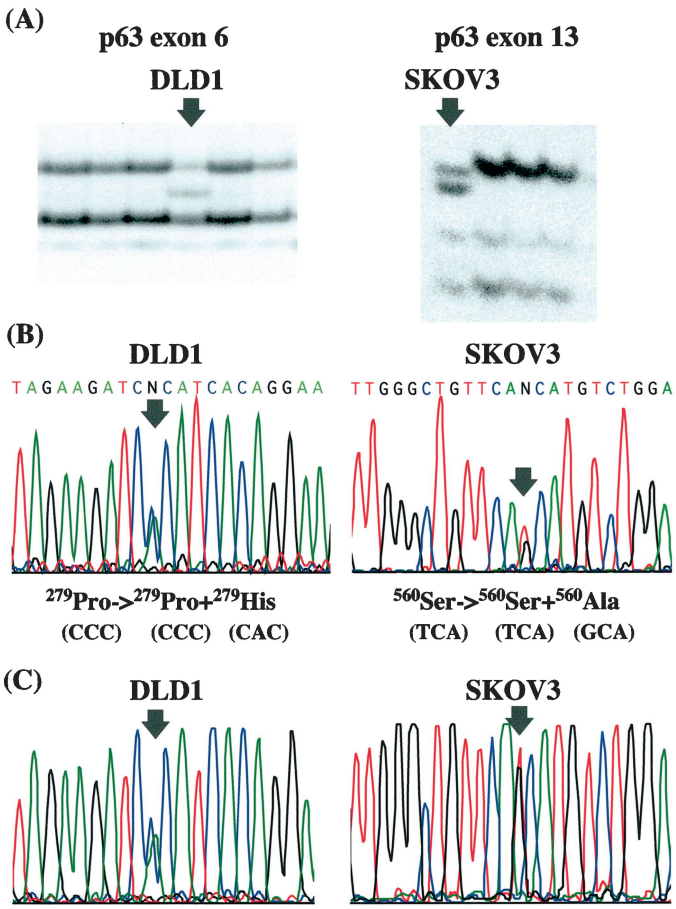

Fig. 2. SSCP-sequencing analysis of the *p63* gene in DLD1 and SKOV3 cells. *A*, SSCP analysis in exon 6 and exon 13 of the *p63* gene. DLD1(*left column, arrow*) and SKOV3 (*right column, arrow*) each show an abnormal band in addition to the common bands, suggesting that both cell lines have an abnormal allele in addition to a normal allele. *B*, direct genomic sequencing of *p63* exon 6 in DLD1 and *p63* exon 13 in SKOV3 cells using the PCR product amplified by the intronic primers for each exon. In both cell lines, both normal and abnormal sequences were seen to overlap. The abnormal allele encodes ^{279}His instead of ^{279}Pro in DLD1 and ^{560}Ala instead of ^{560}Ser in SKOV3. *C*, direct sequencing of the RT-PCR products of *p63* exon 6 in DLD1 and *p63* exon 13 in SKOV3 cells. PCR primer sets spanning the intervening introns allow only cDNA-derived sequences to be amplified. In both cell lines, both normal and abnormal alleles are biallelically expressed.

searched for *p63* mutations in 54 human cell lines that we had previously analyzed for mutations in the *p73* and *p53* genes (19). Of these 54 cell lines, only DLD1 and SKOV3 showed single nucleotide changes in one allele each. DLD1 was changed in exon 6, and SKOV3 was changed in exon 13. As shown in Fig. 2, each cell line showed an abnormal band on the SSCP gel in addition to the normal band, indicating that each cell line is heterozygous for the altered exon. Direct sequencing analysis showed that the nucleotide change in exon 6 of DLD1 causes an amino acid change from ^{279}Pro to ^{279}His. The change in exon 13 of SKOV3 causes an amino acid change from ^{560}Ser to ^{560}Ala. Both changes are located in the putative DNA binding domain of the p63 protein. *p73* has been reported to be monoallelically expressed in neuroblastoma cells (2). To determine the pattern of allelic expression in *p63*, RT-PCR direct sequencing analysis was performed using primers spanning an intron to avoid amplification of contaminating genomic DNA. As shown in Fig. 2*C*, biallelic expression was found in both DLD1 and SKOV3.

The nucleotide change in each cell line may be either a rare polymorphism or a somatic mutation. Regardless, these genetic changes are likely to have functional consequences because the amino acid substitution produced by either change would result in a significant change in the three-dimensional structure of the protein. In DLD1, the resulting amino acid substitution of a histidine for a proline, an amino acid that does not have the ability to form hydrogen bonds, would now allow the formation of an α-helix or a β-sheet, which was precluded previously. In SKOV3, the loss of a serine would mean the loss of polarity and the loss of a possible phosphorylation site. Amino acid substitutions that result in tertiary structural changes can cause significant functional alterations, even when they are defined as polymorphisms. Taken together, functional analysis of these genetically altered *p63* genes and the search for somatic mutations or genetic polymorphisms in primary human cancers are warranted. In *p53*, several "dominant-negative" mutants with altered DNA binding capacity have been shown to compromise the function of the normal coexisting protein (20). The nucleotide changes observed in DLD1 and SKOV3 are both located in the putative DNA binding domain of *p63* and are both expressed, giving rise to the possibility that they could be dominant-negative mutants that suppress the function of the p63 protein produced from the normal allele.

In the literature, one *p63* somatic mutation (^{187}Ala to ^{187}Pro) was found in 66 primary human tumors, and two mutations (^{184}Ser to ^{184}Leu in Ho-1-U-1 cells and ^{204}Gln to ^{204}Leu in SKG-III cells) were observed in 67 human cell lines evaluated by RT-PCR-SSCP analysis. The authors did not define the mutations as homozygous or heterozygous (4). Another group (13) found no mutations in 45 primary lung cancers but found a heterozygous frameshift mutation in one cell line (EBC1) of the 44 lung cancer cell lines examined. In EBC1, the abnormal allele has an insertion of an adenine after the ^{233}Lys codon. All of these mutations, including ours, are located in the putative DNA binding domain of the p63 protein. We conclude that mutations in *p63* are not frequent in the human tumors or in the human cell lines examined. The high percentage of homology among the oligomerization domains of *p53*, *p63*, and *p73* suggests that they may form heterotetramers. Indeed, it has been reported that *p53* and *p73* interact in the yeast two-hybrid system (8). Hypothetically, for the precise regulation of cell proliferation, *p53*, *p63*, and *p73* may all need to be functionally and physically intact. If this were the case, the mutations would be expected to show a mutually exclusive pattern in cancer cells. In the 54 cell lines that we investigated, 36 of these have mutations in *p53*, whereas 18 are wild-type. Two (A427 and NCI-H1155) have mutations in *p73*, whereas 52 are wild-type (19). A427 has a mutation in *p73*, but has wild-type *p53* and *p63* genes. NCI-H1155 and DMS92 have mutations in both *p73* and *p53* genes but are wild-type for *p63*. DLD1 has a mutation in *p53* and *p63* (heterozygous) but is wild-type for *p73*. SKOV3 has a mutation in *p63* (heterozygous) but is wild-type for *p73* and *p53*. Other cell lines have wild-type *p73* and *p63*, with or without *p53* mutations. Therefore, mutations in *p53* and *p63*, as well as in *p53* and *p73*, are not mutually exclusive. The relationship between *p63* and *p73* mutations is difficult to evaluate because of the low mutation rates in both genes.

We detected one single nucleotide polymorphism in intron 10, as shown in Table 3. This polymorphism can be detected by SSCP using primer pair exon 10-F and exon 10-B (Table 2) and is thus useful for detecting a loss of heterozygosity in the *p63* locus.

Table 3 *A polymorphism in intron 10*

The single nucleotide polymorphism seen in intron 10 of the *p63* gene is shown. The 41st nucleotide in intron 10 just after exon 10 shows a polymorphism, which can be detected by primer pair exon 10-F and 10-B (see Table 2). "*A*" allele has the sequence ATGAGTGACA, whereas the "*G*" allele has ATGAGTGACG.

Allele	A + A	A + G	G + G
Number of cell lines	4	9	41
Cell lines	THLE-5B, AsPC-1 MDA-MB-468, SW480	DLD1, SKOV3 HA22T/VGH, HUH4 A2182, NCI-H292, SW620 U118 MG, RKO	Others

In this study, we analyzed the human *p63* gene and found that it has a longer NH_2 terminus than was initially reported and that it is highly homologous with murine *p63*. By SSCP, we studied the mutational status in 54 human cell lines using intronic primers and found possible heterozygous mutations or polymorphisms in two cell lines, DLD1 and SKOV3. RT-PCR analysis revealed that *p63* is biallelically expressed in these cell lines. We compared mutations in the *p53*, *p63*, and *p73* genes in these 54 cell lines to test the hypothesis that these gene products may function in the same tumor suppressor pathway so that the mutations would be mutually exclusive. Our data are not consistent with this hypothesis. We conclude that *p63* mutations are uncommon in the human cancers examined and that *p53* and *p63* are not in the same tumor suppressor pathway. Although *p63* or *p73* somatic mutations seems to be uncommon in human cancer, transcriptional silencing after DNA methylation would be an alternative mechanism of inactivating these members of the *p53* gene family (21, 22). Additional studies are required to elucidate the role of the *p63* gene in human carcinogenesis.

Acknowledgments

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