A naturally occurring p73 mutation in a p73-p53 double-mutant lung cancer cell line encodes p73α protein with a dominant-negative function

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p73, a close homolog of p53 tumor suppressor, induces growth arrest and apoptosis. However, its role in cancers is controversial because of the rarity of p73 mutations, lack of tumors in p73-knockout mice, and the presence of multiple isotypes, among which ΔN isotypes inhibit the function of TA isotypes. We analyzed three naturally occurring p73 mutants found in lung cancer cell lines, NCI-H1155, DMS 92 and A427. NCI-H1155 is a cell line that has a p73 mutation [p73(G264W)] in the DNA-binding domain, as well as a p53 mutation [p53(R273H)], which is frequently found in human cancers and has a "gain-of-function" characteristic. p73α(G264W) not only lacks transactivation activity itself, but also suppressed the transactivation activity of the wild-type p73α in a dose-dependent manner, indicating that p73α(G264W) is a dominant-negative mutant. p73α(G264W) failed to suppress colony formation. We tested two other mutations, p73(Del418) in DMS 92 and p73(Del603) in A427. Both mutants retained similar levels of transactivation activity and suppression of colony formation to those of wild-type p73. The biological significance of these two mutations is unclear. In NCI-H1155 cells the coexistence of mutations that abrogate the normal functions of p73 and p53 may indicate that each mutation confers an additive growth advantage upon the cells. (Cancer Sci 2003; 94: 718–724)

p73 is a close relative of p53, a prototypic tumor suppressor. Relatives of p53 were long considered improbable, but p73 was coincidentally cloned in an experiment to clone other genes.[1)] Another relative, p63, was then cloned, based on the homology with p73.[2, 3)] p73, p63, and p53 form the *p53* gene family, and from the results of the human genome project, they are the only members.[4)] They share three functional domains, i.e., the transactivation domain, the DNA-binding domain and the oligomerization domain.[1–3, 5)] Within the family, p73 and p63 are the most closely related.[6)] They are differentiated from p53 by a higher level of amino-acid sequence homology, by their more conserved genomic structures,[2, 7)] by the presence of multiple splicing variants,[1–3, 8, 9)] and by the presence of transactivation domain-deficient forms (ΔN isotypes) that are transcribed from independent promoters.[2, 3, 10, 11)]

p73 activates the same target protein as p53,[1, 5)] although it is somewhat different in both potency and specificity.[12)] p73 cooperates with c-abl to induce apoptosis following DNA damage,[13–15)] and is involved in the E2F-1 induced, p53-independent apoptosis pathway.[16–18)] These observations support the role of p73 as a tumor suppressor. However, its role is still controversial because (i) mutation of p73 is rarely found in cancers,[19–22)] (ii) p73-knockout mice are not prone to neoplasms,[10)] (iii) in several types of cancers, a higher level of expression of wild-type p73 protein is observed than in normal tissue counterparts[23–27)] and (iv) isotypes that lack the transactivation domain (ΔN isotypes) act as dominant-negative proteins that suppress transactivation activity of both p73 with the transactivation domain (TA isotypes) and p53.[11, 28)] It has recently been reported that the pro-apoptotic function of p53 following DNA damage is dependent on both p73 and p63 function,[29)] suggesting a complicated interdependence among the p53 family member proteins.

Accumulated knowledge about cancer has resulted in general acceptance of the clonal expansion theory of carcinogenesis, i.e., gene mutations or alterations in gene expression patterns produce cells with a selective growth advantage. Repetitions of this step ultimately yield a full-blown cancer with the ability to expand autonomously. Mutated genes found in cancer cells may thus be associated with a growth advantage of the cells, allowing them to increase their relative proportion over successive cell divisions. Investigation of the naturally occurring p73 mutants may help to elucidate the role of p73 in cancers.

We have previously reported three p73 mutants in lung cancer cell lines that have known p53 mutations. The cell lines and corresponding mutations are as follows: NCI-H1155 has G264W for p73 and R273H for p53, DMS 92 has Del 418 for p73 and M237I for p53, and A427 has Del603 for p73 and p53 is wild-type.[30)] p63 is wild-type in all three cell lines.[7)] The NCI-H1155 cell line was of particular interest because both its p73 and p53 DNA-binding domains have point mutations. In this study, we performed functional analysis of the p73 mutants seen in these cell lines. Our results should lead to a better understanding of the role of p73 in carcinogenesis.

Materials and Methods

Cell culture. COS-7, a SV40-transformed monkey kidney fibroblast cell line, and SAOS-2, a human osteogenic sarcoma cell line, were obtained from the Cell Resource Center for Biomedical Research, Tohoku University. NCI-H1155 and A427, non-small cell lung cancer cell lines, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). DMS 92, a small cell lung cancer cell line, was obtained from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). Cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (FCS).

DNA sequencing. Genomic DNA (50 ng) isolated from cell lines was amplified by PCR using *Taq* DNA polymerase (TaKaRa Shuzo, Kyoto). The primers used were 5′-ACCTCTTAACCTGTGGCTTCTC-3′ and 5′-GGAGAGGAGCTGGTGTTGTTG-3′ for exon 8 of the *p53* gene, and 5′-TTGGGGCTGCGTGCTGATGCTA-3′ and 5′-CCTGCAGGTCTCCATGACAGCT-3′ for exon 6 of the *p73* gene. The resultant PCR fragments were sequenced by an automatic DNA sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA).

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Plasmids. Expression plasmids pcDNA-p73α and pcDNA-p73β that have hemagglutinin (HA)-tagged, wild-type p73α and p73β cDNAs in pcDNA3 (Invitrogen, Carlsbad, CA) were gifts from Dr. Gerry Melino (Department of Experimental Medicine and Biochemical Sciences, Rome, Italy).[8] An MDM2 promoter-luciferase construct, MDM2-Luc, was a gift from Dr. Moshe Oren (The Weizmann Institute, Rehovot, Israel).[31] p21Waf1 promoter-luciferase and Bax promoter-luciferase constructs, p21Waf1-Luc and Bax-Luc, were gifts from Dr. Shuntaro Ikawa (Institute of Development, Aging and Cancer, Tohoku University, Sendai).[11] A Renilla-luciferase expression plasmid, pRL-CMV, was purchased from Promega, Madison, WI. Mutant p73 cDNAs corresponding to the mutations in NCI-H1155 cells (G264W), in DMS 92 cells (Del418), and in A427 cells (Del603) were made by introducing individual mutations into wild-type p73 cDNAs by the megaprimer PCR method.[32] The resultant constructs were named pcDNA3-p73α(G264W), pcDNA3-p73β(G264W), pcDNA3-p73α(Del418), pcDNA3-p73β(Del418), and pcDNA3-p73α(Del603). These plasmids have the following common structure: cytomegalovirus immediate-early gene promoter (CMV), bacteriophage T7 RNA polymerase promoter (T7), a cDNA, and the polyadenylation signal of the bovine growth hormone gene (*BGH pA*).

DNA transfection. COS cells were seeded at $2\times10^4/cm^2$ surface area into 6-well plates (for western blotting) or into chamber slides (for subcellular localization) 16 h before transfection. SAOS-2 cells (6×10^4 cells/well) were seeded at $3\times10^4/cm^2$ surface area into 24-well plates (for luciferase assays) or into 6-well plates (for colony formation assays) 16 h before transfection. The transfection cocktail was made by mixing plasmids (3.5 μg unless otherwise specified) and Lipofectamine2000 (Invitrogen: 5 μl) in 160 μl of DMEM for 20 min, and diluting the mixture with 840 μl of DMEM containing 10% FCS to make a total volume of 1 ml. The transfection was performed by replacing culture medium with transfection cocktail (300 μl/cm^2 surface area). After 6 h, the transfection cocktail was replaced with DMEM containing 10% FCS.

Western blotting. The cell extract was prepared 24 h after transfection, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride filter. The filter was serially treated with a p73 monoclonal antibody and horseradish peroxidase-conjugated anti-mouse antibody. The p73-specific bands were visualized using an ECL kit (Amersham Bioscience, Uppsala, Sweden). Antibodies used were anti-p73α (Ab-1: Oncogene Science, Cambridge, MA), or anti-p73β (Ab-3: Oncogene Science).

Subcellular localization. COS cells were grown on chamber slides (Nunc, Naperville, IL) and transfected with the indicated vectors. After 24 h, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) at 4°C for 1 h, and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min. After having been blocked with 1% bovine serum albumin (BSA) in PBS, the slides were incubated with an anti-HA monoclonal antibody (12CA5 diluted 1:200: Roche Diagnostic, Basel, Switzerland) at 37°C for 1 h, then with rhodamine-conjugated goat anti-mouse antibody (diluted 1:200: Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Specimens were examined under a fluorescence microscope.

Luciferase assay. SAOS-2 cells were transfected with one of the p73 expression vectors (1.6 μg/ml transfection cocktail), one of the reporter vectors (p21Waf1-Luc, MDM2-Luc or Bax-Luc) (1.6 μg/ml cocktail), and pRL-CMV (160 ng/ml cocktail). After 24 h the luciferase activity in the cell lysate was measured using the dual luciferase reporter assay kit (Promega). Relative luciferase activity (RLA) was calculated by normalizing luciferase activity with respect to Renilla luciferase activity. To test the dominant-negative function, increasing amounts of the pcDNA-p73α(G264W) or pcDNA-p73β(G264W) (0–2.5 μg/ml transfection cocktail) and a constant amount of pcDNA-p73α or pcDNA-p73β (320 ng/ml cocktail), together with one of the reporter vectors (p21Waf1-Luc, MDM2-Luc or Bax-Luc) (320 ng/ml cocktail) were transfected. The total amount of transfected DNA was kept constant (3.2 μg/ml cocktail) by adding empty pcDNA3 vector.

***In vitro* translation and electrophoretic mobility shift assay (EMSA).** *In vitro* translation was done using the TNT translation system (Promega) according to the manufacturer's protocol. In EMSA, a 27-mer oligonucleotide corresponding to the p53 DNA-binding site in the *GADD45* gene (5′-TACAGAACATGTCTAAGCATGCTGGGG-3′)[33] and its complementary oligonucleotide were radiolabeled with [γ-^{32}P]ATP (Amersham Bioscience) using T4 polynucleotide kinase (Promega), and then annealed to make a double-stranded DNA probe. *In vitro* translation products were mixed with the probe in the binding

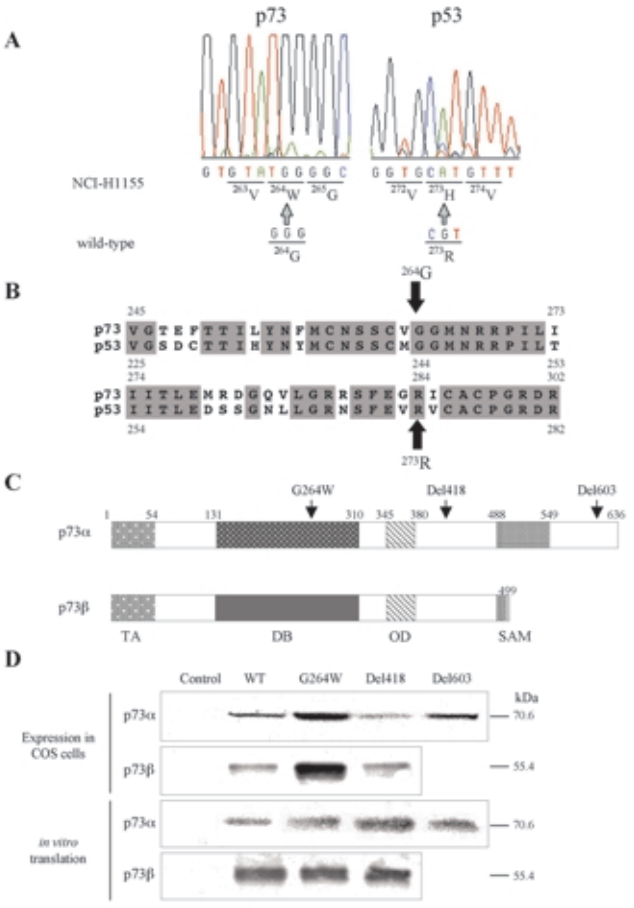

Fig. 1. Materials used in this study. (A) An example of nucleotide sequencing of *p53* and *p73* genes in the cell lines. NCI-H1155 has mutations in both the *p73* and the *p53* genes. (B) Nucleotide sequences of p73 and p53 around the mutations in NCI-H1155 cells. Amino-acid sequences are highly conserved. (C) Schematic presentation of the p73 variants (α and β) and the positions of mutations seen in the cell lines (arrows). The N-terminal transactivation domain (TA; residues 1–54), the site-specific DNA-binding domain (DB; residues 131–310), the oligomerization domain (OD; residues 345–380) and the SAM domain (SAM; residues 484–549)[55, 56] are indicated by different gray boxes. (D) Expression of p73 proteins and their mutants. Upper panels: COS cells were transiently transfected with the individual plasmids. The specific bands in the whole-cell lysate were detected by anti-p73α or p73β monoclonal antibodies. WT, wild-type; kDa, kilodaltons. Lower panels: synthesis of p73 proteins and their mutants by *in vitro* translation. The protein products were detected by western blotting.

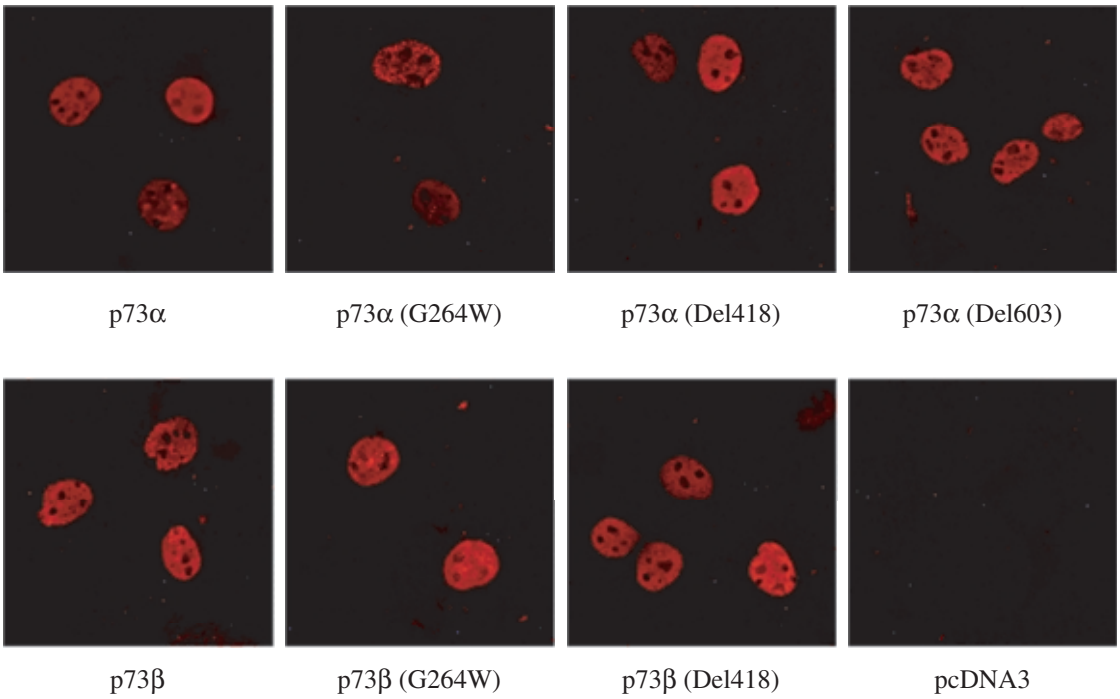

Fig. 2. Subcellular localization of p73 proteins. COS cells were transfected with one of the p73 expression plasmids. After 24 h exogenous proteins were detected with anti-HA monoclonal antibody and with rhodamine-conjugated secondary antibody, and observed under a fluorescence microscope.

buffer (20% glycerol, 5 m*M* $MgCl_2$, 2.5 m*M* EDTA, 250 m*M* NaCl, 0.25 mg/ml poly dI-dC (deoxyinosinic acid-deoxycytidylic acid), 50 m*M* Tris-HCl (pH 7.5), 2.5 m*M* dithiothreitol (DTT)), and run on a 6% DNA retardation gel (Invitrogen) in 0.5× TBE (Tris-borate EDTA) buffer at room temperature. The gel was dried and the bands were visualized with the BAS 2000 system (Fuji Film, Tokyo).

Colony formation assay. SAOS-2 cells (2×10^5 cells/well) were transfected in 6-well plates with 4 μg per well of one of the p73 expression plasmids, the mutant constructs or the empty vector control (for a total of 8 constructs), using Lipofectamine2000. Drug-resistant colonies were selected in DMEM containing 600 μg/ml of G418 for 2 weeks. The G418-resistant colonies were stained with Giemsa's solution and the numbers of the colonies were counted. To investigate the dominant-negative effect of pcDNA-p73α(G264W) in colony formation assay, pcDNA-p73α and pcDNA-p73α(G264W) were transfected (0 μg and 0 μg for a negative control, 0.25 μg and 0 μg for the effect of pcDNA-p73α, 0.25 μg and 1.5 μg for the effect of pcDNA-p73α plus pcDNA-p73α(G264W), and 0 μg and 1.5 μg for the effect of pcDNA-p73α(G264W)), together with 0.5 μg of pcDNA3. The total amount of transfected DNA was adjusted to 2 μg for each transfection by adding pBluescript (Stratagene, La Jolla, CA) as a carrier DNA.

Data analysis. The significance of differences in mean values was tested using Student's unpaired, two-tailed *t* test.

Results

Construction of the expression plasmids for the mutant p73s. First, the previously reported mutations in the cell lines[30] were confirmed (Fig. 1A). NCI-H1155 has p73 and p53 mutations, both of which are located in the DNA-binding domains (Fig 1B). We then constructed expression plasmids encoding HA epitope-tagged p73 mutants corresponding to those seen in the cell lines (Fig. 1C). After transfection into COS cells or in *in vitro* translation experiments, the plasmids produced proteins with the expected sizes (Fig. 1D).

Subcellular localization of the mutant p73 proteins. The p73 expression plasmids (pcDNA-p73α, pcDNA-p73β, and their mutants) or empty plasmid (pcDNA3) were transiently transfected into COS cells and the subcellular localization of the mutant proteins was investigated. Mutant p73 proteins were detected only in the nucleus (Fig. 2), indicating that the subcellular localization is not altered by the mutations G264W, Del418 or Del603.

Transactivation activity of the mutant p73 proteins. The effects of individual p73 mutations on transactivation activity were investigated by means of luciferase assay. Here, pcDNA-p73α, pcDNA-p73β or their mutants were co-transfected with a p53-responsive reporter construct (p21Waf1-Luc, MDM2-Luc or Bax-Luc) into SAOS-2 cells (a p53-deficient human osteogenic sarcoma cell line), and the luciferase activity was measured. Both pcDNA-p73α and pcDNA-p73β transactivate all promoters (Fig. 3). The G264W mutation abolishes the activity, except for the combination of pcDNA-p73β(G264W) and MDM2-Luc, which retains partial activity. The G264W mutation did not affect the nuclear accumulation of p73 (Fig. 2), indicating that the change in the transactivation activity is due to a change in their action in the nucleus. The two other mutations, Del418 and Del603, did not affect the activity.

DNA-binding activity of the p73 mutants. The DNA-binding activity of p73 proteins was studied by means of EMSA. A radiolabeled probe with a p53-binding sequence was incubated with each of the *in vitro*-translated p73s, and the protein-DNA complexes were resolved on a gel. Wild-type p73α and p73β bind to the p53-binding sequence (Fig. 4), as do mutants Del418 and Del603. However, the G246W mutation abolishes the DNA-binding activity of p73, thereby abolishing the transactivation activity.

Effect of G246W mutants on wild-type p73 function. Some p53 mutants have been shown to be dominant-negative mutants,

i.e., they suppress the function of the wild-type p53 protein. To test whether G246W is a dominant-negative mutant, increasing amounts of mutant p73 plasmids (pcDNA-p73α(G264W) or pcDNA-p73β(G264W)) were co-transfected with constant amounts of wild-type plasmids (pcDNA-p73α or pcDNA-p73β), and the changes in transactivation activity were measured by means of the luciferase activity. As shown in Fig. 5, pcDNA-p73α(G264W) suppresses pcDNA-p73α in a dose-dependent manner, indicating that p73α(G264W) is a dominant-negative mutant. pcDNA-p73β(G264W) enhances the luciferase activity at ratios of 1:1 and/or 1:3, but suppressed it as the ratio increases, showing that pcDNA-p73β(G264W) has a dominant-negative effect when transfected at a large molar ratio. The reason for increased activity at ratios of 1:1 or 1:3 should be investigated.

Colony formation assay. The effect of the p73 mutations on cell growth was examined by colony formation assay. Individual p73 expression plasmids or pcDNA3 (negative control)

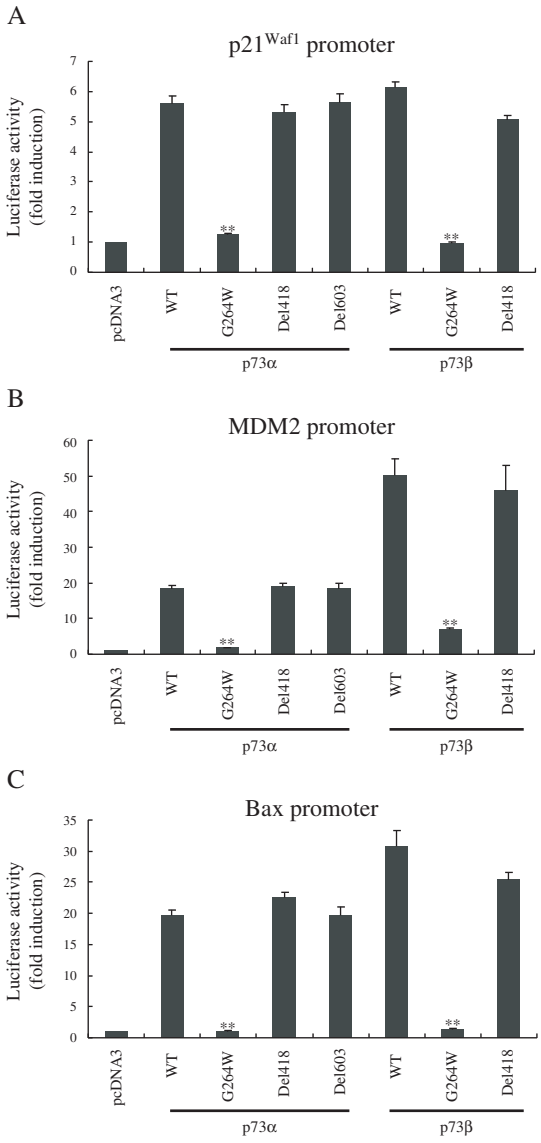

Fig. 3. Transactivation activity of wild-type and mutant p73s. SAOS-2 cells were transiently transfected with one of the p73 expression plasmids (pcDNA-p73α, pcDNA-p73β, and their mutants) or an empty plasmid (pcDNA3) together with one of the reporter plasmids (p21Waf1-Luc, MDM2-Luc or Bax-Luc). Luciferase activity is shown in an arbitrary unit. The means+the standard deviations from triplicate experiments are shown. Significant differences compared with the values in the wild-type are marked by asterisks (** $P<0.01$).

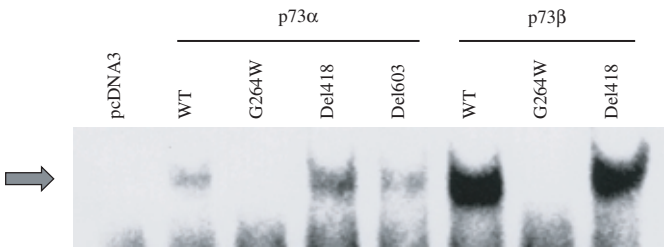

Fig. 4. Electrophoretic mobility shift assay (EMSA). A ^{32}P-radiolabeled probe that has a p53-binding sequence was incubated with *in vitro* translation products of wild-type p73α, p73β or their mutants. The arrow indicates the p73 protein-oligonucleotide complex.

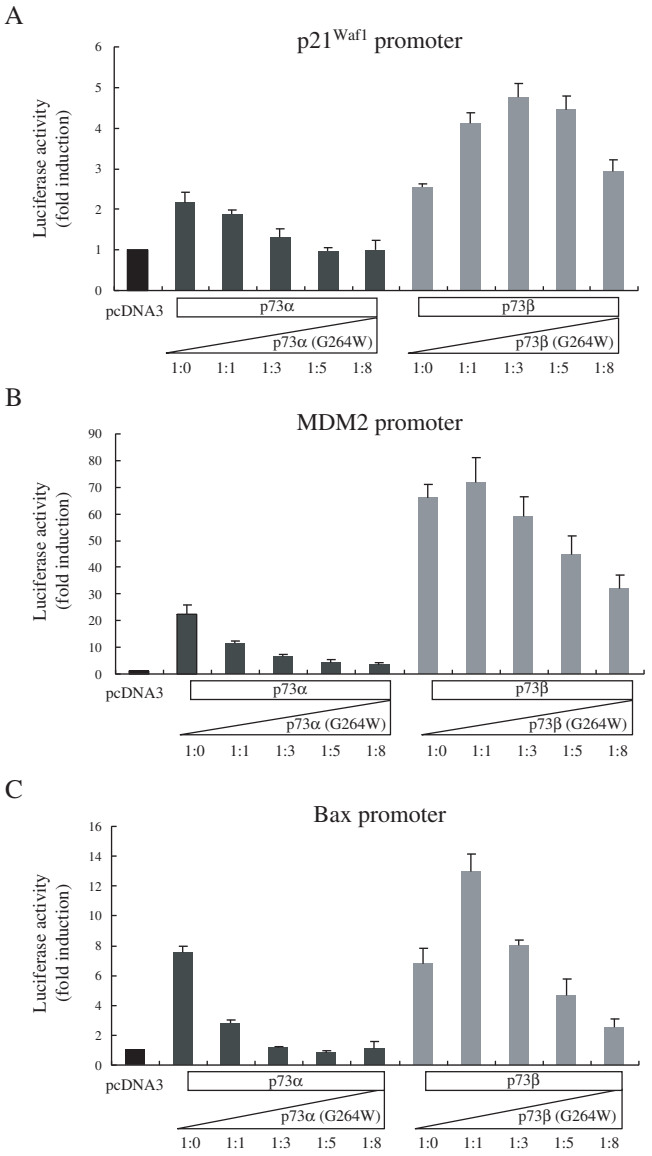

Fig. 5. Effect of mutant G264W on the transcriptional activity of wild-type p73s. SAOS-2 cells were transfected with increasing amounts of pcDNA-p73α(G264W) or pcDNA-p73β(G264W) and a constant amount of either pcDNA-p73α or pcDNA-p73β together with one of the reporter plasmids (p21Waf1-Luc, MDM2-Luc or Bax-Luc). Luciferase activity is shown in an arbitrary unit. The means+standard deviations from triplicate experiments are shown.

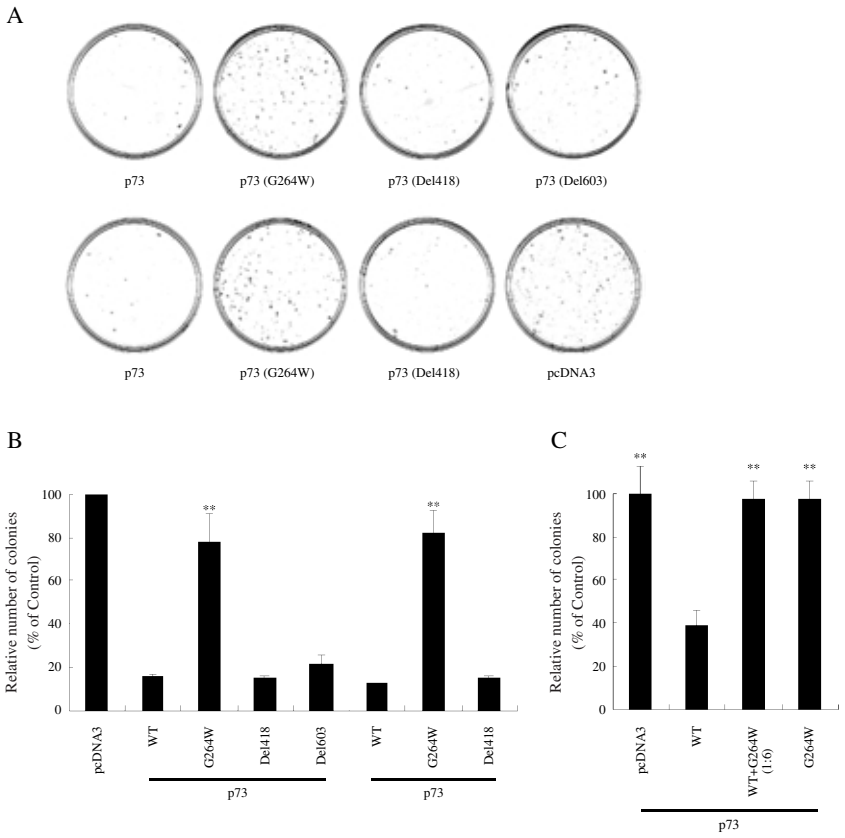

Fig. 6. Colony formation assay. (A) SAOS-2 cells were transfected with one of the p73 expression plasmids (pcDNA-p73α, pcDNA-p73β or their mutants) to study the effect of each p73 cDNA on colony formation. After 2 weeks of G418 selection, stable transformants were stained with Giemsa's solution and photographed. (B) The numbers of drug-resistant colonies are shown in a bar graph. The means+standard deviations from triplicate experiments are shown. Significant differences compared with the values of the wild-type are marked by asterisks (** $P<0.01$). (C) A dominant-negative effect of p73α(G264W) on colony formation. An empty plasmid (pcDNA3), a wild-type plasmid (WT: pcDNA-p73α), a G264W mutant plasmid (pcDNA-p73α(G264W)), and mixture of pcDNA-p73α and pcDNA-p73α(G264W) at a ratio of 1 to 6 were transfected. The numbers of colonies are graphed as in (B). Significant differences compared with the values of wild-types are marked by asterisks (** $P<0.01$).

were transfected into SAOS-2 cells. Then, G418-resistant colonies were selected and their numbers were counted. The transfection of the wild-type p73 plasmids (pcDNA-p73α or pcDNA-p73β) markedly suppressed colony formation (Fig. 6, A and B). p73α(Del418), p73α(Del603) and p73β(Del418) were as effective as wild-type p73s in reducing the numbers of colonies. In contrast, a G264W mutant pcDNA-p73α(G264W) abolished this effect and produced numbers of colonies similar to those of pcDNA3, showing that co-existence of p73α(G264W) abrogated the colony-suppressive function of p73α (Fig. 6C). This demonstrates a dominant-negative effect of p73α(G264W) in the assay.

Discussion

Epidemiological analysis has shown that, although the number is small, mutations in the *p73* gene are found in cancers.[30, 34–38] In hematological malignancies hypermethylation of the p73 promoter is found.[39, 40] Biochemically, p73 is involved in the E2F-1 induced, p53-independent apoptosis pathway,[16–18] and the pro-apoptotic function of p53 after DNA damage has been reported to depend on p73 and p63 function.[29] So, although *p73* is not a typical tumor suppressor gene fitting the classical Knudson's two-hit model,[41] abnormalities in p73 function may have some role in cancer development.

In NCI-H1155 cells, the p73 and p53 mutations are located in the respective DNA-binding domains (Fig. 1B). Mutations in the *p53* gene are observed in about 50% of human cancers and the positions of mutations are clustered in the DNA-binding domain.[42] p73 codon 264 corresponds to p53 codon 244, for which dominant-negative mutations (G244D and G244S) in human cancers have been described.[43] In this study, we found that p73 codon 264 (G264W) is also a dominant-negative mutation. Codon 273 of p53 is one of the hot spots, and the Arg to His amino-acid change (R273H) comprises about 50% of the codon 273 mutations (474 out of 1016 occurrences reported in the IARC TP53 Mutation Database R6 release[44]). R273H is a gain-of-function mutation[45] that enhances tumorigenicity in nude mice,[46] enhances the plating efficiency of the cells in soft agar,[46] up-regulates the expression of epidermal growth factor receptor (EGFR),[47] proliferating cell nuclear antigen (PCNA)[48] and c-myc,[49] and confers cisplatin resistance upon the cells.[50] Therefore, normal functioning of both p73 and p53 is defective in NCI-1155.

Mutually exclusive mutation patterns are observed in two molecules that are located in tandem in the same cancer-related signaling pathway, e.g., p16 and RB,[51] and TGF-β type II receptor and Smad4.[52] Instead, mutations in two genes coexist and cells with both mutations are positively selected when each mutation has an additive effect on cancer development.[53] The coexistence of *p73* and *p53* genes in NCI-H1155 may have an additive effect, and thus may result in a cell line with p73-p53 double mutations.

We did not find functional aberrations in either the Del418 or Del603 mutant. These mutations delete one and three amino acids, respectively, from the COOH-terminal side of the oligo-

merization domain. Takada *et al*. found that two mutations in that region (P405R and P425L) impair the transactivation activity of the COOH-terminal domain.[54] We found no effect of Del418 or Del603 on the transactivation activity. Whether Del418 or Del603 has biological significance needs to be examined.

In this study, we performed functional analyses of three p73 mutants observed in lung cancer cell lines, and found one to be a dominant-negative mutation. Studying the role of p73 in cancers is difficult because of the presence of multiple isotypes, and because of the complicated interactions of other p53 family member proteins. The results we report here provide valuable information and should encourage further study in this field.

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1. Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, Minty A, Chalon P, Lelias JM, Dumont X, Ferrara P, McKeon F, Caput D. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* 1997; **90**: 809–19.
2. Osada M, Ohba M, Kawahara C, Ishioka C, Kanamaru R, Katoh I, Ikawa Y, Nimura Y, Nakagawara A, Obinata M, Ikawa S. Cloning and functional analysis of human p51, which structurally and functionally resembles p53. *Nat Med* 1998; **4**: 839–43.
3. Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, Andrews NC, Caput D, McKeon F. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell* 1998; **2**: 305–16.
4. Futreal PA, Kasprzyk A, Birney E, Mullikin JC, Wooster R, Stratton MR. Cancer and genomics. *Nature* 2001; **409**: 850–2.
5. Jost CA, Marin MC, Kaelin WG Jr. p73 is a simian [correction of human] p53-related protein that can induce apoptosis. *Nature* 1997; **389**: 191–4.
6. Strano S, Rossi M, Fontemaggi G, Munarriz E, Soddu S, Sacchi A, Blandino G. From p63 to p53 across p73. *FEBS Lett* 2001; **490**: 163–70.
7. Hagiwara K, McMenamin MG, Miura K, Harris CC. Mutational analysis of the p63/p73L/p51/p40/CUSP/KET gene in human cancer cell lines using intronic primers. *Cancer Res* 1999; **59**: 4165–9.
8. De Laurenzi V, Costanzo A, Barcaroli D, Terrinoni A, Falco M, Annicchiarico-Petruzzelli M, Levrero M, Melino G. Two new p73 splice variants, gamma and delta, with different transcriptional activity. *J Exp Med* 1998; **188**: 1763–8.
9. Tani M, Shimizu K, Kawahara C, Kohno T, Ishimoto O, Ikawa S, Yokota J. Mutation and expression of the p51 gene in human lung cancer. *Neoplasia* 1999; **1**: 71–9.
10. Yang A, Walker N, Bronson R, Kaghad M. Oosterwegel M, Bonnin J, Vagner C, Bonnet H, Dikkes P, Sharpe A, McKeon F, Caput D. p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature* 2000; **404**: 99–103.
11. Ishimoto O, Kawahara C, Enjo K, Obinata M, Nukiwa T, Ikawa S. Possible oncogenic potential of DeltaNp73: a newly identified isoform of human p73. *Cancer Res* 2002; **62**: 636–41.
12. Lee CW, La NB. Thangue promotor specificity and stability control of the p53-related protein p73. *Oncogene* 1999; **18**: 4171–81.
13. Agami R, Blandino G, Oren M, Shaul Y. Interaction of c-Abl and p73alpha and their collaboration to induce apoptosis. *Nature* 1999; **399**: 809–13.
14. Gong JG, Costanzo A, Yang HQ, Melino G, Kaelin WG Jr, Levrero M, Wang JY. The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. *Nature* 1999; **399**: 806–9.
15. Yuan ZM, Shioya H, Ishiko T, Sun X, Gu J, Huang YY, Lu H, Kharbanda S, Weichselbaum R, Kufe D. p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage. *Nature* 1999; **399**: 814–7.
16. Irwin M, Marin MC, Phillips AC, Seelan RS, Smith DI, Liu W, Flores ER, Tsai KY, Jacks T, Vousden KH, Kaelin WG Jr. Role for the p53 homologue p73 in E2F-1-induced apoptosis. *Nature* 2000; **407**: 645–8.
17. Lissy NA, Davis PK, Irwin M, Kaelin WG, Dowdy SF. A common E2F-1 and p73 pathway mediates cell death induced by TCR activation. *Nature* 2000; **407**: 642–5.
18. Stiewe T, Putzer BM. Role of the p53-homologue p73 in E2F1-induced apoptosis. *Nat Genet* 2000; **26**: 464–9.
19. Mai M, Yokomizo A, Qian C, Yang P, Tindall DJ, Smith DI, Liu W. Activation of p73 silent allele in lung cancer. *Cancer Res* 1998; **58**: 2347–9.
20. Nimura Y, Mihara M, Ichimiya S, Sakiyama S, Seki N, Ohira M, Nomura N, Fujimori M, Adachi W, Amano J, He M, Ping YM, Nakagawara A. p73, a gene related to p53, is not mutated in esophageal carcinomas. *Int J Cancer* 1998; **78**: 437–40.
21. Sunahara M, Ichimiya S, Nimura Y, Takada N, Sakiyama S, Sato Y, Todo S, Adachi W, Amano J, Nakagawara A. Mutational analysis of the p73 gene localized at chromosome 1p36.3 in colorectal carcinomas. *Int J Oncol* 1998; **13**: 319–23.
22. Takahashi H, Ichimiya S, Nimura Y, Watanabe M, Furusato M, Wakui S, Yatani R, Aizawa S, Nakagawara A. Mutation, allelotyping, and transcription analyses of the p73 gene in prostatic carcinoma. *Cancer Res* 1998; **58**: 2076–7.
23. Kovalev S, Marchenko N, Swendeman S, LaQuaglia M, Moll UM. Expression level, allelic origin, and mutation analysis of the p73 gene in neuroblastoma tumors and cell lines. *Cell Growth Differ* 1998; **9**: 897–903.
24. Chi SG, Chang SG, Lee SJ, Lee CH, Kim JI, Park JH. Elevated and biallelic expression of p73 is associated with progression of human bladder cancer. *Cancer Res* 1999; **59**: 2791–3.
25. Imyanitov EN, Birrell GW, Filippovich I, Sorokina N, Arnold J, Mould MA, Wright K, Walsh M, Mok SC, Lavin MF, Chenevix-Trench G, Khanna KK. Frequent loss of heterozygosity at 1p36 in ovarian adenocarcinomas but the gene encoding p73 is unlikely to be the target. *Oncogene* 1999; **18**: 4640–2.
26. Tannapfel A, Wasner M, Krause K, Geissler F, Katalinic A, Hauss J, Mossner J, Engeland K, Wittekind C. Expression of p73 and its relation to histopathology and prognosis in hepatocellular carcinoma. *J Natl Cancer Inst* 1999; **91**: 1154–8.
27. Zaika AI, Kovalev S, Marchenko ND, Moll UM. Overexpression of the wild type p73 gene in breast cancer tissues and cell lines. *Cancer Res* 1999; **59**: 3257–63.
28. Kartasheva NN, Contente A, T.Lenz-Stoppler C, Roth J, Dobbelstein M. p53 induces the expression of its antagonist p73 Delta N, establishing an autoregulatory feedback loop. *Oncogene* 2002; **21**: 4715–27.
29. Flores ER, Tsai KY, Crowley D, Sengupta S, Yang A, McKeon F, Jacks T. p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. *Nature* 2002; **416**: 560–4.
30. Yoshikawa H, Nagashima M, Khan MA, McMenamin MG, Hagiwara K, Harris CC. Mutational analysis of p73 and p53 in human cancer cell lines. *Oncogene* 1999; **18**: 3415–21.
31. Zauberman A, Barak Y, Ragimov N, Levy N, Oren M. Sequence-specific DNA binding by p53: identification of target sites and lack of binding to p53-MDM2 complexes. *EMBO J* 1993; **12**: 2799–808.
32. Ke SH, Madison EL. Rapid and efficient site-directed mutagenesis by single-tube 'megaprimer' PCR method. *Nucleic Acids Res* 1997; **25**: 3371–2.
33. Hollander MC, Alamo I, Jackman J, Wang MG, McBride OW, Fornace AJ Jr. Analysis of the mammalian gadd45 gene and its response to DNA damage. *J Biol Chem* 1993; **268**: 24385–93.
34. Alonso ME, Bello MJ, Gonzalez-Gomez P, Lomas J, Arjona D, de Campos JM, Kusak ME, Sarasa JL, Isla A, Rey JA. Mutation analysis of the p73 gene in nonastrocytic brain tumours. *Br J Cancer* 2001; **85**: 204–8.
35. El-Naggar AK, Lai S, Clayman GL, Mims B, Lippman SM, Coombes M, Luna MA Lozano G. p73 gene alterations and expression in primary oral and laryngeal squamous carcinomas. *Carcinogenesis* 2001; **22**: 729–35.
36. Han S, Semba S, Abe T, Makino N, Furukawa T, Fukushige S, Takahashi H, Sakurada A, Sato M, Shiiba K, Matsuno S, Nimura Y, Nakagawara A, Horii A. Infrequent somatic mutations of the p73 gene in various human cancers. *Eur J Surg Oncol* 1999; **25**: 194–8.
37. Lomas J, Bello MJ, Arjona D, Gonzalez-Gomez P, Alonso ME, de Campos JM, Vaquero J, Ruiz-Barnes P, Sarasa JL, Casartelli C, Rey JA. Analysis of p73 gene in meningiomas with deletion at 1p. *Cancer Genet Cytogenet* 2001; **129**: 88–91.
38. Ichimiya S, Nimura Y, Kageyama H, Takada N, Sunahara M, Shishikura T, Nakamura Y, Sakiyama S, Seki N, Ohira M, Kaneko Y, McKeon F, Caput D, Nakagawara A. p73 at chromosome 1p36.3 is lost in advanced stage neuroblastoma but its mutation is infrequent. *Oncogene* 1999; **18**: 1061–6.
39. Corn PG, Kuerbitz SJ, van Noesel MM, Esteller M, Compitello N, Baylin SB, Herman JG. Transcriptional silencing of the p73 gene in acute lymphoblastic leukemia and Burkitt's lymphoma is associated with 5′ CpG island methylation. *Cancer Res* 1999; **59**: 3352–6.
40. Kawano S, Miller CW, Gombart AF, Bartram CR, Matsuo Y, Asou H, Sakashita A, Said J, Tatsumi E, Koeffler HP. Loss of p73 gene expression in leukemias/lymphomas due to hypermethylation. *Blood* 1999; **94**: 1113–20.
41. Knudson AGJ. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci USA* 1971; **68**: 820–3.
42. Hollstein M, Moeckel G, Hergenhahn M, Spiegelhalder B, Keil M, Werle-Schneider G, Bartsch H, Brickmann J. On the origins of tumor muta-

tions in cancer genes: insights from the p53 gene. *Mut at Res* 1998; **405**: 145–54.
43. Brachmann RK, Vidal M, Boeke JD. Dominant-negative p53 mutations selected in yeast hit cancer hot spots. *Proc Natl Acad Sci USA* 1996; **93**: 4091–5.
44. Olivier M, Eeles R, Hollstein M, Khan MA, Harris CC, Hainaut P. The IARC TP53 database: new online mutation analysis and recommendations to users. *Hum Mutat* 2002; **19**: 607–14.
45. van Oijen MG, Slootweg PJ. Gain-of-function mutations in the tumor suppressor gene p53. *Clin Cancer Res* 2000; **6**: 2138–45.
46. Dittmer D, Pati S, Zambetti G, Chu S, Teresky AK, Moore M, Finlay C, Levine AJ. Gain of function mutations in p53. *Nat Genet* 1993; **4**: 42–5.
47. Ludes-Meyers JH, Subler MA, Shivakumar CV, Munoz RM, Jiang P, Bigger JE, Brown DR, Deb SP, Deb S. Transcriptional activation of the human epidermal growth factor receptor promoter by human p53. *Mol Cell Biol* 1996; **16**: 6009–19.
48. Kawamura M, Yamashita T, Segawa K, Kaneuchi M, Shindoh M, Fujinaga K. The 273rd codon mutants of p53 show growth modulation activities not correlated with p53-specific transactivation activity. *Oncogene* 1996; **12**: 2361–7.
49. Frazier MW, He X, Wang J, Gu Z, Cleveland JL, Zambetti GP. Activation of c-myc gene expression by tumor-derived p53 mutant requires a discrete C-terminal domain. *Mol Cell Biol* 1998; **18**: 3735–43.
50. Blandino G, Levine AJ, Oren M. Mutant p53 gain of function: differential effects of different p53 mutant on resistance of cultured cells to chemotherapy. *Oncogene* 1999; **18**: 477–85.
51. Okamoto A, Demetrick DJ, Spillare EA, Hagiwara K, Hussain SP, Bennett WP, Forrester K, Gerwin B, Serrano M, Beach DH, Harris CC. Mutations and altered expression of p16INK4 in human cancer. *Proc Natl Acad Sci USA* 1994; **91**: 11045–9.
52. Schutte M, Hruban RH, Hedrick L, Cho KR, Nadasdy GM, Weinstein CL, Bova GS, Isaacs WB, Cairns P, Nawroz H, Sidransky D, Casero RA Jr, Meltzer PS, Hahn SA, Kern SE. DPC4 gene in various tumor types. *Cancer Res* 1996; **56**: 2527–30.
53. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759–67.
54. Takada N, Ozaki T, Ichimiya S, Todo S, Nakagawara A. Identification of a transactivation activity in the COOH-terminal region of p73 which is impaired in the naturally occurring mutants found in human neuroblastomas. *Cancer Res* 1999; **59**: 2810–4.
55. Chi SW, Ayed A, Arrowsmith CH. Solution structure of a conserved C-terminal domain of p73 with structural homology to the SAM domain. *EMBO J* 1999; **18**: 4438–45.
56. Wang WK, Bycroft M, Foster NW, Buckle AM, Fersht AR, Chen YW. Structure of the C-terminal sterile alpha-motif (SAM) domain of human p73 alpha. *Acta Crystallogr D Biol Crystallogr* 2001; **57**: 545–51.