\textbf{\Delta Np63\textalpha{} Up-Regulates the \textit{Hsp70} Gene in Human Cancer}

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Abstract

\textit{HSP70}, a stress response protein, is known to be a determinant of cell death and cell transformation. We show that different isoforms of \textit{p63} have different transcriptional activities on \textit{hsp70} genes. \textit{\Delta Np63\textalpha{}} is an abundantly expressed isoform of \textit{p63}, activates \textit{(in vitro and in vivo)}, whereas T\textit{Ap63}\gamma{} down-regulates the expression of \textit{hsp70}. We further show that the transactivation domain at the NH\textsubscript{2} terminus of \textit{p63} represses, whereas the COOH terminus activates \textit{hsp70} transcription. In addition, \textit{\Delta Np63\textalpha{}} regulates transcription of the \textit{hsp70} gene through its interaction with the CCAAT binding factor and NF-Y transcription factors which are known to form a complex with the CCAAT box located in the \textit{hsp70} promoter. Moreover, \textit{\Delta Np63\textalpha{}} expression correlates with \textit{HSP70} expression in all head and neck cancer cell lines. Finally, we show colocalization of \textit{\Delta Np63\textalpha{}} and \textit{HSP70} in the epithelium and coexpression of both proteins in 41 primary head and neck cancers. Our study provides strong evidence for the physiologic association between \textit{\Delta Np63\textalpha{}} and \textit{hsp70} in human cancer, thus further supporting the oncogenic potential of \textit{\Delta Np63\textalpha{}}. (Cancer Res 2005; 65(3): 758-66)

Introduction

The \textit{p53} gene family consists of three members, \textit{p53}, \textit{p63}, and \textit{p73}. Both \textit{p63} and \textit{p73} exhibit high amino acid identity with \textit{p53} including their transactivation (25\%), DNA binding (65\%), and tetramerization domains (35\%). Unlike \textit{p53}, \textit{p63} and \textit{p73} each generate two major protein isoforms, transactivation (TA) and \textit{\Delta N}, through two different promoters and three alternative splicing sites at the 3' end, denoted by \textit{\alpha{}}, \textit{\beta{}}, and \textit{\gamma{}} (Fig. 1A; refs. 1–3). Despite their structural similarity, it is thought that \textit{p63} and \textit{p73} play quite different biological roles compared with \textit{p53}. In line with this reasoning, \textit{p53} plays a major role in tumorigenesis, whereas \textit{p63} and \textit{p73} are involved mainly in normal development. These differences notwithstanding, recent research has brought to light several instances of functional overlap amongst the \textit{p53} family members. Both \textit{p63} and \textit{p73} can bind to \textit{p53} DNA binding sites \textit{in vitro} and transcriptionally regulate common downstream target genes (2–4). In general, the TA isoforms of \textit{p63} and \textit{p73} act more like \textit{p53}, whereas the \textit{\Delta N} isoforms display a dominant-negative function with other TA isoforms and \textit{p53}, perhaps modulating the function of \textit{p53}. This observation raised the possibility that \textit{p63} and/or \textit{p73} might be involved in the same \textit{p53}-regulated pathways and the hypothesis that cell integrity might ultimately depend on the balance between all \textit{p53} gene family members.

\textit{p53} can be activated in response to malignancy-associated stress signals that in turn provoke various responses including cell cycle arrest, differentiation, senescence, DNA repair, and apoptosis (5, 6). Many of the downstream target genes of \textit{p53} are involved in the apoptosis signaling pathway, including \textit{p21}, \textit{BAX}, \textit{PUMA}, and \textit{hsp70} (7–12). \textit{HSP70} is one of the most abundant heat shock proteins (HSP) and accounts for as much as 1\% to 2\% of total cellular protein. \textit{HSP70} and other chaperones are also known to be determinants of cell death and cell transformation. The overexpression of \textit{HSP70} is associated with metastasis (13), whereas the repression of \textit{HSP70} results in the inhibition of tumor cell proliferation and the induction of apoptosis (14). \textit{HSP70} chaperone activity may also influence tumorigenesis by regulating the activity of proteins that are involved in the cell cycle machinery. The antiapoptotic function of \textit{HSP70} has prompted research into the relationship between \textit{hsp70} and the proapoptotic function of \textit{p53}. \textit{HSP70} family members participate in the cytoplasmic sequestration of wild-type (WT) \textit{p53} in cancer cells (neuroblastoma, breast cancer, colon cancer, and retinoblastoma) as well as in embryonic stem cells. In addition, \textit{p53} represses transcription from the human \textit{hsp70} promoter via a direct protein-protein interaction with a specific CCAAT binding factor (CBF; ref. 15). The adenovirus E1A and c-MYC oncogenes also bind to CBF but (as opposed to \textit{p53}) induce the expression of \textit{hsp70} (16, 17).

We recently analyzed the downstream target genes of two \textit{p63} isoforms and found that \textit{HSP70} was significantly up-regulated by \textit{\Delta Np63\textalpha{}} but not by T\textit{Ap63}\gamma{} (4). We now show that the transactivation of \textit{hsp70} by \textit{\Delta Np63\textalpha{}} is structure-related and provide evidence of the physiologic association between \textit{\Delta Np63\textalpha{}} and \textit{hsp70} in human cancer.

Materials and Methods

\textbf{Cell Culture.} Human osteosarcoma cell line Saos2 cells and head and neck cancer cell lines including O11, O12, O13, O28, and O29 were cultured in RPMI medium with 10\% bovine calf serum and 1\% penicillin-streptomycin. Cells were cultured at 37°C with 5\% \textit{CO}_{2}.

\textbf{Establishment of Inducible Cell Line and Reverse Transcription-PCR.} \textit{\Delta Np63\textalpha{}} and \textit{WTp53} Flp-in inducible Saos2 cell lines were generated according to the manufacturer’s instructions, (Invitrogen, Carlsbad, CA). Briefly, pFRT/Laczeo Flp-target site vector was transfected into Saos2 cells and the clones were screened by \gamma{}-galactosidase assay. Then \textit{\Delta Np63\textalpha{}} or \textit{WTp53} and POG 44 were cotransfected into Flp-in host cell lines and positive clones were selected with hygromycin. Total RNA was extracted using Trizol reagent (Invitrogen) at different time periods adding

\textbf{Note:} Supplementary data for this article are available at \textit{Cancer Research} Online (http://cancerres.aacrjournals.org/).

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Cancer Res 2005; 65: (3). February 1, 2005 758 www.aacrjournals.org

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1 μg/mL tetracycline to induce the expression of ΔNp63a or WTp53. Reverse transcription-PCR analysis was carried out as described previously (4).

**Plasmids.** The expression constructs for p63 including TAp63c, TAp63γ, TAp63α, ΔNp63α, ΔNp63β, and ΔNp63γ were cloned into the pRC/cytomegalovirus vector. The deletion constructs ΔNp63α and MWp63α were generated by PCR and subcloned into the pRC/cytomegalovirus vector. WT ΔNp63α was cloned into pAd84 vector and mutant ΔNp63α constructs mutants 518, 534, and 541 were made using the Quick-change kit (Stratagene, La Jolla, CA) using the WT ΔNp63α construct as template. The sequences of these primers used for generating the constructs are available on request. WT p53 and mutant p53 (Arg273His) were cloned into the pCDNA3.1 vector. All constructs were confirmed by sequencing.

**Luciferase Reporter Assay.** Plasmid DNA for transient transfection was isolated using the plasmid maxi kit (Qiagen, Valencia, CA). Saos2 cells were plated at a density of 1 × 10^5 cells/well in six-well plates and grown overnight prior to transfection. All transfections were carried out using FuGene-6 (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Each transfection experiment was done in duplicate and repeated at least thrice. For cotransfection experiments, cells received 0.5 μg of hsp70 gene promoter construct, 0.1 μg of pRL-TK Renilla luciferase vector (Promega), and 1 μg of the indicated expression plasmids and carrier DNA (empty vector). Firefly luciferase and Renilla luciferase assays were done using the Dual-Luciferase Reporter Assay System (Promega). Forty-eight hours after transfection, cells were washed with 1× PBS and harvested with 500 μL of passive lysis buffer (Promega). Cell lysates were cleared by centrifugation, and 10 μL was added to 50 μL of firefly luciferase substrate, and light units were measured in a luminometer. Renilla luciferase activities were measured in the same tube after addition of 50 μL of Stop and Glo reagent.

**Immunofluorescence Analysis.** Cos 7 cells were transiently transfected with different p63 expression constructs. After 36 hours, cells were fixed, hybridized with p63 4A4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1,000 dilution and carrier DNA (empty vector). Secondary antibodies were HSP70, HSP40 (Lab Vision, Freemont, CA), p63 4A4 (Santa Cruz Biotechnology), β-actin (Sigma), NF-YA, NF-YB (Rockland Immunocemicals, Gilbertsville, PA), NF-YC (Santa Cruz Biotechnology), and HSP-CBF. Immunoprecipitation and Immunoblotting. NETN buffer [140 mmol NaCl, 20 mmol NaPO₄ (pH 7.4), 5 mmol EDTA, 1% NP40] was used for cell lysis. For a typical immunoprecipitation reaction, 1 to 2 mg of whole cell extract in about 500 μL was incubated with 2 μg of antibody and 30 μL of antiserum or antirabbit IgG agarose gel (Sigma, St. Louis, MO) at 4°C overnight. Beads were washed thrice with 1× PBS. Protein bound to the beads was eluted by boiling in SDS gel sample buffer, separated by 10% SDS-PAGE, and transferred to a nitrocellulose membrane (Schleicher and Schuell, Riviera Beach, FL). Immunoblotting was done using the ECL kit according to the manufacturer's instructions (Amer sham, Piscataway, NJ). The primary antibodies were routinely used at a concentration of 1 μg/mL, and the horseradish peroxidase-conjugated secondary antibodies (anti-mouse or antirabbit IgG; Amersham) at a 1:1,000 dilution. The primary antibodies were HSP70, HSP40 (Lab Vision, Freemont, CA), p63 4A4 (Santa Cruz Biotechnology), β-actin (Sigma), NF-YA, NF-YB (Rockland Immunocemicals, Gilbertsville, PA), NF-YC (Santa Cruz Biotechnology), HSP-CBF.

**Electrophoretic Mobility Shift Assays.** Double-strand oligonucleotides generated from single-strand oligonucleotides were used as electrophoretic mobility shift assay probes. The sequence of the oligonucleotides corresponded to the CCAAT binding box on the HSP70 gene promoter. The sense strand oligonucleotide is, ttt ccc tgt ctt 3′ aga tca tca acg cag tcg atg. The gel shift assay and the competition experiments were carried out according to standard protocol (18). For supershift experiments, 2 μL of antibodies against NF-YA, NF-YB (Rockland Immunocemicals), NF-YC (Santa Cruz Biotechnology), and HSP-CBF were added to each binding reaction.

**Immunohistochemistry Assay.** Five-micron sections were stained using the ErVision System (Dako Cytomation, Carpinteria, USA). Briefly, sections were deparaffinized in xylene and rehydrated by graded ethanol before performing antigen retrieval using heat-induced epitope retrieval with 10 mmol citrate buffer. Endogenous peroxidase was inhibited by incubating the slides in 3% H₂O₂ for 10 minutes. Sections were then incubated with a rabbit polyclonal antibody against p40 (which recognizes all ΔNp63 isoforms but not TA p63 isoforms; Oncogene Research Products, Boston, USA) at a 1:400 dilution for 45 minutes or HSP70 (Santa Cruz Biotechnology) at a 1:25 dilution at 4°C overnight. After washing, the peroxidase labeled polymer was applied for 30 minutes. Visualization of the antibody-antigen reaction was done by incubation with diaminobenzidine.

The extent of staining was scored using a four-tiered system. A complete absence of staining was scored as 0. Staining that was weak and limited to < 20% of the tumor cells was scored as 1. Strong staining in 20% to 50% of tumor cells was scored as 2. Strong staining in over 50% of the tumor cells was scored as 3. Only nuclear staining was regarded as positive staining.

**Results**

**HSP Family Members are Up-regulated by ΔNp63a.** Based on cDNA microarray analysis, we previously found consistent up-regulation of HSP genes after transduction of ΔNp63a in an adenovirus system. There were five HSP members found to be up-regulated by ΔNp63a (but not by TAp63 isoforms), including three hsp70 and two hsp40 genes (Supplementary data A). The most prominent of these were the hsp70-2 and hsp70B genes demonstrating a 28-fold change. To explore the effects of more physiologic controlled p63 expression, we established TAp63a and ΔNp63a FLP-in inducible systems based in Saos2 cells (p53 and p63 null) and used reverse transcription-PCR and Western blot to confirm the induced expression of hsp70 and hsp40 family members (Fig. 1B and C). The change in hsp70 and hsp40 gene expression in the inducible system parallels the different expression levels of ΔNp63a but not ΔTAp63a, even though the fold change is not as dramatic as the microarray results. It is known that p53 can transcriptionally down-regulate HSP70 (15) and combined with our findings, it is likely that hsp genes are common targets for p53 family members.

To investigate whether the up-regulation of HSP70 is transcriptionally regulated by p63, we did a Luciferase reporter assay. ΔNp63a activated the hsp70B promoter more than 10-fold, whereas TAp63a transfection resulted in only 2-fold to 3-fold activation (Fig. 1D). As a control, we did the luciferase assay with both WT and mutant p53 (Arg273His). As expected, WT p53 down-regulated the hsp70B promoter more than 5-fold, whereas mutant p53 activated the hsp70B promoter approximately 2-fold (Fig. 1E), a much weaker effect than ΔNp63a on the hsp70B promoter. Transactivation of hsp70 by mutant p53 was also confirmed by Western blotting (Supplementary data B). To further confirm the effect of ΔNp63a on hsp70B, we did a kinetic study. The relative luciferase activity of hsp70B was found to increase proportionally to increasing amounts of ΔNp63a and mutant p53 (Fig. 1F). In addition, we also observed a synergistic effect of ΔNp63a and mutant p53 on hsp70 expression in Saos2 cells (Supplementary data B). These data support the notion that activation of hsp70B by ΔNp63a is through transcriptional regulation in a dose-dependent manner.

**p63 Isoforms Display Differential Transcriptional Effects on the HSP70 Promoter.** p63 produces six main isoforms from two
different promoters and differential COOH-terminal splicing (Supplementary data B). In order to explore this structural relationship further, we studied the transactivation potential of all six isoforms of p63 on Hsp70 activation. As shown in Fig. 2A, ∆Np63α displayed the highest transactivation effect on the hsp70B promoter; TAp63α, a low effect; and TAp63β, ∆Np63β, and ∆Np63γ, no transactivation effect (almost equal to empty vector). Moreover, TAp63γ actually suppressed the hsp70B promoter. In order to confirm the suppressive effect of TAp63γ, we did a kinetic analysis with WT p53. The luciferase analysis showed that TAp63γ like WT p53 suppressed the hsp70 gene (Supplementary data C). We thus showed that (a), the transcriptional regulation of p63 on the hsp70 gene was structurally dependent, (b) the NH2-terminal of p63 contained a suppression domain because the activation effect of TAp63α on hsp70 was significantly lower than that of ∆Np63α, and (c) the COOH-terminal of p63 contained an activation domain because the activation effect of all α isoforms was stronger than the β and γ isoforms.

The NH2 Terminus of TAp63 Contains a Repression Domain and the COOH Terminus of p63α Contains a Transactivation Domain. As seen in Fig. 2A, the transactivation activity of ∆Np63α was higher than TAp63α (approximately 5-fold) and the activity of ∆Np63γ was higher than TAp63γ. This observation suggested that the TA domain might specifically repress transactivation of hsp70 or affect overall protein stability leading to decreased p63 levels. To address this issue, we tested the protein stability of all isoforms of p63 by transfecting 1 μg of each construct into Saos2 cells. As shown in Fig. 2B, TAp63β, and TAp63γ were indeed unstable. However, TAp63α and ∆Np63α showed similar stable protein expression, indicating a specific contribution of the TA domain to transcriptional down-regulation of hsp70 by p63 isoforms.

To further test this hypothesis, we generated an artificial isoform of the p63 gene, termed MWp63α. The start codon of the MWp63α construct is amino acid 68 of TAp63α (Supplementary data D). We then compared the transcriptional activity of TAp63α, ∆Np63α, and MWp63α on the hsp70B gene promoter and measured their protein stability by Western blotting. TAp63α and ∆Np63α constructs generated the same level of expression, whereas MWp63 showed a more than 10-fold increase in protein level (Fig. 2C), clearly indicating that MWp63α is more stable. On the other hand, the luciferase assay with equivalent amounts of MWp63α and ∆Np63α (1 μg) showed similar level of activity on the hsp70B gene promoter (approximately 10-fold), compared with...
a 2-fold activation for TAp63α (Fig. 2D). We then decreased the MWp63α plasmid to 0.5 μg and found a similar protein expression level to 1 μg of ΔNp63α. The luciferase assay results showed that 0.5 μg MWp63α had only half the activity on the hsp70 gene promoter compared with 1 μg of MWp63α or ΔNp63α (data not shown). This data clearly indicates that protein stability is not the significant element in regulation of hsp70 by p63. Moreover, immunostaining results showed consistent nuclear localization of TAp63, ΔNp63α, and MWp63α indicating that the introduced structural alteration had no impact on subcellular localization (Supplementary data H). Taken together, our data suggest the existence of an NH2-terminal repression domain in TAp63 isoforms.

To assess the transactivation potential of the p63 COOH-terminal region, we generated a series of deletion constructs, termed hereafter Del 574, Del 548, Del 493, Del 400, Del 346, from the COOH-terminal end of ΔNp63α (Supplementary data E). Equal expression of all constructs was confirmed by Western blotting, showing that these constructs are structurally correct and that there is no protein degradation involved (Fig. 2E). We then checked the transactivation activity of all these constructs and found that the hsp70B promoter was strongly activated by WT ΔNp63α but not by any of the deletion constructs (Fig. 2F). These results clearly indicate that the minimal activation domain of ΔNp63α on the hsp70B promoter rests within the last 12 amino acids of the gene. In addition, using luciferase analysis, we reveal that the sterile α motif domain, a protein-protein interaction domain located in the COOH-terminal of both p63 and p73 proteins but not in p53, is not involved in hsp70 transcription regulation (Supplementary data F and G).

ΔNp63α Expression Correlates with HSP70 Transcription In vitro and In vivo. Because ΔNp63α and WT p53 display opposite transcriptional effects on the hsp70 promoter, we hypothesized that the balance of ΔNp63α and p53 proteins might regulate hsp70 gene expression. We cotransfected ΔNp63α and WT p53 constructs to judge the effects of both proteins on the hsp70 promoter. hsp70 activation by ΔNp63α was decreased with the addition of incremental amounts of WT p53 (Fig. 3A). Repression by WT p53 on the hsp70 gene promoter was diminished in parallel with increasing amounts of ΔNp63α in Saos-2 cells (Fig. 3B).

**Figure 2.** The regulation effect of p63 on hsp70 gene is structure-related. A, six isoforms of p63 gene differentially regulate the hsp70 gene promoter. Saos2 cells were transfected with 1 μg each of the indicated plasmid DNA along with 0.5 μg hsp70 gene promoter. Protein lysates were collected at 24 hours after transfection (all transitory transfection studies were done under the same conditions unless specifically indicated). B, Western blot analysis indicating protein stability of different isoforms of p63. β-Actin was used as a protein loading control. C, Western blot shows the size and amount of transfected proteins. Saos2 cells were transiently transfected with 1 μg of the indicated plasmids. β-Actin was used as a protein loading control. D, luciferase reporter assay showing differential reporter activity among TAp63α, MWp63α, and ΔNp63α. Saos2 cells were transfected with 1 μg each of the indicated plasmid DNA along with 0.5 μg hsp70 gene promoter. E, Western blot showing protein stability of ΔNp63α and the five deletion derivates. One microgram of each indicated expression plasmid DNA was transfected into Saos2 cells. F, transactivation potential of ΔNp63α and the five COOH-terminal deletion constructs. Different amounts of expression plasmids for ΔNp63α and its five deletion derivates (up to 1 μg) were cotransfected with HSP70 reporter plasmids in Saos2 cells.
These data indicate that in Saos2 cells (p53 and p63 null), ΔNp63α and WT p53 can mutually interfere with each other in their regulation of the hsp70 gene promoter.

To further address these opposing regulatory functions of p53 and ΔNp63α in vivo, we first investigated whether HSP70 expression correlates with p53 and ΔNp63α in five head and neck cancer cell lines (O11, O12, O13, O28, and O29). The different cell lines vary greatly in their expression patterns for p53 and ΔNp63α. Most of the cells have abundantly expressed ΔNp63α and lack expression of p53. As a rule, WT p53 is unstable and mutant p53 is stable. Among our tested cells, O13 is ΔNp63α-positive and p53 mutant (positive); the others are ΔNp63α-negative and p53-positive (even though it is WT). HSP70 expression was closely correlated with ΔNp63α expression levels but not with the p53 status in all cell lines tested (Fig. 3C).

This observation indicates that ΔNp63α is one of the dominant regulators for HSP70 expression in cancer cells. Then variable amounts of adenovirus p53 and ΔNp63α were infected into O11, O12, O13, O28, and Saos2 cells to see their effect on HSP70 expression. We observed up-regulation of HSP70 expression after ΔNp63α infection (Fig. 3D). Interestingly, p53 could not repress endogenous and induced HSP70 expression in these cells. To further confirm our observation, we did a detailed time course study on head and neck cancer cell lines and inducible Saos2 cells. The results consistently show that WTP53 has no effect on hsp70 expression (Supplementary data 1). Taken together, our data suggest that it is ΔNp63α expression and not p53 expression which always correlates with hsp70 gene expression in squamous cell carcinoma of the head and neck.

ΔNp63α Regulate hsp70 through CBF, NF-Y and CCAAT Binding Box. We have shown that ΔNp63α could transcriptionally activate hsp70 gene expression in vitro and in vivo. In order to localize the cis-response element of hsp70 to ΔNp63α, we generated a series of deletion constructs on all hsp70B, hsp70A, and hsp70-2 gene promoters. These constructs were then cotransfected with ΔNp63α expression constructs into Saos2 cells and luciferase analyses were done to detect activity. As shown in Fig. 4, the activity of the hsp70B gene promoter was proportional to the size of the deletion with the critical region located between −55 to −142 in the hsp70B promoter. The same critical regions were delineated on the hsp70A and hsp70-2 promoters (data not shown). By searching the Motif database, we found that these regions contain a CCAAT binding box that was also shown to be essential for p53 repression of hsp70 (15). These data suggest that ΔNp63α and p53 transcriptionally regulate the hsp70 gene through the CCAAT binding box located in the hsp70 promoter. The decrease in activity when additional regions on the hsp70 promoter are deleted may be due to other factors such as HSF (19).

Several transcriptional factors including HSP-CBF, NF-YA, NF-YB, and NF-YC form a transcriptional complex that binds to a CCAAT box on several gene promoters (20, 21); p53 interaction with HSP-CBF was reported previously (15). We tested the interaction between ΔNp63α and HSP-CBF or NF-Y by cotransfection of the pRC/cytomegalovirus-ΔNp63α expression construct and the pMT2-HSP-CBF or NF-YA expression constructs in Saos2 cells. Immunoprecipitation was done with the p63 antibody and the control anti-FLAG antibody, followed by Western blotting with HSP-CBF or NF-YA antibodies. Specific bands were identified with the p63 but not by the FLAG antibody precipitation after Western
Figure 4. Regulation of ΔNp63α on the hsp70 is through CCAAT box in hsp70 promoter. Schematic diagram of a 1.4-kb fragment of the hsp70B gene promoter cloned into the pGL3 basic reporter construct. A series of deletion constructs is shown. The position of the most proximal nucleotide from the reporter region relative to ATG is shown for each construct. Right, luciferase activity of the hsp70 gene reporter constructs in Saos2 cells. The differences in relative luciferase activity between different constructs are indicated. –77 to –81 is the location of CCAAT box.

Figure 5. Physical interaction of ΔNp63α with CBF and NF-YA complex. A, Saos2 cell lysates were isolated following transient transfection of ΔNp63α with pMT2-CBF, or ΔNp63α with NF-YA (lanes 1 and 3) and were immunoprecipitated (IP) with p63 4A4 (lane 2) or a control Flag antibody (lane 4). Immunoblotting was done with either the CBF antibody (top) or the NF-YA antibody (bottom). Reciprocal IP-WB is presented on right panel. Cell lysates were immunoprecipitated with HSP-CBF and NF-YA antibodies and blotted with anti-p63 antibody. Lanes 1, 3, and 5, show input cell lysates. B, electrophoretic mobility shift assays were done using hsp70 CCAAT box oligonucleotide probes and Saos2 cell protein extracts. The single protein complex band is indicated (left). Protein complex binds specifically to hsp70 oligonucleotides. A competitive assay was done using increasing amounts of cold unlabeled oligonucleotide probes (competitors). Increasing amounts of cold to hot probes (oligonucleotides) were added as indicated (middle). CBF and NF-Y bind to the hsp70 CCAAT box. Super-shift assays were done with one of the following antibodies (anti-CBF, -NF-YA, -NF-YB, -NF-VC, or -GATA-1) and the hsp70 CCAAT box oligonucleotides probes together with Saos2 cell lysates. Right, the super-shifted complex containing the anti-CBF, anti-NF-YA, anti-NF-YB, and the gel shift complex. C, WT p53 decreases and ΔNp63α enhances the binding of CBF and NF-Y protein complex to the hsp70 CCAAT oligonucleotides. Lanes 1-6, cell lysates from different Saos2 cells. Lanes 1 and 4 do not contain protein extracts. Lanes 2 and 5 contain normal Saos2 cell lysates. Lane 3 contains the cell lysates from Saos2 infected with WT p53. Lane 6 contains the cell lysates from Saos2 infected with ΔNp63α.
This result was confirmed by immunoprecipitation with the HSP-CBF or NF-YA antibodies, followed by immunoblotting with the p63 antibody (Fig. 5A). These data clearly indicate a specific interaction between ΔNp63α and the HSP-CBF and NF-Y transcription factors and support the hypothesis that p53 and ΔNp63α regulate the hsp70 gene through a common interaction with the HSP-CBF/NF-Y complex.

We next did gel shift analysis to verify the existence of the transcription complex in hsp70 promoter. As was expected, the transcription factors do form a complex with the hsp70 promoter CCAAT binding box in Saos2 cell lysates. This binding was specific because the intensity of the band decreased with increasing amounts of unlabeled oligonucleotide (Fig. 5B). These complexes were also super-shifted by anti-HSP-CBF, anti-NF-YA and anti-NF-YB antibodies but not by anti-NF-YC or negative control GATA-1 antibodies (Fig. 5B). Taken together, these results show that CBF and NF-Y form a complex that binds to the CCAAT box located in the hsp70 promoter.

We then tested whether p53 and ΔNp63α expression could influence the binding of the CBF and NF-Y complex to the CCAAT box in the hsp70 promoter. Gel shift results show that ΔNp63α can significantly increase the binding of the protein complex to the promoter.

Table 1. p53 status and expression level of Np63 and HSP70 in head and neck tumors

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<td>Mutant</td>
<td>Larynx</td>
<td>SCC</td>
</tr>
</tbody>
</table>

*3 = strong and diffuse in over 50% of tumor cells; 2 = medium in 20% to 50% of tumor cells; 1 = weak, focal. 0 = negative; N/D = not done.
and a significant association was observed (p53 been widely accepted as a gene expression mediated through the CCAAT box in the hsp70 gene promoter. The integration of our data and other previous studies leads to a comprehensive model in which TA isoforms of p63 and p73 have different effects on hsp70 gene expression.

**Colocalization and Coexpression of ΔNp63α and Hsp70 Protein in Head and Neck Cancers.** To further confirm the physiologic interaction between ΔNp63α and hsp70, we next investigated the localization of both ΔNp63α and HSP70 in normal oral epithelium from human head and neck tissue. Both the HSP70 antibody and ΔNp63α antibody showed strong nuclear staining concentrated in the basal cells of the epithelium (data not shown). We then analyzed nine primary head and neck cancers to check for correlation of expression between these two proteins (Fig. 6). In all nine cases, ΔNp63α and HSP70 showed coordinate staining: strong in four cases, medium in three cases, and weak in two cases. In addition, the p53 status in eight of these head and neck cancer samples was checked by sequence analysis of p53. Two cases with p53 mutations were identified. When we compared the p53 status and HSP70 expression levels in these eight cases, we found no correlation between p53 status and HSP70 expression (Table 1).

This observation is consistent with our findings in head and neck cancer cell lines (see above). As a follow-up to this study, 32 additional cases of head and neck primary tumors were analyzed for their ΔNp63α and HSP70 expression without p53 sequencing data (Supplementary data J). The association between ΔNp63α and HSP70 expression in these 41 cases was calculated using the test and a significant association was observed (P < 0.0001; Supplementary data K). Thus, our study provides strong evidence to support the physiologic association between ΔNp63α and HSP70 in human head and neck cancer.

**Discussion**

Numerous studies have investigated the transcriptional activity of the different isoforms of the p63 gene in different systems. These data consistently show that TA isoforms of p63 and p73 transactivate p53 downstream target genes by binding to p53 recognition elements in the promoter of the target genes. ΔNp63 or ΔNp73 can act as dominant-negative molecules in blocking binding and thereby inhibiting their transactivation activity (2, 3). In contrast to these observations, our microarray analysis and the current study reveal an unanticipated function of the ΔNp63 protein in up-regulation of the hsp70 gene. In addition, in comparing the WT p53 and mutant p53 activity on the hsp70 promoter, we find that mutant p53 acts in the same way as ΔNp63α. These data clearly indicate that, in certain situations, proteins considered as dominant-negative in function also act as functional proteins in vivo.

The integration of our data and other previous studies leads to a comprehensive model in which p53 family members influence transcription of target genes through two different mechanisms: (a) binding to cis elements directly, or (b) interacting with other transcription factors to influence the transcription of downstream targets. It is interesting to point out that the structural domains of the p63 gene play totally opposite roles in these two different types of mechanisms. The NH2-terminal TA domain of TAp63 has been widely accepted as a trans-activation element in up-regulating some p53 downstream targets such as p21, JAG2, PUMA, etc. (2, 3, 22), although we show that it contains a repression domain for regulating HSP70. Although several reports suggest that the carboxyl terminal of p63α contains a repression domain for p53 target genes (23), we show that the carboxyl terminal of p63α contains an activation domain for the hsp70 gene. Our findings and these other studies show that multiple transcription mechanisms might exist in parallel for p53 and p63, thereby influencing different sets of target genes that control cell integrity and normal function.
documented that overexpression of HSPs including hsp90, hsp70, and the small HSP, hsp27, is closely correlated with chemotherapeutic resistance (26). Inhibition of hsp90, using 17-allylamo, 17-demethoxygeldanamycin, increases the sensitivity of the cancer cells to chemotherapy and has been used in clinical trials (27). Therefore, our discovery of ΔNp63α activation of hsp70 in cancer cells may provide a new broad therapeutic target for cancer treatment.

Acknowledgments

Received 8/13/2004; revised 10/18/2004; accepted 11/18/2004.

Grant support: National Cancer Institute’s Lung Cancer SPORE grant #CA 58184-01 and the National Institute of Dental and Craniofacial Research grant #ROI-DE 012588-0.

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We thank Dr. D. Linzer, Northwestern University, Evanston, IL, for providing HSP-CBF antiserum.

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ΔNp63α Up-Regulates the Hsp70 Gene in Human Cancer
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