Overexpression of the Wild Type *p73* Gene in Breast Cancer Tissues and Cell Lines[1]

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ABSTRACT

The *p73* gene is a structural and, in overexpression systems, functional p53 homologue. Ectopic p73 expression can activate a broad subset of *p53*-responsive genes, induce apoptosis, and act as a growth suppressor. Yet, viral oncoproteins that antagonize p53 (adenovirus E1B 55K, SV40 large T, and human papillomavirus E6) do not antagonize p73. This could suggest that inactivation of p73, in contrast to p53, is not required for tumorigenesis. Also, p73 is not activated by DNA damage. Because intragenic p73 mutations in tumors have not been reported and imprinting is idiosyncratic, tumor-specific changes in wild-type p73 expression levels become the most reliable guide toward identifying the normal function of p73 and its role in tumorigenesis.

We analyzed 77 invasive breast cancers and 7 breast cancer cell lines for p73 mRNA expression levels, allelic origin, intragenic mutations, and COOH-terminal splice variants. A range of normal tissues, including breast, showed very low p73 expression, with little variation from tissue to tissue. In contrast, 38% (29 cases) of breast cancers had elevated p73 mRNA ranging from 5–25-fold above normal, with the remaining tumors (64%) falling within the normal range. Moreover, five of seven cell lines (71%) also exhibited p73 overexpression (13–73-fold). Yet, no correlation with p21 mRNA and protein levels was present, although four of the five lines were mutant for p53. Mutation analysis of the eight highest expressers showed wild type status. Eight of 14 informative samples were biallelic, whereas the remaining 6 samples showed monoallelic expression. Tumors and cell lines with p73 overexpression tended to exhibit a complex profile of up to six different COOH-terminal splice variants, whereas normal and transformed tissues with low p73 mRNA predominantly expressed p73 α. We confirm the previously described variants p73 γ and δ in breast tissue and describe two novel isoforms, p73 ϵ and ϕ, thereby further enlarging combinatorial possibilities. Together, our *in vivo* data show that p73 does not have a role as a classic Knudson-type tumor suppressor in breast cancer.

INTRODUCTION

Two structural homologues of the *p53* tumor suppressor gene were recently identified in human tissues and termed p73 and p63/p51/KET (1–4). Overall, p73 shares 29% identity in the NH_2-terminal transactivation domain of p53, 63% in the specific DNA-binding region with conservation of all DNA contacting residues, and 38% in the tetramerization domain. Structure comparisons show that the *p63/KET* and *p73* genes are closer related to each other than they are to p53 and that both genes are ancestral to p53 (4).

Both p63 and p73 are characterized by encoding multiple isoforms, of which only some have significant transactivating activities in p53 reporter or response gene assays. For example, p63 has six variants, generated by combining three different COOH-terminal variants with either a full-length or a truncated (δ) NH_2 terminus (with or without the transactivation domain; Ref. 4). Only full-length p63 γ shows strong p53-reporter activity and apoptotic activity, whereas all others

do not. In the case of p63, an interesting possibility arose with the demonstration of transdominant inhibition of p53 and p63 γ by δ N-p63 in a p53-reporter assay. Delta N-p63 is strongly expressed in basal cells of various epithelia, which are the proliferating stem cells of these tissues (4). Rare somatic mutations in p63/p51 were found in some human epidermal tumors (3). The expression status of p63 in tumors is unknown. p73, when ectopically overexpressed, also mimics p53 activities in certain transcriptional and growth control assays *in vitro*. Ectopic p73 β and, to a lesser extent, p73 α transactivate many p53-responsive promoters, including the endogenous *Waf1* gene (1, 5–7). Overexpression of wt[4], but not transcriptionally inactive mutant p73 suppresses focus formation in several cell types and promotes apoptosis (1, 5, 6). Moreover, p73 maps to a genomic region (chrom 1p36.3) that frequently undergoes allelic loss (loss of heterozygosity) in NB, breast and colon carcinoma, and melanoma (see Ref. 8 for review). For these reasons, p73 was postulated to be a tumor suppressor gene that is targeted during tumorigenesis and undergoes loss of expression. However, the lack of inactivating mutations in all human tumors thus far studied, and the fact that the *p73* gene is neither induced nor activated by DNA damage, questions this model. There are other reasons to believe that the biological role of p73 is distinct from that of p53. Homozygous p73 $-/-$ mice exhibit a striking lack of tumor susceptibility. These mice have no cancer phenotype after almost 3 years (9). On the other hand, they have severe developmental abnormalities. Conversely, the strikingly mild developmental phenotype of homozygous p53 $-/-$ mice suggests the existence of ancestral developmental genes that specifically substitute for p53 and carry these animals through this period. Furthermore, three classic viral oncogenes that target and inactivate the p53 protein to allow host cell transformation do not target the p73 protein physically or functionally. SV40 large T antigen, Ad E1B 55 kDa protein, and human papillomavirus E6 protein do not interact with p73. E1B 55 kDa does not inhibit p73-mediated transcription, and HPV E6 does not degrade p73 α and β (10–13). Also, adenoviral E1B 55 kDa plus E4 34 kDa do not promote p73 β degradation, although p73 α might be susceptible to E4 34 kDa alone (11, 13). Moreover, p73 binds only weakly to the p53 antagonist mdm-2, but more strongly to mdm-x (9), the precise function of which remains to be defined (14). We previously reported overexpression of wt p73 in NB and related tumors and NB cell lines (15). Here we report similar data in breast cancer, adding further evidence that an increase rather than a loss of wt p73 expression is associated with tumorigenesis. Together, the lack of inactivating mutations in human tumors, the absence of tumor susceptibility in the mouse knock-out model, increased expression of wt p73 in some tumors, and the lack of viral and cellular oncogene interaction does not support a role of the *p73* gene as a classic Knudson-type tumor suppressor like p53.

MATERIALS AND METHODS

Tissues and Cell Lines. Primary tissues of 77 invasive breast cancers were collected at University Hospital at State University of New York at Stony Brook from 1993–1998. Freshly harvested tumors (>60% tumor cells) were immediately snap frozen in liquid nitrogen and stored at -80°C, until needed.

Freshly harvested normal tissue from 14 individuals consisted of breast (two cases), uterus (two cases), lymphocytes (two cases), neutrophils (three cases), thyroid, kidney, ovary, and placenta. A polycystic kidney (adult type) was also included. Normal tissues were processed immediately. Human breast cancer cell lines MDA 468, MDA 361, MDA 231, MDA 435, MCF-7, T47D, and SKBR3 were grown in 10% FCS containing DMEM at 5% CO_2. Human NB lines LAN-5, SK-N-SH, and SK-N-AS were grown in RPMI/10% FCS and used as positive and negative controls for expression analysis (15). Waf-1 immunoblotting was performed, as described previously (15), using a rabbit Waf-1 IgG (a gift from David Beach). MDA 361 cells grew very poorly and did not provide enough material for immunoblotting.

RNA and DNA Extraction. Snap frozen tissue was homogenized under liquid nitrogen in 2 ml of RNA STAT-60 (Tel-Test, Inc., Friendswood, TX). After adding chloroform, total RNA was precipitated in isopropanol, washed twice in 75% ethanol, and dried. Concentrations of reconstituted RNA were measured in triplicate by UV spectrophotometry and adjusted to 1 μg/μl. To obtain corresponding DNA, DNA reverse extraction from the same samples was performed by using DNA STAT-60 (Tel-Test, Inc.).

Semiquantitative RT-PCR. To determine p73 expression, the Titan One Tube RT-PCR system (Boehringer Mannheim) was used as we described previously (15). Rat mRNA and rPK primers were added, and rPK served as internal standard as described (16, 17). For this, total RNA was prepared from adult rat liver, triple measured for concentration, and stored in aliquots at -70°C. This strategy ensures that the intensity of the internal standard can be titrated to a low abundance transcript that is comparable with p73 and falls within the linear range of detection, in contrast to the high abundant human β-actin or glyceraldehyde-3-phosphate dehydrogenase transcripts. Also, the exact same mass amount of total reference RNA and, consequently, the same amount of standard transcript is added to each sample. Briefly, exactly 1 μg of human RNA and 0.1 μg of rat RNA were added to 10 μl of total reaction mix containing 10 units of RNAsin (Life Technologies, Inc.), 0.2 mM dNTPs, 0.3 mM each of the p73 primer pair P1 and P2 (exons 2–5; P1 sense 5'-CGG-GACGGACGCCGATG-3' and P2 antisense 5'-CTTGGCGATCTGGCAG-TAG-3'), and 25 μM of the L-type rPK primer pair (sense 5'-AACCAACG-TAGCAGCATGGAAG-3' and the antisense 5'-GGGTCAGTTGAGCC-ACACTCG-3'). Both sense primers had been labeled with [γ-P^{32}]ATP using the T4 kinase reaction. First, the absence of cross-hybridization of primers for rat PK and human p73 to human and rat templates, respectively, were confirmed by running optimized reactions with: (*a*) rat primers, but only human template mRNA; and (*b*) human primers, but only rat template mRNA. Second, the number of cycles at which the amplification of both test and reference fragments were well within the linear phase were determined by analyzing the reaction kinetics, as described (16). Reverse transcription was performed for 30 min at 50°C, followed by 25 cycles of PCR at 94°C for 30 s, 58°C for 30 s, and 68°C for 45 s, including the last 15 cycles with elongation of 5 s for each cycle. To further ensure that each sample received the same amount of human RNA, a 476-bp product of endogenous glyceraldehyde-3-phosphate dehydrogenase mRNA was amplified in a parallel reaction, and bands of equal intensity were obtained in all. The p73-derived 543-bp product and the rPK-derived 67-bp product were analyzed on 7 M urea PAGE gels, dried, and band intensities were quantitated by PhosphorImager analysis (model 445 SI; Molecular Dynamics). Relative p73 expression levels were standardized using the corresponding rPK value of each sample. Tissues were analyzed in batches, and each batch contained LAN-5 and SK-N-SH NB lines as positive controls and SK-N-AS as a very low-expressing line. p73 expression for each sample was normalized by taking the ratio between p73 and rPK. Samples were measured in duplicates, and the average was taken. All gels contained samples from normal tissues, tumors, and cell lines side by side. For p21 mRNA analysis, a p21 primer pair (sense 5'-TGTCCGTCAGAAC-CCATG and antisense 5'TGGGAAGGTAGAGCTTGG) was used and standardized by coamplified human β2 microglobulin (sense 5'-TGTCTTTCAG-CAAGGACTGG and antisense 5'-GATGCTGCTTACATGTCTCG).

Allelic Expression. Allele-specific expression for the *p73* gene was determined by the *Sty*I polymorphism, as we described previously (15). This polymorphism exploits the additional *Sty*I site that results from a double nucleotide substitution (G to A and C to T) at positions 4 and 14 of exon 2 that give rise to *GC* and *AT* alleles. Restriction length analysis for genomic DNA was performed using two nested PCR reactions. Restriction sizes for the *GC* allele are 482 bp (genomic) and 284 bp plus 259 bp (cDNA); for the *AT* allele,

the restriction sizes are 376 bp plus 106 bp (genomic) and 50 bp plus 234 bp plus 259 bp (cDNA).

COOH-terminal Splice Variants. Nested RT-PCR reactions spanning the mid-region of exon 10 to the end of exon 14 were used to determine the existence of COOH-terminal splice variants. External primer set: s5'-GCCGG-GAGAACTTTGAGATC-3'at cDNA position 1192–1211 and as 5'-TCCTT-GATGGGCTGCTTGC-3' at position 1993–1975. RT-PCR was performed for 30 min at 50°C, followed by 25 cycles at 94°C for 30 s, 58°C for 30 s, and 68°C for 45 s with elongation of 5 s/cycle for the last 25 cycles. The first PCR (1 μl) was added to the second PCR with the internal primer set s5'-CAGC-CACTGGTGGACTCCTATC-3' at cDNA position 1257–1278 and as 5'-TAGTCGGGCCCTGCTTCAG-3' at position 1771–1752; 94°C for 30 s, 58°C for 30 s, and 72°C for 90 s for 30 cycles. All products were gel-purified and sequenced for confirmation.

Direct Sequencing of Full-length p73 cDNA. Sequencing was performed in 4 overlapping sections A-D as we previously described (15). In select cases, exons 4–9 of the p53 tumors were sequenced as we previously described (18).

RESULTS

p73 Expression Levels in Breast Cancer Tissues and Cell Lines. We used a semiquantitative RT-PCR assay measuring a 543-bp product from the 5' region of the p73 cDNA. To establish the normal baseline, we first determined p73 expression in a wide range of normal tissue samples from 14 individuals representing eight different tissues (breast, kidney, thyroid, ovary, uterus, placenta, neutrophils, and lymphocytes). All normal tissues, including breast, had very low expression of p73 transcripts (mean relative expression, 0.16; see Fig. 1*A*). This result was consistent with our previous comprehensive survey by quantitative dot blot analysis of 43 different adult and 7 fetal human tissues (15). Among those 50 tissues, expression levels of p73 mRNA varied only 4-fold between the lowest- (amygdala) and the highest (liver)-expressing tissues. Next, we measured p73 expression levels in 77 invasive breast cancers and seven breast cancer cell lines. Elevated expression was arbitrarily defined as a 5-fold or higher increase over mean normal expression. With this criteria, 48 tumors had p73 expression levels within the range of normal tissues when measured side by side (see Fig. 1*A* for summary and Fig. 1*B* for examples). Two tumors (BC 76 and BC 77) had lower, but still detectable, expression, which probably is due to compromised RNA quality in these clinical samples. In contrast, 29 tumors (38%) showed elevated p73 transcripts ranging from 5–25-fold (Fig. 1, *A* and *B*). Moreover, five of seven breast cancer cell lines (71%; MDA 361, MDA 468, T47D, MDA 231, and MCF-7, in decreasing order) also exhibited overexpression of p73 mRNA, ranging from 13–73-fold (Fig. 1, *A* and *B*). Forced expression of ectopic p73 can mimic the ability of p53 to activate transcription of endogenous p21 (1, 5, 7). If the endogenous *p73* gene was an important transcriptional activator of endogenous p21, a correlation between p73 and p21^{Waf-1} expression levels would be expected in these cell lines because all but one line (MCF-7) harbors transactivation-deficient p53 mutants, therefore, eliminating p53 as a transactivator of p21. However, we did not detect such a correlation on the mRNA or protein level (Fig. 1*C*). This finding parallels our earlier observation of a lack of correlation between p73 and p21 levels in NB cell lines (15). Our finding in breast suggest that either the levels of endogenous p73 overexpression present in MDA 231, MDA 468, and T47D are insufficient for p21 activation or that p21 is not an important physiological target of p73.

The *p73* Gene Is Mostly Biallelically Expressed in Breast Cancer. Although the initial analysis on lymphocytes from five healthy individuals and a NB cell line described only monoallelic expression of the *p73* gene (1), subsequent analysis on normal lung (19), thyroid and lymphocytes (15, 20), NB (15), and melanoma (21) demonstrated biallelic expression in the majority of the cases, although a prevalence
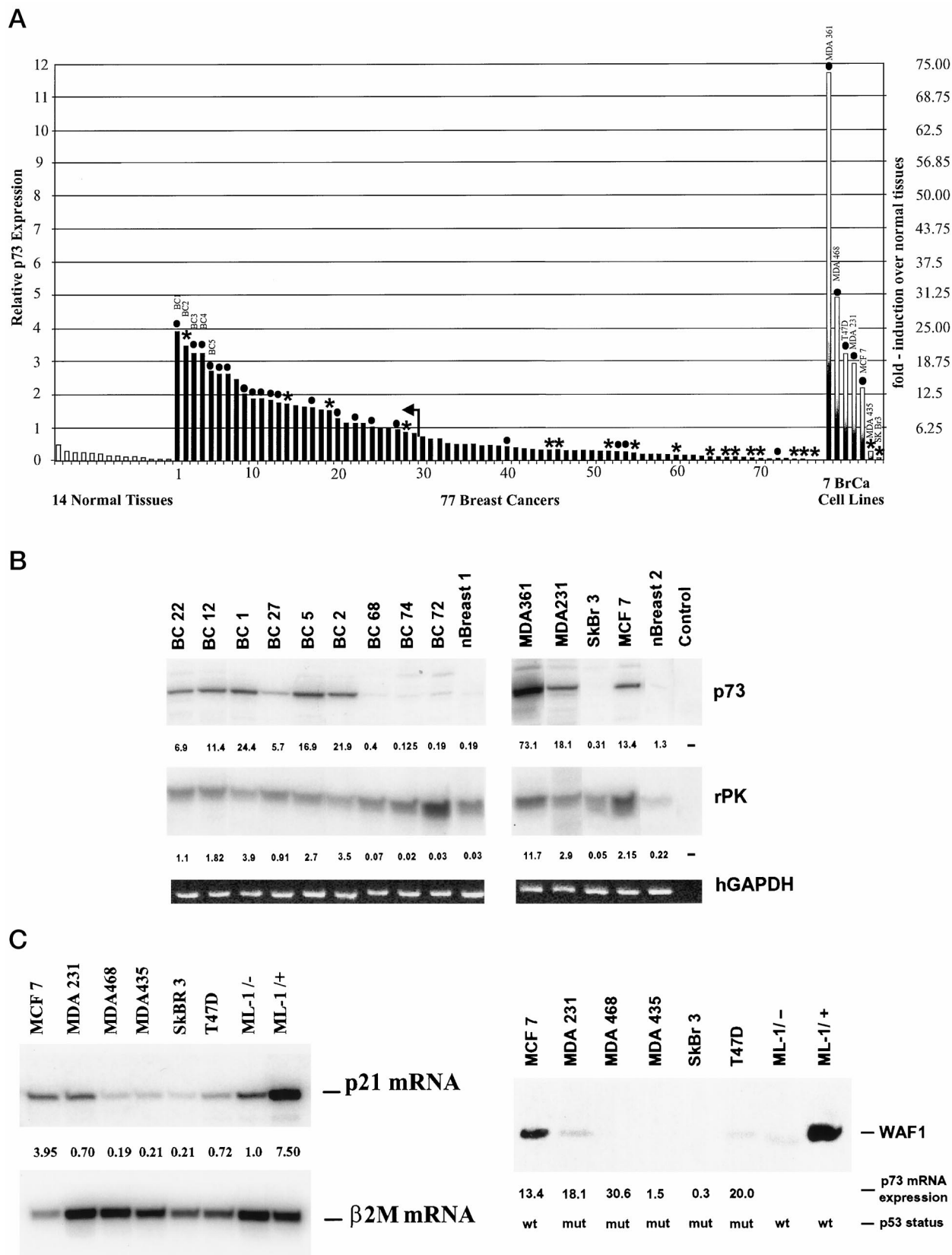
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Fig. 1. p73 expression in primary breast cancers and breast cancer cell lines. *A*, histogram of relative expression of p73 transcripts, as determined by semiquantitative RT-PCR. Expression levels were standardized using the corresponding rPK value of each sample (*left ordinate*). The fold-induction over the mean normal tissue expression (0.16) is indicated (*right ordinate*). *Arrow*, the arbitrary cutoff for tumors with 5-fold or higher p73 expression compared with mean normal expression. Tumors with complex (●) or with simple (∗) COOH-terminal splice variants are indicated. *B*, examples of breast cancers, normal breast tissue, and breast cancer cell lines are shown. Numbers on top indicate the -fold increase over the mean normal tissue expression, whereas numbers below refer to the -fold increase over the internal rPK standard. Control lane contains no template RNA. *C, left,* relative expression of p21[WAF1] transcripts, as determined by semiquantitative RT-PCR. The numbers refer to the -fold increase over the internal β2 microglobulin standard (*β2M*); *right,* p21[WAF1] immunoblot analysis of cell lysates (100 µg/lane) from breast cancer lines MCF-7, MDA 231, MDA 468, MDA 435, SkBr3, and T47D. Their respective fold-increases of p73 mRNA expression (over mean normal tissues) and p53 mutation status are indicated below. Cell lysates (100 µg) from ML-1 cells, either untreated (−) or treated (+) with the p53-activating agent camptothecin (5 µM for 24 h), serve as control for p21 induction.

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of monoallelic expression in kidney was found in one study (22). Moreover, differences between individuals, and even among various tissues of the same individual, indicate the idiosyncratic nature of the allelic expression of p73. For example, Nomoto *et al.* (19) described a patient who expressed his *p73* gene preferentially from the *A1* allele in the lung and liver, from the *A2* allele in the stomach, and from both alleles in small intestine, spleen and kidney. To evaluate *p73* allelism in breast tissue, we determined allele-specific expression of p73 in our heterozygous samples using the *Sty*I polymorphism of exon 2. Of 14 informative samples, 8 samples were biallelic (7 breast cancers and 1 breast cancer cell line) and 6 samples were monoallelic (5 breast cancers and 1 normal breast; Fig. 2). In addition, no correlation existed between p73 overexpression and biallelism. Four of the 14 cancers showed high p73 expression (BC 8, BC 13, BC 18, and BC 22), of which 2 were associated with biallelic and 2 with monoallelic expression (Fig. 2). Taken together, our result shows that the *p73* gene is biallelically expressed in the majority of cases but can, under certain unknown conditions, underlie epigenetic regulation that leads to monoallelic expression, consistent with previous data (15, 19, 20–22).

Absence of *p73* Gene Mutations in Breast Cancer. Full-length sequencing of all 13 coding exons of p73 from the eight highest-expressing samples (tumors BC 1, BC 2, BC 3, BC 5, and BC 8 and cell lines MDA 361, MDA 468, and T47D) failed to show any coding region mutations. MDA 361 and MDA 468 harbored a silent third nucleotide change at codons 245 and 405, respectively, which is likely to be a polymorphism. We conclude that overexpression of wt p73 mRNA in malignant tumors is inconsistent with a role of *p73* as a classic tumor suppressor gene.

Lack of Correlation between wt p73 Overexpression and p53 Mutations. It is conceivable that mutant p53 could inhibit the putative tumor suppressor action of p73 in a dominant negative fashion by generating defective heterooligomers with wt p73. In cotransfection assays, the R175H and R248W mutants of p53 coprecipitates with p73 α (23). Furthermore, in a p53 reporter assay, transactivation activity of p73 α was partially inhibited by the 175 and 248 mutants of p53, which correlated with a reduction in p73 α-mediated apoptosis (23). If such an inhibitory mechanism of p73 by p53 were in place *in vivo*, one would expect a strong correlation with p53 mutations, particularly in breast cancers that exhibit wt p73 overexpression, because in such tumors the selection pressure against p73 would be highest. However, we did not find such a correlation. Of eight breast cancers with p73 overexpression, which we sequenced, five tumors harbored only wt *p53* alleles (BC 7, BC 9, BC 10, BC 14, BC 15), whereas the remaining three cancers harbored p53 mutations (BC 3, V157F; BC 8, ex 6 del; and BC 12, R306stop). This mutation frequency of 38% (3 of 8) is identical to the one we determined in a group of 8 randomly chosen breast cancers with normal levels of p73 expression. Five of the latter tumors had wt p53, while 3 harbored mutant p53 (BC 55, G266R; BC 61, R248G and BC 64, I232S).
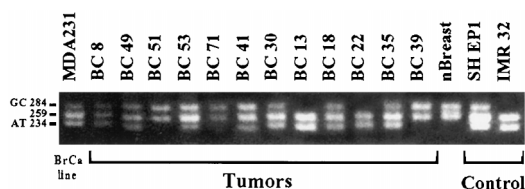
Expression Pattern of p73 COOH-terminal Splice Variants. Originally, an alternatively spliced mRNA termed p73 β was identified together with full-length p73 α. p73 β lacks exon 13, resulting in a frameshift with five unique amino acids, followed by premature termination (1). Recently, De Laurenzi *et al.* (6) reported two novel p73 COOH-terminal splice variants, p73 γ (splicing exon 11) and p73 δ (splicing exons 11, 12, and 13) that were identified in normal lymphocytes, keratinocytes, and several tumor cell lines, including MCF-7 (see Fig. 3*A*). To assess these variants in our samples, we amplified the 3' end of p73 mRNA encompassing the mid-region of exon 10 to the end of exon 14 in 35 breast cancers, 7 breast cancer lines, and 14 normal tissues of various types, including normal breast. We confirmed the expression of p73 γ and δ isoforms, which we detected in breast cancers and cell lines (Fig. 3*B*). In addition to these four isoforms, we report the identification of two additional splice variants, p73 ϵ and p73 ϕ. They appeared as novel amplification products of 227 bp and 273 bp, respectively, together with p73 α-δ in a single PCR reaction (Fig. 3, *Lanes 10* and *13*). We confirmed *p73* ϵ and ϕ isoforms by direct sequencing of the RT-PCR-generated 3'ends of the gene. To further confirm their presence, we cloned out long open reading frames of p73 ϵ and ϕ splice forms from MCF-7 cells, which express the 3' ends of these forms (see Fig. 3*B*, *Lane 1*). The longest products we cloned to date for both forms encompass exons 4–14 (sequence confirmed). This increases the total number of described p73 isoforms to six (see Fig. 3*A*). p73 ϵ is generated by splicing exons 11 and 12, whereas p73 ϕ splices exon 11 and exon 13, but retains exon 12. The splicing of exons 11 and 12 in p73 ϵ results in a frameshift starting at residue 400, followed by 141 novel amino acids and a premature stop codon at residue 540. In contrast, p73 ϕ was identical to p73 γ, but different from all other isoforms between residues 400 and 445, followed by a novel stretch of 110 amino acids and a premature stop at residue 555. Both p73 ϵ and p73 ϕ contain the regions homologous to the transactivation domain, the DNA-binding domain and the oligomerization domain of p53.

In normal tissues (kidney, uterus, ovary, placenta, breast, thyroid, neutrophils, and lymphocytes), p73 α was the sole or predominant isoform, whereas p73 β was less consistent and p73 γ was rarely found (Fig. 3*B*, *Lanes 23–36*; see also Ref. 24). p73 δ, ϵ, and ϕ were not detectable in this assay. We termed this a simple profile, albeit minor expressions of the "lower" forms (alphabetically), as shown for p73 δ in lymphocytes but not in keratinocytes (6), would have escaped detection in this nonradioactive PCR assay. Interestingly, the normal expression profile differs within the same organ from person to person (*e.g.,* uterus 1–3 in Fig. 3B, *Lanes 25*, 26 and 28). In contrast, as a general rule, samples with p73 overexpression (which coincided with transformed cells), tended to exhibit a more complex pattern of isoforms (for example BC 1, BC 11, BC 13, MCF 7, and MDA 231; *Lanes 12*, 13, 11, 1, and 2, respectively), but exceptions were noted (BC 18 and BC 4; *Lanes 8* and *14*). Nevertheless, of 20 highly expressing primary breast cancers, 16 showed a complex pattern (Fig. 1*A*, ● above histograms). This was also true for all five overexpressing cell lines. Within this complex pattern, however, the expression profiles of the six splice variants differed greatly among individual breast cancers and cell lines. Depending on the sample, either all 6 isoforms or various combinations of subsets were detectable (*e.g.,* compare BC 3, BC 27, BC 22, and BC 13; *Lanes 5*, *7*, *10*, and *13*, respectively). In contrast, most but not all breast cancer samples with low p73 expression showed a simple pattern (*i.e.,* they lacked isoforms other than p73 α and β; Fig. 3*B*, *Lanes 19–21* and *37–44*). The exceptions were BC 72 and BC 54, which showed three isoforms each (Fig. 3*B*, *Lanes 17* and *22*). Nevertheless, 13 of 17 low-expressing breast cancers and both low-expressing cell lines showed a simple profile (Fig. 1*A*, ∗ above histograms). Likewise, of 14 normal tissues

Fig. 2. Frequent biallelic expression of the *p73* gene in breast cancer. Allele-specific p73 expression was determined in heterozygous samples by the *Sty*I restriction length polymorphism of exon 2, which distinguishes the *G/C* (284 bp) and the *A/T* (234 bp) alleles. The 259-bp band is constant. Controls are the A/T homozygous IMR 32 and heterozygous SH EP1 neuroblastoma lines.

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                    9                           10
                    |                           |                    414
p73α    DTYYLQVRGRENFEILMKLKESLELMELVPQPLVDSYRQQQQLLQRPSHLQPPSYGPVLSPM
p73β    DTYYLQVRGRENFEILMKLKESLELMELVPQPLVDSYRQQQQLLQRPSHLQPPSYGPVLSPM
p73γ    DTYYLQVRGRENFEILMKLKESLELMELVPQPLVDSYRQQQQLLQRPPRDAQQPWPRSASQR
p73δ    DTYYLQVRGRENFEILMKLKESLELMELVPQPLVDSYRQQQQLLQRPTWGP★
p73ε    DTYYLQVRGRENFEILMKLKESLELMELVPQPLVDSYRQQQQLLQRPFLTGLGCPNCIEYFT
p73ϕ    DTYYLQVRGRENFEILMKLKESLELMELVPQPLVDSYRQQQQLLQRPPRDAQQPWPRSASQR
p53     EYFTLQIRGRERFEMFRELNEALELKDQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFKT
                **|**|**** **  *  * ***    *   *|*         387
                9                          10
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                              11
                              |                    476
p73α    NKVHGGMNKLPSVNQLVGQPPPHSSAATPNLGPVGPGMLNNHGHAVPANGEMSSSHSAQSMV
p73β    NKVHGGMNKLPSVNQLVGQPPPHSSAATPNLGPVGPGMLNNHGHAVPANGEMSSSHSAQSMV
p73γ    RDEQQPQRPVHGLGVPLHSATPLPRRPQPRQFFNRIGVSKLHRVFHLPRVTEHLPPAEPDH★
p73ε    SQGLQSIYHLQNLTIEDLGALKIPEQYRMTIWRGLQDLKQGHDYSTAQQLLRSSNAATISIG
p73ϕ    RDEQQPQRPVHGLGVPLHSATPLPRRPQPRQDLGALKIPEQYRMTIWRGLQDLKQGHDYSTA
p53     EGPDSD★
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                        12                       13
                        |                        |           538
p73α    SGSHCTPPPPYHADPSLVSFLTGLGCPNCIEYFTSQGLQSIYHLQNLTIEDLGALKIPEQYR
p73β    SGSHCTPPPPYHADPSLVRTWGP★
p73ε    GSGELQRQRVMEAVHFRVRHTITIPNRGGPGGGPGPDEWADFGFDLPDCKARKQPIKEEFTEAE
p73ϕ    QQLLRSSNAATISIGGSGELQRQRVMEAVHFRVRHTITIPNRGGPGGGPDEWADFGFDLPDC
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                                              600
p73α    MTIWRGLQDLKQGHDYSTAQQLLRSSNAATISIGGSGELQRQRVMEAVHFRVRHTITIPNRG
p73ε    IH★
p73ϕ    KARKQPIKEEFTEAEIH★
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                                    636
p73α    GPGGGPDEWADFGFDLPDCKARKQPIKEEFTEAEIH★
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B

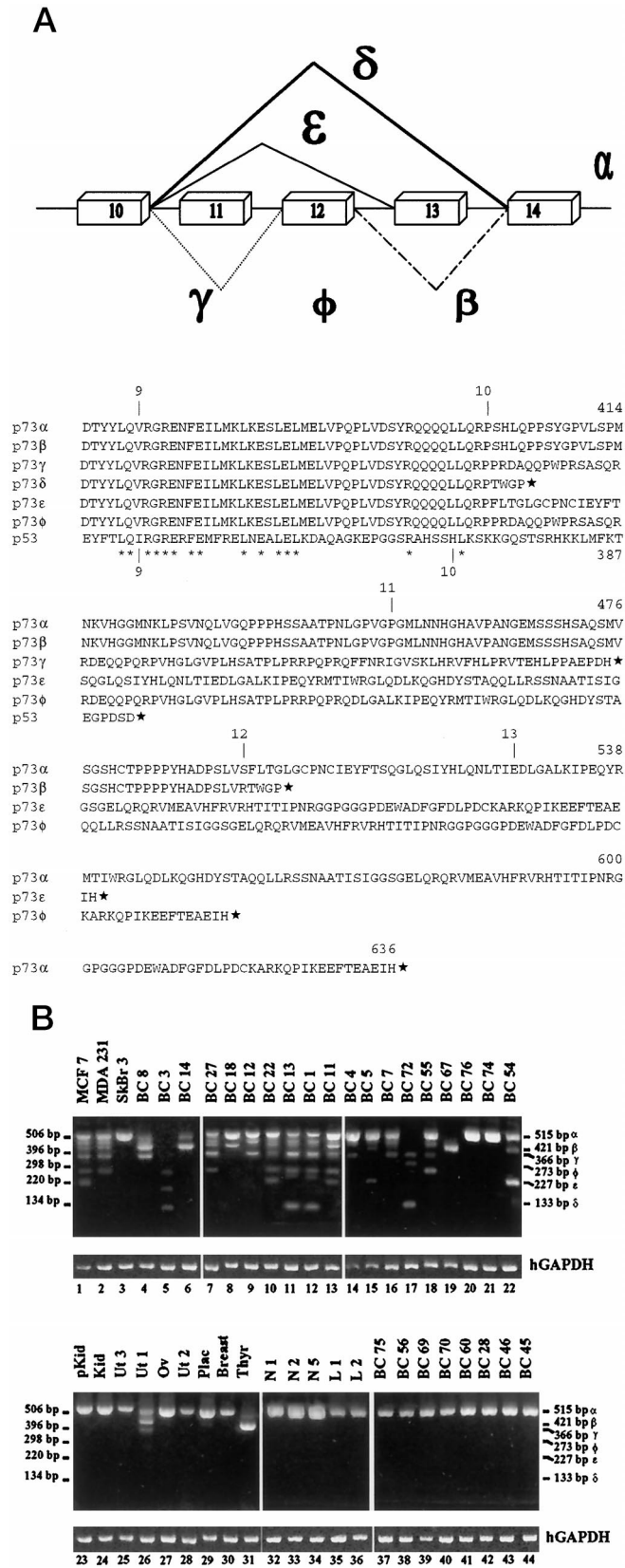Fig. 3. p73 COOH-terminal splice variants of the *p73* gene. *A*, diagram of the six different COOH-terminal splice variants α, β, γ, δ, ε, and ϕ of the human *p73* gene, detected in various normal and malignant tissues including breast cancer, as reported here. The spliced exons (*top*) and the predicted truncations or frameshifts of the amino acid sequence (*bottom*) are shown. *B*, samples of expression profiles among primary breast cancers and breast cancer cell lines and normal tissues. Generally, the low-expressing tissues express predominantly p73 α (*bottom*), whereas overexpressing tumors show a complex pattern (*top*). Length of the nested PCR product used to identify each variant is indicated.

(neutrophils, lymphocytes, placenta, ovary, uterus, breast, and kidney), 10 tissues (including breast) expressed exclusively a simple pattern (*i.e.,* p73 α). Normal thyroid expressed primarily p73 β. The exception was one uterus (of two tested), which expressed p73 α, β, and γ (Fig. 3*B*, *Lane 26*). Interestingly, some tumors (high and low expressers) did not express p73 α or β, but only lower isoforms (Fig. 3*B*, BC 3 and BC 72 in *Lanes 5* and *17*), whereas all normal tissues always expressed α and/or β.

DISCUSSION

In this study, we examined the status of the *p73* gene in breast cancer by analyzing 77 primary tumors and seven cell lines. We found strong overexpression of wt p73 transcripts in 38% of breast cancers (range, 5–25-fold) and 71% of cell lines (range, 13–73-fold). Sequence analysis of the entire open reading frame on a subset of overexpressing samples failed to reveal any mutations. This result is consistent with our previous data on NB and related tumors and NB cell lines, where a subset also showed strong overexpression of wt p73 mRNA, ranging from 8–80-fold in NB tumors and 8–90-fold in NB cell lines (15). In accord with our results, wt p73 overexpression associated with malignant tissue, but not with its matched normal counterpart, has been found in lung cancer (25), prostate cancer (24), and colorectal cancer (26). Using semiquantitative RT-PCR, as we did in our studies, Mai *et al.* (25) found that 9 of 10 lung cancers showed strong p73 mRNA induction compared with matched normal lung from the same patient. Increased wt p73 expression was also found in three of three colorectal cancers compared with their normal mucosa (26) and in prostate cancer (24). Despite an increase in expression, however, the levels of p73 mRNA and protein reached are still too low for reliable Northern and Western blot detection and are best measured with a sensitive radioactive RT-PCR method (10, 26). For example, we were unable to immunoblot p73 protein from overexpressing breast cancer and NB cell lines with p73-specific monoclonal antibodies (from Ref. 10), even with maximally enhanced chemiluminescence and long exposure. Importantly, true tumor-specific loss of p73 expression (rather than low expression within the normal range) has not been described. In summary, there is mounting evidence that the p73 alteration associated with human tumors is increased expression of wt gene products rather than loss of expression. Overexpression of wt p73 mRNA in malignant tumors is inconsistent with a role of *p73* as a classic tumor suppressor gene. Instead, it suggests that inactivation of the *p73* gene is not required during tumorigenesis because it somehow promotes tumorigenesis.

Monoallelic expression of p73 is highly idiosyncratic and varies from tissue to tissue and person to person (15, 19–21, 26). Consistent with this data, we found 8 of 14 (57%) biallelic and 6 of 14 (43%) monoallelic breast cancers in this study. Moreover, Mai *et al.* (22, 25) reported tumor-specific reactivation of the second *p73* allele, which they called "loss of imprinting," in 5 of 5 lung cancers and 8 of 12 renal cancers, whereas their matched normal tissues expressed monoallelically. However, although simple reactivation of the second *p73* allele might be a widespread phenomenon during tumorigenesis, it is unlikely to be the cause of the dramatic rise in expression levels that we observed in NB and breast cancer and that others have observed in lung cancer (25), colorectal cancer (26), and prostate cancer (24). This further supports the notion that overexpression, be it generated by epigenetic and/or transcriptional up-regulation, is associated with transformation.

We also analyzed expression profiles of p73 COOH-terminal splice variants, comparing transformed breast samples with a spectrum of normal tissues. De Laurenzi *et al.* (6) recently reported the existence of p73 γ and δ variants in primary cells and transformed cell lines

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using a highly sensitive radioactive RT-PCR assay. None of our normal tissues expressed isoforms lower than p73 γ in sufficient amounts to become detectable in ethidium bromide-stained agarose gels. Interestingly, BC 3 and BC 72, expressing high and low p73 levels, respectively, expressed only the lower isoforms, but failed to express p73 α and β (Fig. 3B, *Lanes 5* and *17*). Furthermore, at least in this cohort, expression of isoforms γ, δ, ε, and φ roughly correlated with p73 overexpression, which coincided with transformation. However, a larger spectrum of normal and malignant tissues needs to be surveyed to determine whether consistent patterns can be filtered out. Biochemically, neither p73 γ nor δ interact with p53, but form homotypic interactions; p73 γ also forms strong heterotypic interactions with α, β, and δ, whereas p73 δ forms strong interactions with α nd γ, but binds only weakly to β (6). Functionally, p73 γ is very weak compared with p53 and p73 β in suppressing colony formation in SaoS-2 cells and in transactivating Waf-1 (6). Interestingly though, although p73 γ activates transcription poorly by itself, it has no inhibitory effect on coexpressed p73 β, which consistently is the strongest transactivator of p53 targets in ectopic expression (6, 7, 11). p73 δ, like p73 α, shows intermediate strength in transactivation and suppression. (6). Hence, the various isoforms seem to have very different biological effects from weak to strong or neutral. Homo- and heterotypic interactions between p73 ε and φ are likely but need to be shown and, if they exist, their functional consequences *in vivo* need to be established. In addition, further work is necessary to understand the complex regulatory network generated by: (*a*) multiple p73 COOH-terminal isoforms and their idiosyncratic expression profiles that vary among tissues and individuals; (*b*) homo- and heterotypic interactions among themselves; and (*c*) the resulting differentials in their activities. Also, as suggested by p63 and the coexpression data on p73 *in vitro*, *in vivo* interactions between p73 and p53 are conceivable and they might modulate the function of p53 (3, 6, 18). Particularly, dominant negative δ N-p73 variants might exist, and it will become very important to determine whether they occur in a tumor-specific manner.

Currently, data from ectopic p73 overexpression in tissue culture, together with the structural homology of p73 to p53 on the one hand, are not easily reconcilable with genetic, viral, and primary tumor data of p73, on the other hand. Is *p73* a tumor suppressor gene that does not conform to the two-hit hypothesis or does *p73* have oncogenic activities despite its membership in the p53 family and, therefore, is actively selected for in tumors? Several scenarios can be envisioned. First, the simplest interpretation of the observed wt p73 overexpression in multiple human tumors is the assumption that the overall net effect of p73 function *in vivo* is to promote growth and transformation and, therefore, deregulated expression is actively selected for in tumors. Mechanistically, one could envision that some isoforms of p73 might directly transactivate growth-promoting genes and override the neutral or even growth-inhibiting effects of other isoforms. [We note that at least in NB cell lines, p73 does not seem to be involved in differentiation. Although a 4–8-fold increase of p73 mRNA levels was present in all three of the differentiated sublines from N/S pairs of NB cells (15), retinoic acid- mediated neuritic differentiation failed to induce p73 mRNA].[5] Second, alternatively, transdominant inhibiting isoforms, perhaps a δN-p73 or certain COOH-terminal variants, might exist, and it would be those that are the truly important gene products, overexpressed along with phenotypically neutral isoforms. These inhibitors would antagonize the suppressor action of p53 and of its own p53-like isoforms, either by direct competitive binding to the same cognate p53 DNA binding sites or by engaging in heterocom-

plexes that are defective for specific DNA binding. In this case, however, the selection pressure for p53 mutations should be relieved, because a given tumor usually does not exhibit redundant mechanisms of p53 inactivation. Our data, however, does not support the latter model. We found an identical mutation frequency (38%) of p53 in breast cancers with and without p73 overexpression. In any case, both scenarios are consistent with the genetic data of p73. Furthermore, they are also consistent with our observation that overexpression tends to coincide with a complex p73 isoform profile and that high levels of endogenous p73 expression in breast cancer lines fail to induce p21, even in the absence of functional p53. Nevertheless, it is unclear why, in both scenarios, the production of the phenotypically advantageous variants would be so imprecisely regulated that they could generate counteracting or wasteful by-products. A third, rather remote interpretation of wt p73 overexpression in tumors is that p73 does have a suppressor role that might respond to other signals than DNA damage, such as oncogene activation. Overexpression could then be a compensatory, albeit ineffective, cellular response to inhibit oncogenic pathways. Finally, it is also possible that p73 overexpression is secondary to other transforming events and has no phenotypic effects on the tumor. Future work will have to be directed toward understanding whether *in vivo*, under physiological expression levels, the net effect of the activity of p73 is a positive or negative modulation of cell growth and/or differentiation. This might clarify some of the apparent paradox that currently exists between results from forced p73 overexpression *in vitro* and endogenous overexpression in primary tumors. One is tempted to speculate that at least part of the answer might lie in the distorted cellular response that could be obtained with superphysiological overexpression of a single p73 isoform, a situation that differs from p73 overexpressing tumors with lower overall levels and multiple isoforms of different biological effects.

Note Added in Proof

Steegenga *et al.* recently reported that Ad E4orf6 oncoprotein represses p73 but not p53 transactivation, suggesting that p73 protein might be inactivated by a distinct mechanism in adenoviral transformation (Steegenga *et al.*, Distinct regulation of p53 and p73 activity by adenovirus E1A, E1B, and E4orf6 proteins. Mol. Cell Biol., *19:* 3885–3894, 1999), a conclusion that is also reached by Higashino *et al*. (13). Zeng *et al*. recently reported that mdm-2 suppresses p73 function *in vivo* without promoting p73 degradation (Zeng *et al*., MDM2 suppresses p73 function without promoting p73 degradation. Mol. Cell Biol., *19:* 3257–3266, 1999).

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