The p53-homologue p63 may promote thyroid cancer progression

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Abstract

Inactivation of p53 and p73 is known to promote thyroid cancer progression. We now describe p63 expression and function in human thyroid cancer. TAp63α is expressed in most thyroid cancer specimens and cell lines, but not in normal thyrocytes. However, in thyroid cancer cells TAp63α fails to induce the target genes (p21Cip1, Bax, MDM2) and, as a consequence, cell cycle arrest and apoptosis occur. Moreover, TAp63α antagonizes the effect of p53 on target genes, cell viability and foci formation, and p63 gene silencing by small interfering (si) RNA results in improved p53 activity. This unusual effect of TAp63α depends on the protein C-terminus, since TAp63β and TAp63γ isoforms, which have a different arrangement of their C-terminus, are still able to induce the target genes and to exert tumour-restraining effects in thyroid cancer cells. Our data outline the existence of a complex network among p53 family members, where TAp63α may promote thyroid tumour progression by inactivating the tumour suppressor activity of p53.

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Introduction

Thyroid cancer is a common endocrine malignancy and several genetic abnormalities have been identified in the different thyroid cancer histotypes, involving both oncogenes and tumour suppressor genes. Mutations in proto-oncogenes (Ret, BRAF, Ras) are often observed in well-differentiated thyroid tumours (papillary and follicular) (Fagin 2002). Indeed, Ret/PTC rearrangements and BRAF mutations are observed in approximately 10–50% of papillary thyroid cancer, whereas Ras mutations are observed in approximately 20–50% of follicular thyroid cancer (Gimm 2001).

Tumour suppressor gene abnormalities, responsible for thyroid tumour progression, involve PTEN, β-catenin and p53. Decreased PTEN expression, presumably by loss of heterozygosity, has been observed in papillary (10%), follicular (15%) and poorly differentiated thyroid carcinomas (60%), whereas β-catenin mutations have been found in approximately 60% of anaplastic thyroid carcinomas (Gimm 2001). More specifically, p53 mutations are found in more than 80% of the poorly differentiated (anaplastic) thyroid carcinomas (Fagin *et al.* 1993). As a consequence, loss of p53 function is believed to play an important role in thyroid tumour progression from well (papillary and follicular) to poorly (anaplastic) differentiated thyroid cancer (Fagin *et al.* 1993). Moreover, several reports have shown that, even in the absence of inactivating mutations, the p53 protein is inactive in certain thyroid tumours and cell lines (Wyllie *et al.* 1995, Nishida *et al.* 1996), suggesting that other mechanisms may be responsible for p53 inactivation in these tumours. Finally, up-regulation of non-mutated p53 protein has

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been related to a poor clinical outcome in thyroid cancer (Dobashi *et al.* 1993, Nishida *et al.* 1996, Ruter *et al.* 1996).

Two novel members have been added to the p53 family: p63 (Yang *et al.* 1998) and p73 (Kaghad *et al.* 1997). These proteins have remarkable similarities in both structure and function to p53, since they can transactivate p53-responsive genes including p21Cip1, Bax and MDM2, and induce cell cycle arrest and apoptosis (Jost *et al.* 1997, Kaghad *et al.* 1997, Yang *et al.* 1998).

In addition to the transcriptionally active (full length) TAp63 and TAp73 isoforms, p63 and p73 genes, by the use of an inner promoter located in intron 3, may generate the ΔNp63 and ΔNp73 variants, which are N-terminally truncated and exert a dominant negative effect towards p53, TAp63 and TAp73 (Yang *et al.* 1998, 2000, Pozniak *et al.* 2000). Furthermore, p63 and p73 may undergo multiple C-terminal splicing, generating at least six isoforms for p73 (α, β, γ, δ, ε, φ) (Kaghad *et al.* 1997, De Laurenzi *et al.* 1998, Zaika *et al.* 1999), and three isoforms for p63 (α, β, γ) (Yang *et al.* 1998). At variance with p53 null mice, however (Donehower *et al.* 1992), p63 and p73 knockout mice do not develop spontaneous tumours (Mills *et al.* 1999, Yang *et al.* 2000), suggesting that p63 and p73 function is not strictly related to tumour suppressor activity. Moreover, attempts to identify mutations in the p63 and p73 gene in human cancers have been largely unsuccessful. More interestingly, several cancers overexpress the dominant negative isoforms ΔNp63 and ΔNp73 (Zaika *et al.* 1999, 2002) and data obtained from fibroblasts *in vitro* suggest that ΔNp63 and ΔNp73 may display an oncogenic potential (Hibi *et al.* 2000, Petrenko *et al.* 2003).

We have shown that thyroid cancer cells express ΔNp73α and TAp73α (Frasca *et al.* 2003). In these cells, TAp73α tumour suppressor activity is kept latent by several mechanisms including the cytoplasmic entrapment of c-Abl (Vella *et al.* 2003), interaction with p53 mutants and ΔNp73α (Frasca *et al.* 2003).

In the present study, we explored the role of p63 in thyroid tumours and found that TAp63α is expressed in most thyroid cancers but not in the normal thyroid or in follicular adenomas, and it may represent, therefore, a marker of malignancy. In thyroid cancer cells, TAp63α does not elicit p53-like responses. In contrast, TAp63α exerts an unexpected inhibitory effect on the tumour suppressor activity of p53. The absence of TAp63α tumour suppressor activity and the presence of an anti-p53 effect suggest a role for p63 in thyroid tumour progression.

Materials and methods

Cells

The human thyroid cancer cell lines (see Table 2) BC-PAP (papillary) and FRO (follicular) were provided by Drs A Fusco and M Santoro (Naples, Italy); SW-1736 (anaplastic), Hth-74 (anaplastic) and C-643 (anaplastic) cells were provided by Dr N E Heldin (Uppsala, Sweden); FF-1 (anaplastic) and AM-1 (anaplastic) cells were established in our laboratory; 8505-C (papillary) cells were purchased from DMSZ (Braunschweig, Germany); FTC-133 (follicular) and 8305-C (anaplastic) cells were purchased from ECACC (Salisbury, Wilts, UK); C-98 cells, a clone harbouring a mutation in the p53 gene, were established from TPC-1 (a papillary thyroid cancer cell line provided by Dr A Fusco, Naples, Italy). All thyroid cancer cell lines (see Table 2) were grown in RPMI 1640 (Sigma, St Louis, MO, USA) supplemented with 2 mM glutamine, 10% FBS and 100 μg penicillin and streptomycin/ml. Normal thyroid cells in primary culture were obtained from surgical specimens after treatment with 1 mg collagenase IV/ml (Sigma). The human osteosarcoma cell line, Saos-2, and the simian kidney cell line, COS-1, were provided by Dr J Y Wang (La Jolla, CA, USA) and cultured in DMEM (Sigma) supplemented with 10% FBS, and 100 μg penicillin and streptomycin/ml. The human breast cancer cell line, MCF-7 (ATCC, Manassas, VA, USA), and the human oesophagus carcinoma cell line, A431 (Dr Weir, Boston, MA, USA), were grown in MEM supplemented with FBS and antibiotics as described above.

Human thyroid tissue samples

Human thyroid cancer specimens were obtained at surgery and stored in liquid nitrogen until processing.

Immunohistochemistry

In order to set up this technique, p63-positive (FTC-133 and C-643) and p63-negative (C-98) (see Fig. 2) thyroid cancer cells were grown in monolayers, harvested by trypsinization, and centrifuged at 270 ***g*** for 10 min at 4°C. As a positive control, we used the A431 oesophagus cancer cell line, which expresses p63 at a higher level than thyroid cancer cells (see Fig. 2B). Cell pellets were immediately frozen in liquid nitrogen or, alternatively, fixed with paraformaldehyde and paraffin embedded. From these pellets were obtained 7 μm-thick sections of both fixed and unfixed cells, which were subjected to immunohistochemical staining for p63 and p53. In these experiments in A431 cells, p63 was detected in both frozen and

paraffin-embedded sections. In contrast, in p63-positive thyroid cancer cells, p63 immunostaining was observed only in frozen sections, but not in paraffin-embedded sections. Thyroid tissue sections were cut with a cryostat at −30°C, fixed with acetone at −20°C for 10 min and hydrated with PBS at room temperature for 45 min. After blocking in 2% normal serum for 20 min, sections were incubated overnight with the anti-pan-p63 monoclonal antibody 4A4 (1 : 100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the anti-p63γ goat polyclonal antibody C-18 (1 : 100) (Santa Cruz Biotechnology) or the anti-p53 monoclonal antibody DO-1 against the N-terminus of p53 (1 : 200) (Santa Cruz Biotechnology). Specific labelling was detected with biotin-conjugated anti-mouse/anti-rabbit/anti-goat IgG and avidin–biotin peroxidase complex. Sections were counterstained with either haematoxylin QS or Nuclear Fast Red (NFR), examined and photographed using an Olympus BH-2 microscope. In every experiment, sections were incubated with secondary antibody alone to further verify the specificity of the reaction.

Immunofluorescence

Cells were fixed in 3.7% formaldehyde, permeabilized with PBS/0.3% Triton X-100, blocked with PBS/10% normal goat serum and incubated with primary antibodies for 1 h. To detect endogenous p63 we used the anti-pan-p63 monoclonal antibody 4A4 (Santa Cruz Biotechnology). To detect transfected p63 we used the anti-MycTag monoclonal antibody 9E10 (Santa Cruz Biotechnology). Cells were then incubated with Alexa-conjugated (Alexa Fluor 594 or 488) secondary antibodies (Molecular Probes, Leiden, The Netherlands) for 1 h. To visualize the cytoplasm, the cells were also incubated with Alexa-conjugated phalloidin (Molecular Probes) for an additional 30 min. The cells were finally counterstained with Hoechst (Sigma) to colour the nuclei. Epifluorescence microscopy was performed with an Olympus microscope. The images were digitally acquired with an Orca CCD camera (Hamamatsu, Hamamatsu City, Japan) and processed with Image-Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD, USA).

Immunoprecipitation and immunoblot analysis

Cell lysates were prepared in RIPA buffer containing 0.1% SDS and protease inhibitor cocktail (Roche Biochemical Inc., Basel, Switzerland). For immunoprecipitation experiments, 1 mg cell lysate was incubated for 2 h with 2 μg antibody. After incubation with protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden), samples were resuspended in loading buffer, separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% milk–Tris-buffered saline plus Tween (TBST) and then immunoblotted with primary antibodies (1 μg/ml). Appropriate horseradish-peroxidase-conjugated secondary antibodies were added at 1 : 2000 (Amersham Biosciences), and proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

The following antibodies were used for immunoprecipitation: polyclonal anti-p63α antibody H129, polyclonal anti-pan-p63 H137 and monoclonal antibody DO-1 against the N-terminus of p53 (all from Santa Cruz Biotechnology). The following antibodies were used for western blotting: anti-pan-p63 monoclonal antibody 4A4 (Santa Cruz Biotechnology), anti-p53 monoclonal antibody DO-1 (Santa Cruz Biotechnology), anti-p21Cip1 polyclonal antibody (Santa Cruz Biotechnology), anti-β-actin monoclonal antibody (Sigma), anti-GFP monoclonal antibody (Covance Research Product Inc. Princeton, New Jersey, USA) and anti-Myc monoclonal antibody 9E10 (Santa Cruz Biotechnology).

Transcript analysis by RT-PCR

Total RNA (1 μg) was reverse transcribed with Superscript II (Invitrogen, Paisley, Strathclyde, UK) and oligo(dT) primers. Of the synthesized cDNA 2 μl were then combined in a PCR specific for p63α and β, using primers 5′ CGT ACA GGC AAC AGC AAC A 3′ (forward) and 5′ CGT TGC GCT GCT GAG GGT TGA 3′ (reverse) spanning exons 10 and 14 of the p63 gene (fragment size 632 bp). The TAp63 transcript was detected using primers 5′ CCC AGA GCA CAC AGA CAA A 3′ (forward) and 5′ CAC AGA TCC GGG CCT CAA A 3′ (reverse) spanning exons 2 and 8 (fragment size 896 bp). The p63γ transcript was detected using primers 5′ATG CCC AGT ATG TAG AAG A 3′ (forward) and 5′ GGG CTT GGA ATG TCT AAA G 3′ (reverse) spanning exons 6 and 15 of the p63 gene (fragment size 697 bp), and the ΔNp63 transcript was detected using primers 5′AAC AAT GCC CAG ACT CAA 3′ (forward) and 5′ ACA GGC ATG GCG CGG ATA 3′ (reverse) spanning intron 3 and exon 5 (fragment size 392 bp).

Plasmids and transfections

pBOS-H2B-GFP and pCDNA3.1-p53 were provided by Dr J Y Wang (La Jolla, CA, USA);

pCDNA3.1-p53-GFP was a gift from Drs G Wahl and JM Stommel (La Jolla, CA, USA); pCDNA3.0-Myc-TAp63α, pCDNA3.0-Myc-TAp63γ, pCDNA3.0-Myc-ΔNp63α, p21Luc and BaxLuc were donated by Dr G Blandino (Rome, Italy); pCDNA3.0-Myc-TAp63β was kindly provided by Dr L Guerrini (Milan, Italy).

All transfections were performed in 6-well plates with Fugene 6 (Roche Biochemical Inc., Basel, Switzerland) according to the manufacturer's instructions (DNA : Fugene ratio 1 : 3), and cells were processed 24 h after transfection.

We tested the onco-suppressor effect of p53 and p63 constructs by evaluating the reduction of the number of transfected cells (apoptosis plus inhibition of cell growth) as previously reported (Ozaki *et al.* 2003). We transfected p53 and p63 constructs (2 μg/well) together with H2B-GFP (0.2 μg/well). Forty-eight hours after transfection, the GFP-positive cells were scored under a fluorescence microscope and numbers obtained were expressed as a percentage of GFP-positive cells among the total population and were compared with the empty-transfected cells.

Luciferase assay

The p21Luc, BaxLuc and MDM2Luc constructs were co-transfected with pCDNA3.1, pCDNA3.0-Myc-TAp63α, pCDNA3.0-Myc-TAp63β, pCDNA3.0-Myc-TAp63γ, pCDNA3.0-Myc-ΔNp63α and pCDNA3.1-p53 (DNA ratio 1 : 1). A vector coding for the *Renilla* luciferase (provided by Dr E Conte, Catania, Italy) was also co-transfected in all conditions (DNA ratio 1 : 20). Twenty-four hours after transfection, the cells were processed with the Dual Luciferase assay (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Luciferase activity was normalized for transfection efficiency (*Renilla* activity).

Gene silencing by siRNA

Cells were plated onto 6-well plates (10^5 cells/well) and kept in antibiotic-free medium for 24 h. Cells were then transfected with a mixture containing OptiMEM, 8 μl lipofectamine/well (Lipofectamine 2000, Invitrogen) and either 0.5 μg GFP-small interfering (si) RNA or 0.5 μg TAp63-siRNA/well (Dharmacon Research Inc., Lafayette, CO, USA) for 5 h. The sequence of these siRNAs is available from the manufacturer. Cells were then incubated with fresh medium for 48 h and transfected with p53 (0.5–1.0 μg/well) together with p21-luc (1.0 μg) and *Renilla* (0.2 μg), using Fugene 6 reagent (Roche). Twenty-four hours after transfection, cell extracts were analysed for p21 activity by the luciferase assay. Aliquots of these samples were also subjected to western blot for the assessment of p63 status.

Cell cycle evaluation

Cells were synchronized for 36 h in serum/leucine-free medium and released in complete medium for 12 h (cell cycle) or 72 h (apoptosis) in the presence or absence of 2 μM doxorubicin. Adherent and floating cells were harvested and resuspended in 70% ethanol and stored at −20°C. Permeabilized cells were centrifuged and resuspended in PBS containing 20 μg Propidium Iodine (PI)/ml plus 40 μg RNAse/ml (Sigma) for 30 min in the dark. Cells were then subjected to FACS analysis (Coulter Elite flow cytometer, Beckman Coulter, Milan, Italy) and gated for PI (X axis = FL2; Y axis = events). To evaluate the subG1 population, a log scale was applied to the X axis (PI, FL2). Cells transfected with GFP-tagged constructs were not treated with 70% ethanol so as to avoid GFP denaturation and, as a consequence, loss of fluorescent emission. GFP-containing cells were fixed instead with 1% paraformaldehyde in PBS for 2 h at 4°C and permeabilized with 0.3% Triton in PBS for 20 min at room temperature or, alternatively, treated with citrate hypotonic buffer. Triton-permeabilized cells were incubated with PI and RNAse overnight at 4°C, whereas citrate-permeabilized cells were incubated with PI and RNAse for 30 min at room temperature in the dark. Cells were than subjected to FACS analysis and gated for PI (FL2) and GFP (FL1). Cell cycle analysis was performed by placing separate gates in both the transfected (GFP positive), and untransfected (GFP negative) population. The percentage of GFP-positive cells ranged from 2 to 20% of total cells. For GFP-positive cells at least 10^4 events were counted.

Foci formation

C-98, BC-PAP, SW-1736 and C-643 thyroid cancer cells were plated onto 6-well plates (10^5 cells/well) in complete 10% FCS medium. After 24 h, each well was transfected using the Fugene 6 method (Roche) with either 2 μg empty vector, or a mixture containing 1 μg empty + 1 μg p53, 1 μg empty + 1 μg TAp63α, 1 μg empty + 1 μg ΔNp63α, 1 μg p53 + 1 μg TAp63α, or 1 μg p53 + 1 μg ΔNp63α. Cells were allowed to grow until 90% confluent and were split onto 100 mm Petri dishes. Cells were then grown in complete medium containing G418 (Gibco) (concentration range 0.5–1.0 mg/ml, depending on the cell line) to allow antibiotic (geneticin) selection. The foci obtained with

this procedure were fixed in 11% glutaraldehyde, stained with Crystal Violet (BDH, Poole, Dorset, UK) and counted.

Statistical analysis

FACS analysis results were compared by two-way analysis of variance. Significance was obtained by Student's *t*-test (*$P<0.05$, **$P<0.01$, ***$P<0.001$). Statistical analysis was carried out with Microsoft Excel software.

Results

TAp63α is expressed in human thyroid cancer tissue

Previous studies performed in paraffin-embedded specimens indicated that p63 is expressed in a small subset of papillary and anaplastic thyroid carcinomas (Preto *et al*. 2002). We examined 23 frozen thyroid specimens by immunohistochemistry (IHC) (Fig. 1A, Table 1) using the anti-pan-p63 4A4 antibody. In these experiments, p63 immunoreactivity was present in most thyroid cancers (9/9 papillary carcinomas, 7/7 follicular carcinomas, 9/11 anaplastic carcinomas) (Table 1). In contrast, p63 was not detected in normal thyroid (n = 8), whereas it was detected in 1 out of 7 benign adenomas (Table 1). p63 was also detected in vessels of both normal and neoplastic thyroid tissue (Fig. 1A, right panel). In thyroid cancer cells, p63 immunoreactivity was mostly localized in the nucleus (Table 1). Immunohistochemistry with a specific anti-p63γ antibody gave negative results (not shown) and immunohistochemistry in paraffin-embedded tissues provided a weak signal only in a small percentage of thyroid specimens, in accordance with the previous observations (Preto *et al*. 2002). Since the 4A4 antibody used in these experiments recognizes all p63 isoforms, we studied p63 isoform expression in frozen thyroid tissue specimens by RT-PCR (Fig. 1B). As a negative control we used C-98 thyroid cancer cells (p63 negative, see Table 2), while as a positive control we used the oesophagus cancer cell line A431 (Kaelin 1999). In thyroid cancer specimens, we detected the TAp63α transcript but found no mRNA for TAp63β, TAp63γ and ΔN-p63 (Fig. 1B). TAp63α transcript was also found in normal thyroid tissues; its expression, in accordance with the IHC results, must be attributed to the presence of p63 immunoreactivity in blood vessels (Fig. 1A). In the same samples, we then evaluated p63 protein expression by immunoprecipitation with the anti-pan-p63 antibody (H137) and by western blot analysis with the anti-pan-p63 antibody (4A4) (Fig. 1C). As a positive control we used lysates of COS-1 cells transfected with TAp63α, TAp63γ or ΔNp63α (Fig. 1C). In accordance with the RT-PCR results, we found that in thyroid cancer specimens only the TAp63α protein was expressed (Fig. 1C). In contrast to malignant tissue, TAp63α protein was not detected in normal thyroid tissues (Fig. 1C, left panel). Taken together, these data indicate that the TAp63α protein is expressed in most thyroid cancer cells but not in normal thyroid or in follicular adenoma cells. The apparent contrast of the TAp63α transcript presence in normal thyroid is due to the TAp63α expression in endothelial cells. It is also interesting to note that the dominant negative isoform, ΔNp63, which has been reported to be upregulated in several p63-positive malignancies, is not expressed in thyroid tumours.

TAp63α is expressed in thyroid cancer cell lines

To identify an *in vitro* model to study the role of TAp63α in thyroid cancer biology, we explored by RT-PCR the expression of p63 in a panel of thyroid cancer cell lines, representative of the three thyroid cancer histotypes (3 papillary, 2 follicular and 6 anaplastic) (Fig. 2A). Normal thyrocytes in primary culture were also studied (Fig. 2A, B, C, on the left). The TAp63α transcript was present in 2 out of 3 papillary thyroid cancer cell lines (BC-PAP and 8505C), 1 out of 2 follicular thyroid cancer cell lines (FTC-133) and all (6/6) anaplastic thyroid cancer cell lines (FF-1, SW-1736, C-643, Hth-74, 8305C and AM-1) (Fig. 2A). Moreover, 3 cell lines (FTC-133, SW-1736 and C-643) were also faintly positive for the TAp63γ transcript (Fig. 2A). In contrast, the ΔN-p63 transcript was not found in the thyroid cancer cell lines tested (Fig. 2A). In contrast, normal thyrocytes were negative for p63 transcript (Fig. 2A, on the left).

In the same cell lines, we then evaluated p63 protein expression by western blot (Fig. 2B) and found that the TAp63α protein was present in BC-PAP, 8505C, FTC-133, C643 and Hth-74 cells (Fig. 2B). In contrast, in FF-1, SW-1736, 8305C and AM-1 cells, which were positive for TAp63α mRNA, the protein was not detected (Fig. 2B). C-98 and FRO cells expressed neither TAp63α mRNA nor the protein (Fig. 2A, B). Data obtained are summarized in Table 2. The TAp63γ protein was not detected in any of the cell lines expressing the mRNA. A high expression of all three p63 protein isoforms (TAp63α, ΔNp63α and TAp63γ) was observed in A431 cells, used as a positive

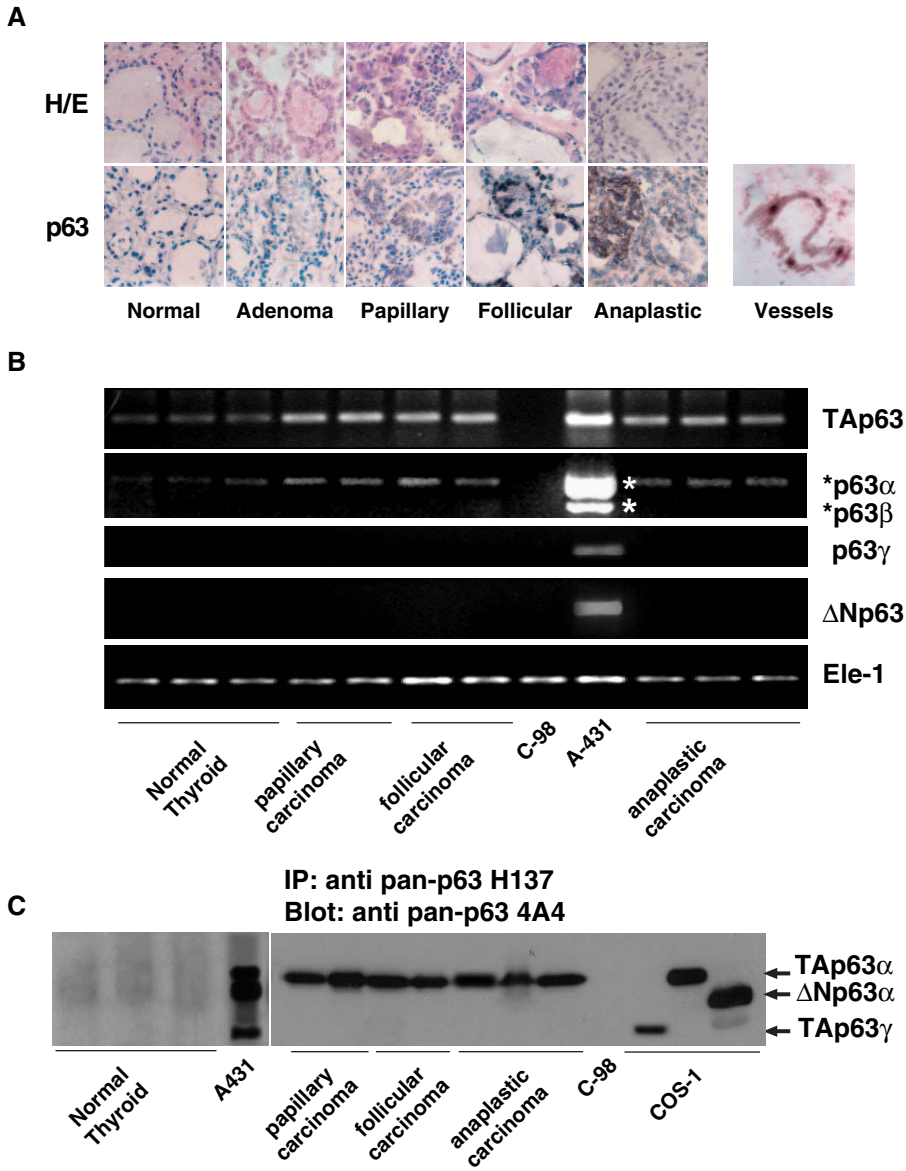

Figure 1 p63 expression in human thyroid cancer specimens. (A) Immunohistochemistry for p63 in human thyroid samples was performed on frozen thyroid specimens using anti-pan-p63 antibody (4A4) (see also Table 1 and the Methods section). Eosin staining (H/E; top panels) and p63 staining (bottom panels) are shown for normal thyroid, follicular adenoma, papillary carcinoma, follicular carcinoma and anaplastic carcinoma at $\times 20$ magnification. A representative p63 immunostaining obtained in blood vessels of normal thyroid tissue is shown (right). (B) Specimens obtained from three normal thyroids, two papillary, two follicular and three anapalstic thyroid carcinomas were analysed by RT-PCR for the presence of TAp63, p63α/β, TAp63γ and ΔNp63 transcripts. The human oesophagus cancer cell line A431 (p63 positive) and the thyroid cancer cell line C-98 (p63 negative) were used as controls. RT-PCR for the ubiquitous gene Ele-1 was also performed (lower panel). (C) Lysates from the same specimens were immunoprecipitated (IP) with an anti-pan-p63 polyclonal antibody (H137) and subjected to Western blot analysis (Blot) with anti-pan-p63 monoclonal antibody (4A4). C-98 cells were used as a negative control; COS-1 cells, transfected with TAp63α, TAp63γ or ΔNp63α, and A431 cells were used as positive controls.

control (Fig. 2B on the left). In primary normal thyrocytes, p63 was always absent at western blot analysis (Fig. 1B, on the left). However, when AM-1 and 8305-C cells, which were positive for the TAp63α transcript and negative for the protein (Fig. 2 and Table 2), were incubated with the proteasomal

Table 1 Immunohistochemistry for p53 and p63 in human thyroid tissue specimens

	p53		p63	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
Normal (*n = 8*)				
74 N	−	−	−	−
81 N	−	−	−	−
89 N	−	−	−	−
143 N	−	−	−	−
212 N	−	−	−	−
390 N	−	−	−	−
560 N	−	−	−	−
361 N	−	−	−	−
Adenomas (*n = 7*)				
196 A	++	−	+	−
206 A	−	−	−	−
212 A	−	−	−	−
218 A	−	−	−	−
232 A	−	−	−	−
387 A	−	−	−	−
392 A	−	−	−	−
Papillary (*n = 9*)				
201 P	++	−	+	−
321 P	++	−	+++	+
340 P	+	−	+	−
354 P	+++	−	++	−
360 P	++	+	++	−
380 P	++	−	++	−
383 P	+	−	++	−
386 P	++	−	++	−
395 P	+++	−	++	−
Follicular (*n = 7*)				
97 P	++	−	++	−
146 P	+	−	+	−
191 P	++	−	+++	−
260 P	+	−	+	−
283 P	+++	−	+++	−
267 P	+++	+	+++	+
371 P	++	−	++	−
Anaplastic (*n = 11*)				
6 P	++	−	−	−
17 P	+	−	+	−
32 P	+++	−	++	−
96 P	++++	−	++	−
103 P	++	−	++	+/−
161 P	+++	−	+++	−
164 P	++++	−	+++	−
219 P	+++	−	+++	−
241 P	++++	++	−	−
238 P	++++	−	+++	−
333 P	+++	−	+++	−

− no expression.
+ low expression.
++ moderate expression.
+++ high expression.
++++ very high expression.

Table 2 p53 family member status in human thyroid cancer cell lines

	p53	TAp63α	TA/ANp73α
Papillary			
C-98	K286E	−	−
BC-PAP	D259Y; K286E	+++	+++
8505C	R248G	+++	+
Follicular			
FRO	−	−	−
FTC-133	R273H	++++	−
Anaplastic			
FF-1	E285K	+	−
SW-1736	−	−	++++
C-643	R248Q; K286E	+++	−
Hth-74	K286E	++	++
8305C	R273C	+	−
AM-1	n.d.	+	−
Saos-2	−	−	+/−
A431	R273H	++++	+/−

− no expression.
+ very low expression, detectable only by RT-PCR.
++ low expression.
+++ moderate expression.
++++ high expression.
Results presented are the combination of previous data (Frasca *et al.*, 2003) and data obtained by RT-PCR and western blot in figure 2.

inhibitor MG132, a faint band corresponding to TAp63α protein was observed (not shown). These findings suggest that in these cells the TAp63α protein is present, but its expression is decreased below detectable levels by proteasomal degradation.

In these thyroid cancer cells, we then evaluated p63 localization by immunofluorescence using the 4A4 antibody (Fig. 2C). In C-98 cells (p63 negative, see Fig. 2A, B and Table 2) we did not detect any p63 immunoreactivity (Fig. 2C), whereas in FTC-133 and C-643 cells (p63 positive, see Fig. 2A, B and Table 2), the p63 signal was localized in the nucleus, in a manner similar to that observed in the p63-positive cells A431 (Fig. 2C). In accordance with the results obtained by immunohistochemistry, normal thyrocytes in primary culture did not display any p63 immunoreactivity (Fig. 2C, on the left).

These data indicate that TAp63α is present in most thyroid cancer cells in permanent culture, but not in normal thyrocytes, and confirm the data obtained in thyroid tissue specimens.

Endogenous p63 does not exert p53-like functions in thyroid cancer

p63 is able to activate a pool of genes, which are also common targets of p53 and p73 (Sasaki *et al.* 2001),

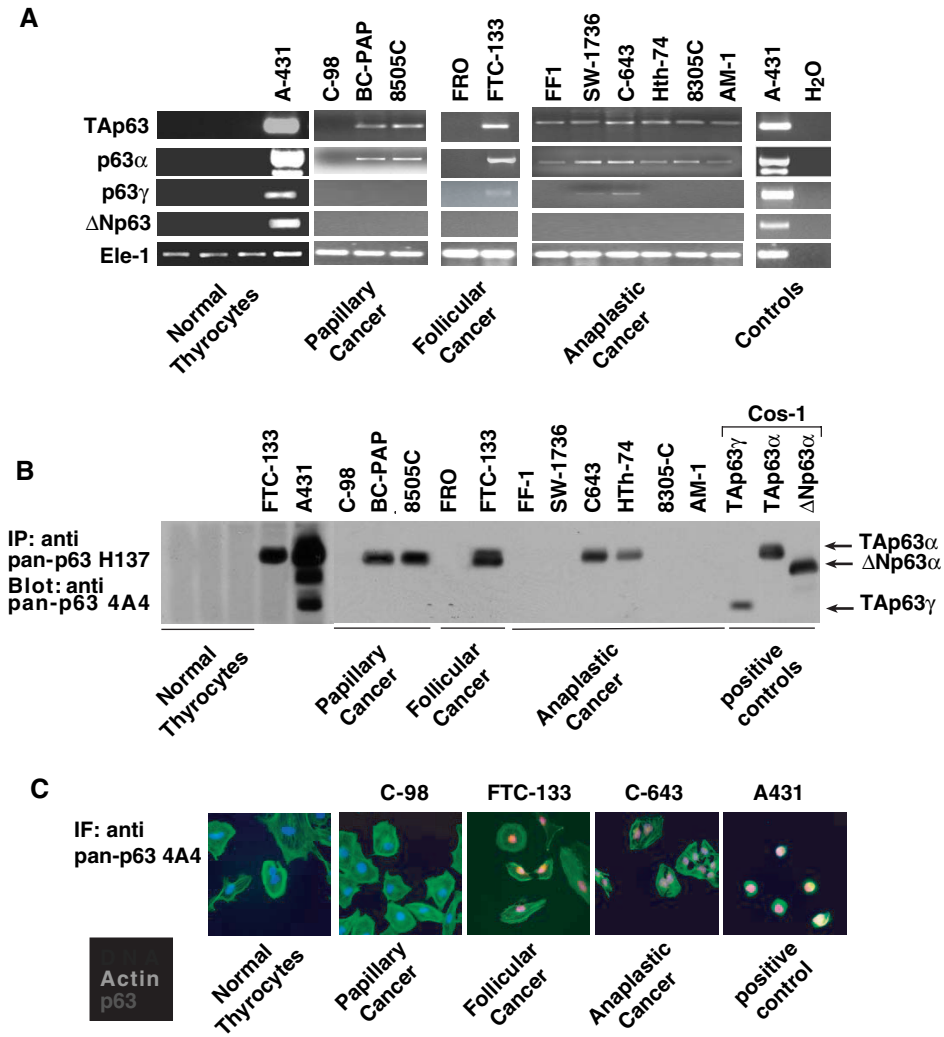

Figure 2 Expression and localization of p63 in human thyroid cancer cells. (A) Three primary cultures of normal thyrocytes, 3 papillary (C-98, BC-PAP, 8505C), 2 follicular (FRO, FTC-133), and 6 anaplastic (FF1, SW-1736, C-643, Hth-74, 8305C, AM-1) thyroid cancer cell lines were screened by RT-PCR for p63 isoform expression. The A431 cell line was used as a positive control. (B) Lysates from the same cell lines were subjected to immunoprecipitation (IP) with anti-pan-p63 polyclonal antibody (H137) and then blotted (Blot) with an anti-pan-p63 monoclonal antibody (4A4). COS-1 cells, transiently transfected with TAp63γ, TAp63α or ΔNp63α, were used as a positive control. (C) Cellular localization of p63 by immunofluorescence. Primary thyrocytes, C-98 (papillary, p63 negative), FTC-133 (follicular, p63 positive) and C-643 (anaplastic, p63 positive) thyroid cancer cell lines were plated onto cover slips, fixed and stained for p63 (4A4 antibody, red) and filamentous actin (phalloidin, green). Nuclei were visualized with Hoechst (blue). A431 cells were used as a positive control.

including p21Cip1, Bax and MDM2 (Zhu *et al.* 1998, Lee & La Thangue 1999, Nakano *et al.* 2000). Doxorubicin is an effective DNA damaging agent, which leads to the activation of p53 and p73 and, as a consequence, to the transactivation of target genes. Hence, to explore the onco-suppressor activity of TAp63α, we exposed thyroid cancer cells to doxorubicin. To this end, we used cell lines with a genetic background suitable for our experiments, i.e. we selected FTC-133 and C-643 cells, which express TAp63α and p53 inactive mutants but not p73 (Fig. 2 and Table 2). Hence, in these cells doxorubicin is expected to induce p21Cip1/Bax expression exclusively via TAp63α. As a positive control we employed the human breast cancer cell line MCF-7, which has a wild-type p53 and maintains a normal response to doxorubicin. As a negative control we used FF-1 thyroid cancer cells, harbouring mutated p53 (Frasca

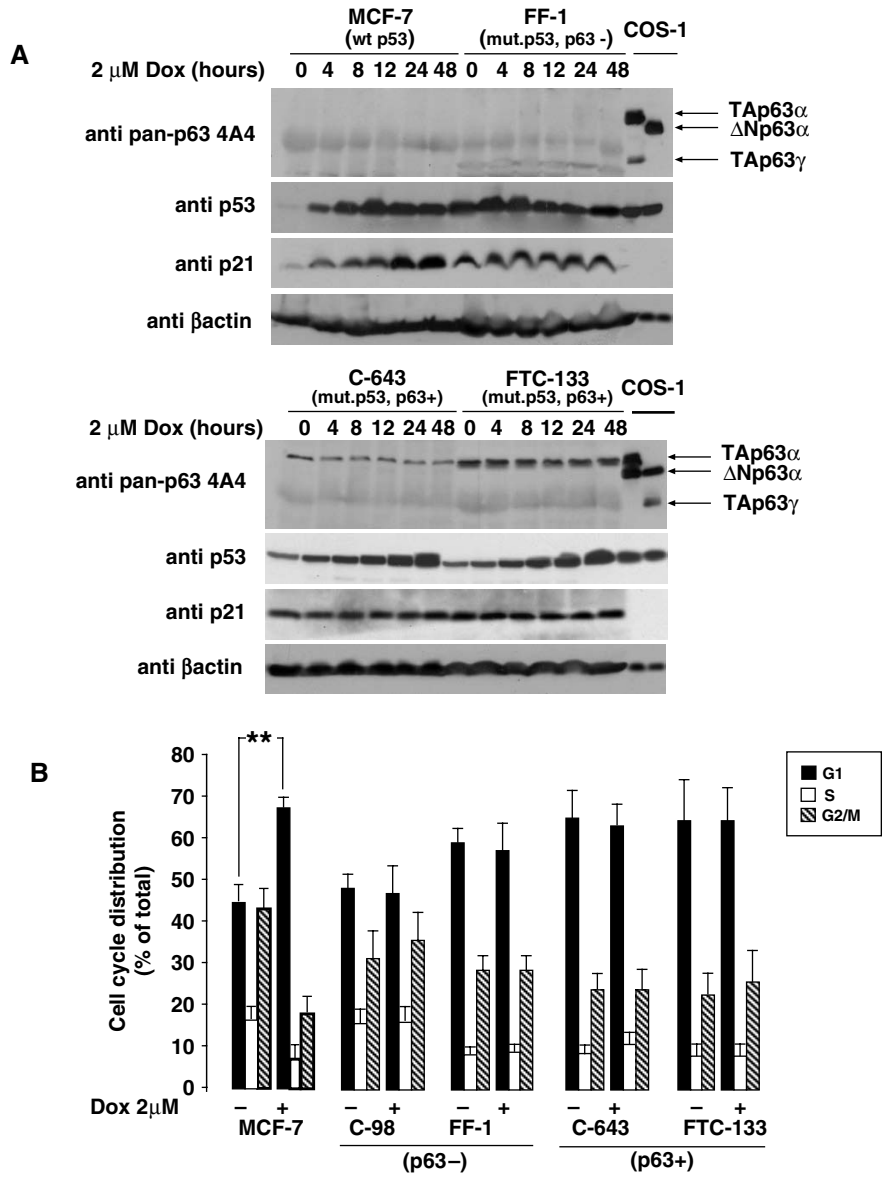

Figure 3 Activity of endogenous TAp63α in thyroid cancer cells. (A) FF-1 (p63 negative), C-643 (p63 positive) and FTC-133 (p63 positive) thyroid cancer cells were incubated with 2 μM doxorubicin (Dox) for the indicated times. MCF-7 cells, which, unlike thyroid cancer cells, express wild-type (wt) p53, were used as a positive control. Cells were lysed and blotted with anti-pan-p63 monoclonal antibody 4A4, anti-p53 (DO-1), anti-p21, and anti-β-actin as a loading control. COS-1 cells, transiently transfected with TAp63γ, TAp63α or ΔNp63α, were used as a positive control. mut, mutant. (B) C-98, FF-1 (p63 negative), C-643, FTC-133 (p63 positive) thyroid cancer cell lines were analysed for variations in their cell-cycle profiles before and after treatment with 2 μM doxorubicin. The bars represent average and standard deviation of the cell-cycle distribution from FACS analysis (G1, solid bars; S, open bars; G2/M, hatched bars) of three separate experiments. The MCF-7 cell line was used as a positive control. $^{**}P<0.01$.

et al. 2003) and lacking both p73 (Frasca *et al*. 2003) and p63 proteins (Fig. 2 and Table 2).

In MCF-7 cells, exposure to doxorubicin increased the expression level of p53 and, as a consequence, led to the induction of the target gene p21 (Fig. 3A). In contrast, doxorubicin neither significantly affected the TAp63α level in C-643 and FTC-133 cells (Fig. 3A), nor caused the appearance of p63 protein in FF-1 cells (Fig. 3A) and C-98 cells (not shown). As a consequence, doxorubicin failed to increase p21Cip1 content

in FF-1, C643, C-98 and FTC-133 thyroid cancer cell lines (Fig. 3A). In accord with the failure of p21Cip1 induction (Fig. 3A), doxorubicin failed to cause a significant G1 arrest in thyroid cancer cells (Fig. 3B), independently of p63 status, whereas it did do so in MCF-7 cells, as expected. Moreover, doxorubicin treatment failed to induce the pro-apoptotic gene Bax and apoptosis in thyroid cancer cells (data not shown). In accordance with the abrogation of p53 function (Blagosklonny 2002), exposure to doxorubicin for 72 h induced a G2/M arrest in all four thyroid cancer cell lines (not shown), indicating that doxorubicin was used at an effective concentration.

Taken together, these results indicate that in thyroid cancer cells TAp63α is not involved in the DNA damage response.

TA63α function is not restored by ectopic expression

Since DNA damage, following exposure to doxorubicin, was not able to increase the TAp63α level in thyroid cancer cells, we suspected a defect upstream of p63 induction. To address this issue, we induced TAp63α overexpression by transient transfection in C-98 (p63 negative, see Table 2) and FTC-133 (p63 positive, see Table 2) thyroid cancer cells. As a control, p53/p63 null human osteosarcoma cell line Saos-2 cells, which are commonly used as a model to study p53 family member functions (Yang *et al.* 1999, Ghioni *et al.* 2002), were also included. In all cell lines p53 was also transfected as a positive control (Fig. 4A).

Immunofluorescence staining of transfected cells with anti-myc antibody revealed a correct localization of the ectopic TA-p63α in the cell nuclei (not shown). Western blot analysis showed that ectopic TAp63α was expressed in transfected cells and it was effective in inducing p21Cip1 (Fig. 4A) in Saos-2 cells. In contrast, TAp63α was almost ineffective in thyroid cancer cells (Fig. 4A).

We then tested the effect of overexpressed TAp63α on cell cycle distribution in C-98 (p63 negative, see Table 2), FTC-133 (p63 positive, see Table 2) and Saos-2 (control) cells (Fig. 4B). To this end, TAp63α or p53 were transiently transfected together with H2B-GFP to mark the transfected population. FACS analysis performed in the GFP-positive cells showed that TAp63α did not induce G1 arrest in thyroid cancer cells, whereas p53 did, in accordance with p21Cip1 induction (Fig. 4B). In contrast, both TAp63α and p53 were able to elicit G1 arrest in control Saos-2 cells (Fig. 4B). In addition, FACS analysis revealed that TAp63α did not increase the apoptotic population (subG1) in thyroid cancer cells (Fig. 4C), whereas p53 did (Fig. 4C). This increase, however, was less than 10% of the total population, in accordance with previous reports indicating that thyroid cancer cells are refractory to p53-induced apoptosis (Moretti *et al.* 1997, 2000). In control Saos-2 cells, both TAp63α and p53 significantly increased apoptosis (subG1 was approximately 50% of the total population) (Fig. 4C).

These data indicate that even ectopic overexpression of TAp63α is not able to elicit p53-like responses in thyroid cancer cells and raises the question why this protein does not exert any tumour suppressor activity in these cells.

TAp63α does not interact with p53 mutants in thyroid cancer cells

Since transient transfection experiments suggested a possible defect intrinsic to TAp63α, we tested whether TAp63α protein in thyroid cancer cells is inhibited by the direct interaction with p53 mutants (Gaiddon *et al.* 2001, Strano *et al.* 2002). To this end, we performed co-immunoprecipitation experiments in p63 positive (C-643 and FTC-133), and p63 negative (C98) thyroid cancer cell lines, and in the A431 oesophageal cancer cell line (Park *et al.* 1994) as a positive control (Fig. 5). In A431 cells we were able to detect p63 in anti-p53 immunoprecipitates (Fig. 5), whereas we did not find p53/p63 co-immunoprecipitation in any thyroid cancer cell line (Fig. 5).

These data indicate that the p53 mutants expressed in these thyroid cancer cell lines do not interact with p63.

p63 function is selectively abrogated in the TAp63α isoform

The p63 protein exists in various C-terminal splicing isoforms (α, β and γ) and, at variance with p53, contains a C-terminal domain, which is a protein interaction module endowed with inhibitory function (Moll *et al.* 2001). Such a domain is present in TAp63α but absent in TAp63β and TAp63γ. Moreover, it has been reported that exon 13 alone, located at the p63 C-terminus and missing in TAp63β, may inhibit TAp63α transcriptional activity by interaction with unknown proteins (Ghioni *et al.* 2002). To test whether the C-terminal inhibitory domain is involved in the inactivation of TAp63α in thyroid cancer cells, we compared the transactivation activity of ectopic TAp63α, TAp63β (lacking exon 13), TAp63γ (lacking exons 11-14), ΔNp63α and p53 on the target genes p21Cip1, Bax and MDM2 in thyroid cancer cells and

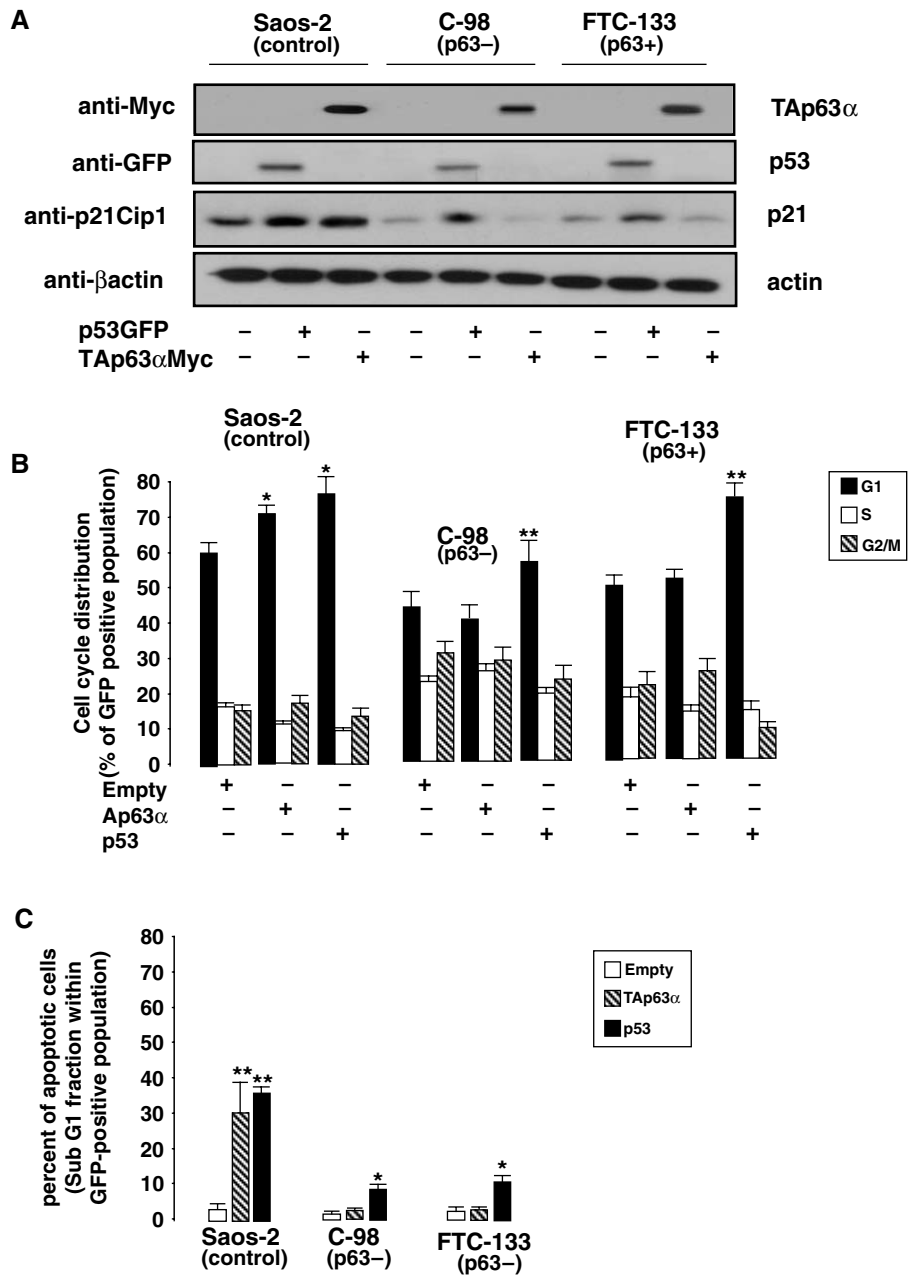

Figure 4 Activity of ectopic TAp63α in thyroid cancer cells. (A) TAp63α-Myc and p53-GFP were transfected in C-98 (p63 negative) and C-643 (p63 positive) thyroid cancer cells. The human osteosarcoma cell line, Saos-2 (p53 and p63 negative) was used as a control. Twenty-four hours after transfection, cells were lysed and subjected to Western blot analysis with either anti-Myc or anti-GFP monoclonal antibodies (two upper panels). Aliquots of the same samples were analysed for the expression of the downstream target protein p21Cip1 and reprobed with an anti-β-actin antibody (two lower panels). (B) The same cells were transfected with an empty vector, TAp63α-Myc, or p53 together with H2B-GFP to mark the transfected population. Twenty-four hours after transfection, the GFP-positive cells were analysed by FACS analysis to determine their cell-cycle profile. The bars shown (G0/G1, solid bars; S, open bars; G2/M, hatched bars) represent the average plus standard deviation from three separate experiments. $^{*}P<0.05$, $^{**}P<0.01$. (C) The same cells were transfected with an empty vector, TAp63α-Myc, or p53 together with H2B-GFP to mark the transfected population. Twenty-four hours after transfection, the GFP-positive cells were analysed by FACS analysis to determine the apoptotic population (subG1). The bars shown (empty, open bars; TAp63α, hatched bars; p53, solid bars) represent the average and standard deviation from three separate experiments. $^{*}P<0.05$, $^{**}P<0.01$.

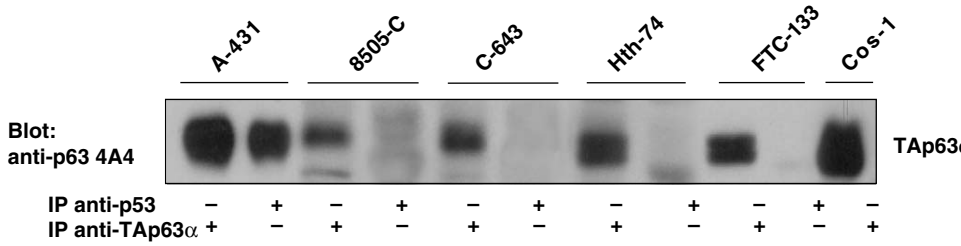

Figure 5 Co-immunoprecipitation experiments for p53 and p63. Lysates from p63-positive 8505C, C-643, Hth-74 and FTC-133 thyroid cancer cells were immunoprecipitated (IP) with either anti-p63α (H129) or anti-p53 (DO-1) antibody and blotted with an anti-p63 monoclonal antibody (4A4). A431 cells were used as a positive control (on the left), whereas COS-1 cells transfected with TAp63α were used as a standard (on the right). No co-immunoprecipitation is evident in thyroid cancer cell lines.

Table 3 Transcriptional activity of ectopic p63 and p53 in thyroid cancer cells on p21, Bax and Mdm2 promoters

	TAp63α	TAp63β	TAp63γ	ΔNp63α	p53
Saos-2					
p21	92±7	95±15	100	0±12	91±4
Bax	60±9	72±7	90±12	2±4	100
Mdm2	49±12	67±12	100	0±6	50±15
C-98					
p21	11±8	84±5	100	9±4	42±10
Bax	34±7	96±11	100	8±4	76±11
Mdm2	6±9	82±14	100	5±8	57±8
FF-1					
p21	6±12	64±14	100	2±8	49±7
Bax	3±7	80±8	100	4±2	76±5
Mdm2	12±3	74±6	100	3±5	58±9
C-643					
p21	24±7	87±9	100	0±6	72±9
Bax	4±9	47±15	100	6±4	30±14
Mdm2	6±8	100	97±6	8±5	87±11
FTC-133					
p21	10±4	81±6	100	2±10	68±8
Bax	10±9	74±13	100	5±8	76±12
Mdm2	7±5	89±14	100	13±6	93±9

p63 and p53 transcriptional activity on reporter genes was evaluated by the luciferase assay as described in methods. Numbers indicate the mean±S.E. of three separate experiments performed in duplicates and are expressed as percent of maximal induction.

p53/p63-null Saos-2 cells (Table 3). Luciferase assays (expressed as a percent of maximal induction) revealed that TAp63α, TAp63β, TAp63γ and p53 displayed comparable transactivation activity in Saos-2 cells, as previously reported (Table 3) (Ghioni *et al.* 2002). In contrast, TAp63α displayed a weak transactivation activity in thyroid cancer cells (C-98, FF-1, C-643 and FTC-133), similar to that observed with the dominant negative isoform ΔNp63α (Table 3).

In accordance with the data obtained by the luciferase assay, western blot analysis of p21Cip1 showed that TAp63γ consistently increased the level of p21Cip1 protein in both Saos-2 control cells and in C-98 and FTC-133 thyroid cancer cells, whereas TAp63α was effective in Saos-2 cells but did not increase p21Cip1 in thyroid cancer cells (Fig. 6A). Anti-Myc western blots indicated that both ectopic TAp63α and TAp63γ were expressed at a comparable level in transfected cells (Fig. 6A, middle panel).

To test the onco-suppressor function of p63, we transfected C-98 (p63 negative) and FTC-133 (p63 positive) with p53, TAp63α and TAp63γ together with H2B-GFP to mark the transfected population. Forty-eight hours after transfection, FACS analysis revealed that TAp63γ arrested the cells in G1 to an extent similar to that of p53 (Fig. 6B), whereas TAp63α was not effective, in accordance with the results obtained with the luciferase assays. In control Saos-2 cells, TAp63α, TAp63γ and p53 were all effective, although to a variable extent, in inducing a G1 arrest (not shown).

These results suggest that TAp63α does not act as a typical p53 family member in thyroid cancer cells, which maintain a high responsiveness to the tumour suppressor activity of TAp63β and TAp63γ.

TAp63α exerts a dominant negative effect on p53 activities

It is known that ΔNp63, the N-terminal truncated p63 isoform, is devoid of transactivation activity and is able to exert a dominant negative effect towards p53 family members (Yang *et al.* 1998). Since TAp63α displayed very poor transactivation activity in thyroid cancer cells, comparable to that of ΔNp63α, we tested whether TAp63α could antagonize p53 activities.

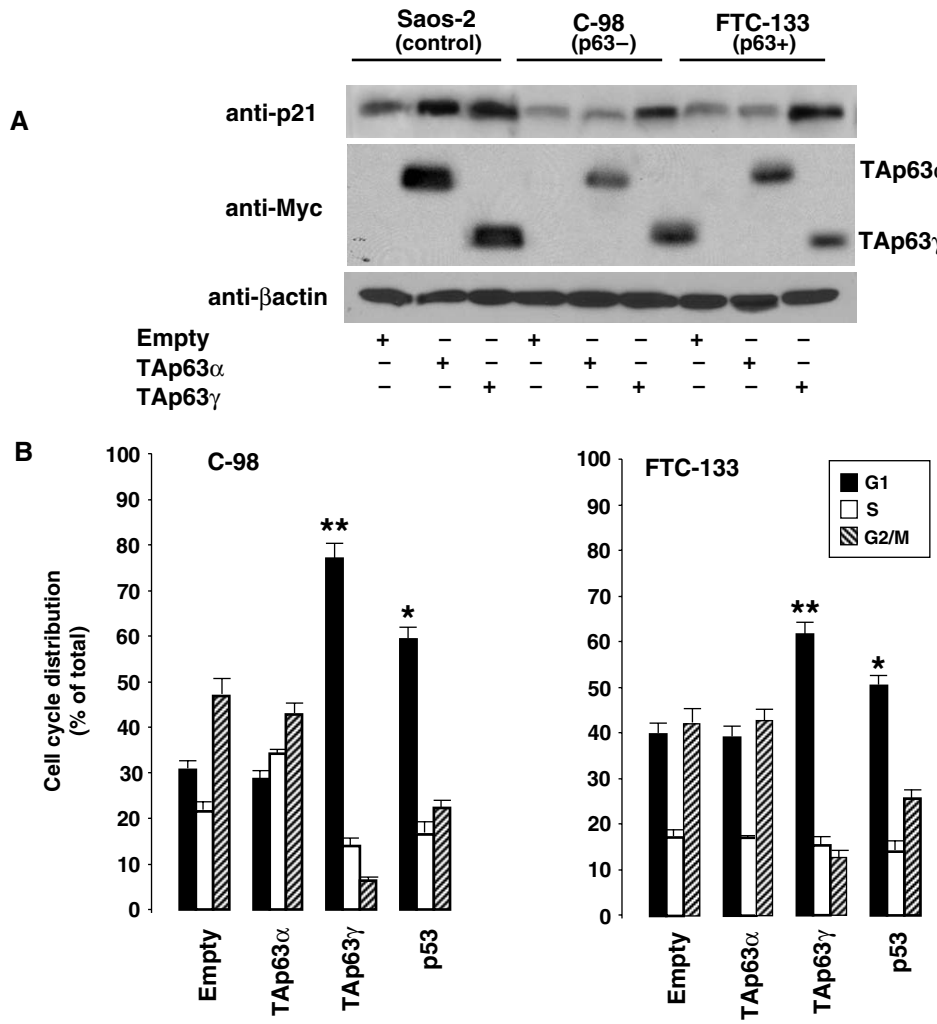

Figure 6 Comparison of TAp63α and TAp63γ activity in thyroid cancer cells. (A) TAp63α and TAp63γ were transfected in C-98 (p63 negative) and FTC-133 (p63 positive) thyroid cancer cells and Saos-2 (p53 and p63 negative) osteosarcoma cells. Twenty-four hours after transfection, cells were lysed and subjected to western blot analysis with anti-p21Cip1 (upper panel), anti-Myc (middle panel) and anti-β-actin antibody (lower panel). (B) C-98 (p63 negative) and FTC-133 (p63 positive) thyroid cancer cells were transfected with the indicated p53 and p63 constructs together with H2B-GFP to mark the transfected population. Twenty-four hours after transfection, the GFP-positive cells were analysed by FACS to determine their cell-cycle profile. Bars show the profile of transfected cells expressed as a percentage of the total population (G1, solid bars; S, open bars; G2/M, hatched bars) and represent the average ± S.E. from three separate experiments. $^{*}P<0.05$, $^{**}P<0.01$.

We transfected p21Cip1, Bax and MDM2 promoters together with p53, TAp63α and ΔNp63α in C-98, FF-1 (p63 negative, see Table 2), C-643, FTC-133 (p63 positive, see Table 2) thyroid cancer cells and in p53-null Saos-2 cells. The ability of TAp63α to antagonize the effect of p53 was evaluated by luciferase assay (Table 4). In Saos-2 cells, TAp63α did not significantly affect the activity of p53 on reporter genes (Table 4), whereas ΔNp63α exerted an antagonistic effect, as expected (Table 4). However, it was interesting to note that in Saos-2 cells the effect of TAp63α was not additive or synergistic with p53 (Table 4). Surprisingly, TAp63α antagonized p53 transactivation activity to a variable extent in thyroid cancer cells (C-98, FF-1, C-643, FTC-133; Table 4), in a manner similar to that of ΔNp63α (Table 4).

Therefore, we tested whether TAp63α could also antagonize the effect of p53 on thyroid cancer cell viability (Fig. 7A) (Ozaki *et al.* 2003). Transient co-transfection experiments with H2B-GFP to mark

Table 4 Inhibition of p53 transcriptional activity on p21, Bax and Mdm2 promoters by TAp63α and ΔNp63α in thyroid cancer cells

	p53+Empty	p53+TAp63α	p53+ΔNp63α
Saos-2			
p21	100	111±7	55±16
Bax	100	87±9	33±1
Mdm2	100	95±8	25±13
C-98			
p21	100	32±14	0±0
Bax	100	28±8	12±5
Mdm2	100	32±10	14±4
FF-1			
p21	100	38±12	21±13
Bax	100	29±10	17±5
Mdm2	100	35±12	16±8
C-643			
p21	100	38±8	24±7
Bax	100	47±9	19±11
Mdm2	100	39±12	40±13
FTC-133			
p21	100	44±6	57±5
Bax	100	35±14	23±13
Mdm2	100	39±8	19±13

The ability of p63 to antagonize the p53 transcriptional activity on reporter genes was evaluated by the luciferase assay as described in methods. Numbers indicate the mean±S.E. of three separate experiments performed in duplicates and are expressed as percent of maximal induction.

the transfected population revealed that TAp63α attenuated the effect of p53 on the reduction of cell number, in a manner similar to that of ΔNp63α (Fig. 7A). In Saos-2 cells, used as a control, TAp63α did not influence this effect of p53 (Fig. 7A) whereas ΔNp63α did, in accordance with the data obtained with the luciferase assays (Table 4).

We then tested the effect of TAp63α on foci formation of C-98, FF-1, C-643 and FTC-133 thyroid cancer cells. Hence, we transfected p53, TAp63α or ΔNp63α into the above-mentioned cell lines and subjected them to antibiotic selection (Fig. 7B, C). Transfection with p53 drastically reduced the number of foci (Fig. 7B, C). No significant reduction of foci was observed in cells transfected with either TAp63α or ΔNp63α (Fig. 7B, C). Interestingly, co-transfection with TAp63α inhibited the effect of p53 on foci, in a manner similar to that of ΔNp63α (Fig. 7B, C).

Since these data were obtained by p63 overexpression, we tried an alternative approach aimed at reducing the expression of TAp63α (Fig. 8). To this end, we subjected the p63-positive FTC-133 cells, which display the highest level of TAp63α (Fig. 3A), and the p63-negative C-98 cells (as a negative control) to p63 silencing by the siRNA technique (Fig. 8). Forty-eight hours after incubation with siRNAs, we transfected p53 together with the p21Cip1 reporter. The luciferase assays revealed that p63 silencing in FTC-133 cells enhanced the p21Cip1 promoter response to increasing doses of p53 cDNA, whereas no difference was observed in the p63-negative C-98 cells, as expected (Fig. 8). Taken together, these data suggest that in thyroid cancer cells TAp63α may have a tumour-promoting role by antagonizing p53 in a manner similar to that of the dominant negative isoform, ΔNp63α.

Discussion

The present results indicate that most thyroid cancers express TAp63α, while normal thyroid cells and benign adenomas do not. The expression of TAp63α in thyroid cancer has been assessed by different techniques, including RT-PCR, western blot and immunohistochemistry. Data obtained in malignant thyroid tissues were confirmed in thyroid cancer cell lines, which were then used as *in vitro* models to study TAp63α function in thyroid cancer. In these cells, we found that endogenous TAp63α does not play a p53-like role, fails to induce the target genes p21Cip1, Bax and MDM2, and does not cause cell growth arrest and apoptosis. Even ectopic overexpression of TAp63α is not able to exert any tumour suppressor activity in thyroid cancer cells. These observations raise several questions about the possible role of TAp63α in thyroid tumorigenesis.

Evidence of p63 expression in human cancers is not novel and has already been reported in a variety of human malignancies, including thymomas, non-Hodgkin's lymphomas, bladder, breast, prostate and lung cancers (Moll *et al.* 2001). It is noteworthy that p63 has also been detected in a small subset of papillary and anaplastic thyroid carcinomas (Preto *et al.* 2002). Immunohistochemistry experiments performed in those studies, however, might have underestimated the real prevalence of p63 expression in thyroid tumours, because of the use of paraffin-embedded specimens, in which the antigenic properties of p63 can be altered. In contrast, by examining frozen samples of thyroid cancer we found that the large majority was positive for p63 expression by immunohistochemistry, a probable effect of the better preservation of the p63 epitopes with the freezing process.

p63 up-regulation in human tumours (Okada *et al.* 2002) is often concomitant with the overexpression of the dominant negative isoform ΔNp63 (Crook *et al.* 2000, Hibi *et al.* 2000, Park *et al.* 2000). In some

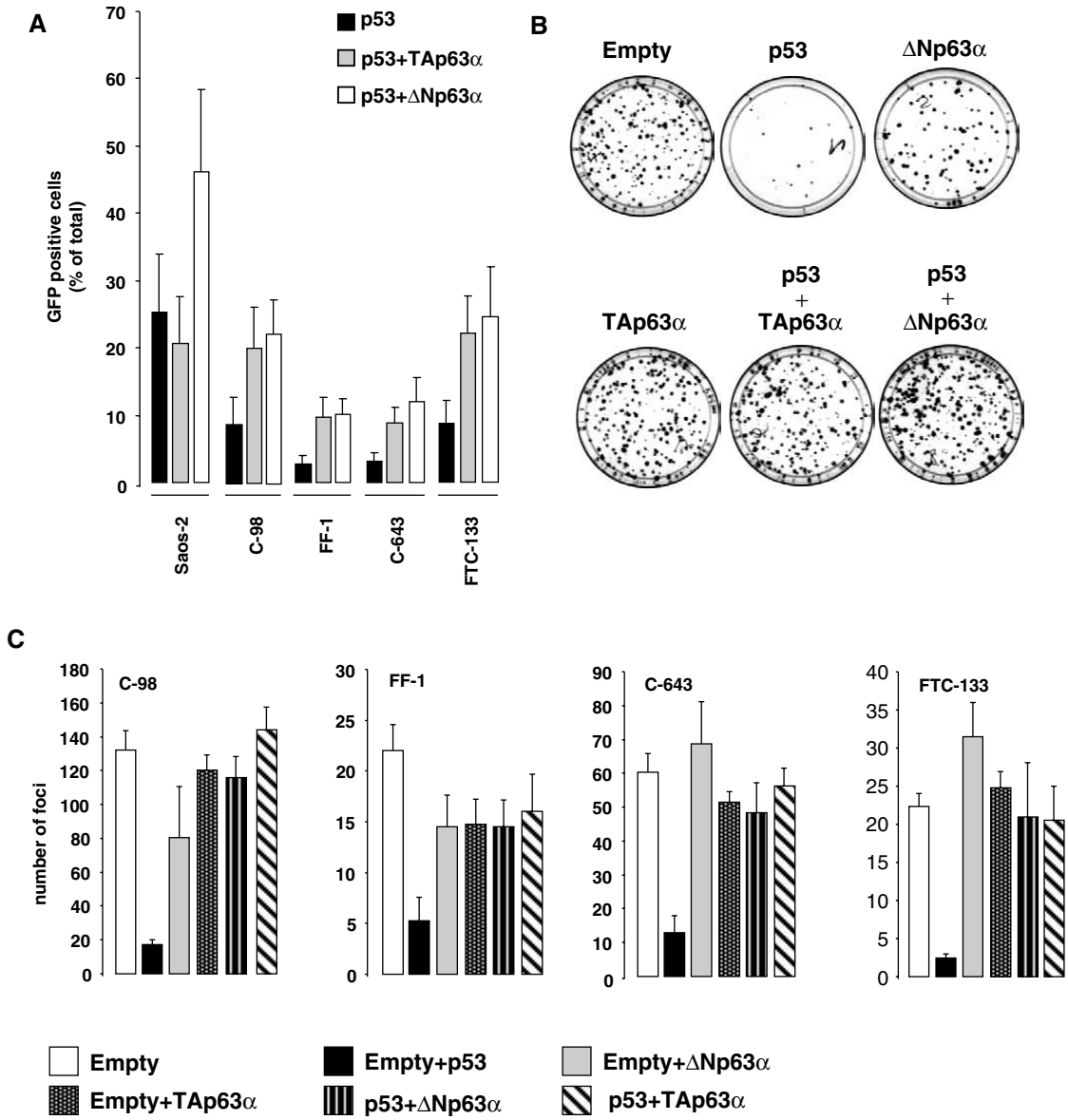

Figure 7 Effect of TAp63α on p53-mediated tumour suppression in thyroid cancer cells. (A) Saos-2 osteosarcoma cancer cells (p53 and p63 negative), C-98, FF-1 (p63 negative), and C-643, FTC-133 (p63 positive) thyroid cancer cells were transfected with the indicated constructs together with H2B-GFP. Forty-eight hours after transfection, GFP-positive cells were counted under a fluorescence microscope and expressed as a percentage of total cells. Values represent means ± S.D. of three separate experiments performed in duplicate. (B) C-98 (p63 negative) thyroid cancer cells were transfected with the indicated constructs. Cells were than split onto 100 mm Petri dishes, subjected to antibiotic (geneticin) selection for 2–4 weeks, and foci were stained with Crystal Violet. (C) C-98, FF-1 (p63 negative), C-643 and FCT-133 (p63 positive) thyroid cancer cells were transfected with the indicated constructs. Cells were than split onto 100 mm Petri dishes, subjected to antibiotic (geneticin) selection for 2–4 weeks, and foci were stained with Crystal Violet, as above. Bars represent the number of foci contained in each plate (mean ± S.D. from eight different plates).

tumours, ΔNp63 is preferentially expressed, suggesting that this p63 isoform may act as an oncogene (Crook *et al*. 2000, Hibi *et al*. 2000, Park *et al*. 2000). However, a previous report showing TAp63 expression in gastric cancer suggests that TAp63 isoforms may also be involved in tumour progression (Tannapfel *et al*. 2001). This might be the case for thyroid tumours, where ΔNp63 is not expressed.

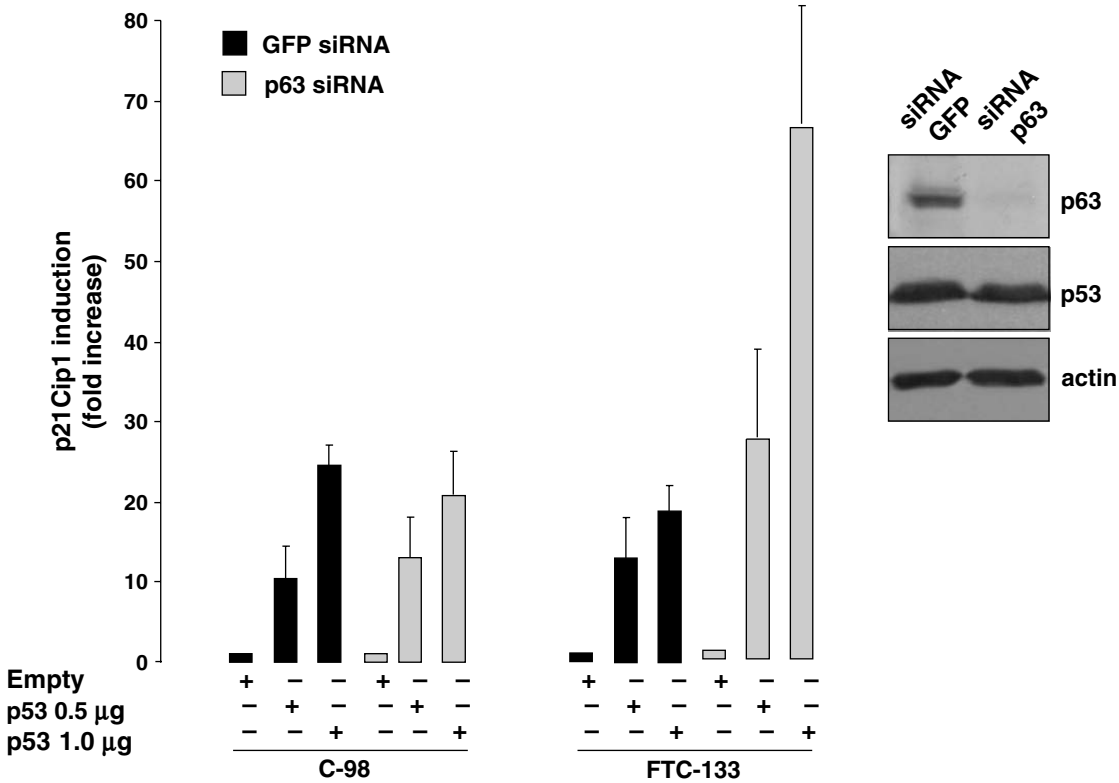

Figure 8 Effect of TAp63α silencing on p53 transcriptional activity in thyroid cancer cells. C-98 (p63 negative) and FTC-133 (p63 positive) thyroid cancer cells were transfected with the indicated siRNAs. Forty-eight hours after transfection, cells were transfected with the indicated doses of pCDNA3.1p53 together with p21Cip1 gene reporter. After 24 h cells were lysed and samples were subjected to luciferase assay. Values represent means ± S.D. of three separate experiments performed in triplicate and are expressed as fold induction over empty-transfected cells arbitrarily set at 1. A representative blot for p63 in FTC-133 cells treated with the indicated siRNAs is shown on the right.

Our studies indicate that in thyroid cancer TAp63α is devoid of any onco-suppressor activity. In fact, genotoxic stress caused by doxorubicin was not able to activate TAp63α in malignant thyroid cancer cells, suggesting that this p63 isoform was not involved in p53-like activities. Since no increase in TAp63α expression was observed after doxorubicin treatment, we first hypothesized an impaired signalling upstream of TAp63α itself. However, TAp63α overexpression, obtained by transient transfection, also failed to induce the target genes p21Cip1, Bax and MDM2, suggesting that the defect was either downstream or intrinsic to TAp63α itself. A defect downstream of p63 was ruled out with the ectopic expression of p53, TAp63β and TAp63γ, which share similar downstream pathways with TAp63α. All these onco-suppressors were still able to transactivate target genes and to exert tumour suppressor activity in these thyroid cancer cells.

One possible inactivation mechanism intrinsic to the p63 protein is the interaction with p53 mutants (Gaiddon *et al.* 2001, Strano *et al.* 2002). In our hands, however, co-immunoprecipitation experiments performed in thyroid cancer cells excluded such interaction. Moreover, experiments performed in the p53-null thyroid cancer cell line SW1736 also ruled out the possibility of a p53 interference with TAp63α (not shown).

In thyroid cancer cells, only TAp63α is transcriptionally weak, since TAp63β and TAp63γ display transcriptional activity similar to that of p53. Therefore, the onco-suppressor activity defect is restricted to the TAp63α isoform. One difference between p53 and p63 resides in the carboxyl tail: p63, in a similar manner to p73, contains a carboxyl terminus that undergoes alternative splicing and gives rise to different isoforms. Deletion studies have shown that the last 71 amino acids at the C-terminal domain (TI domain), which are missing in both TAp63β and TAp63γ, are endowed with inhibitory properties towards p63 transcriptional activity (Serber *et al.* 2002). This inhibition occurs by

an intramolecular interaction between the TI domain and the transactivation domain (TA) located at the N-terminus of p63 (Serber *et al*. 2002). This interaction is responsible for the occupancy of the TA domain, which, consequently, is no longer available for transactivation (Serber *et al*. 2002). Moreover, previous evidence established a dominant negative capability of the TI domain, since ΔNp63α (endowed with TI), but not ΔNp63γ (devoid of TI) is able to inhibit TAp63γ activity (Yang *et al*. 1998). These results outline, therefore, the importance of the TI domain in the dominant negative activity of ΔNp63 isoforms. A previous report has also shown that the p63 C-terminal domain encoded by exon 13 (which is missing in TAp73β) exerts an inhibitory effect on TAp63α activity, possibly by interacting with various proteins (Ghioni *et al*. 2002). It is also known that, although endowed with a very weak transcriptional activity, TAp63α is still able to bind DNA in a manner similar to that of p53 and the other more active p63 isoforms (Yang *et al*. 1998). Taken together, this evidence suggests that in some cell contexts, such as in thyroid cancer cells, TAp63α may occupy the DNA binding sites of p53 responsive elements, thereby preventing occupancy by more transcriptionally active p53 family members. This interpretation could partially explain why TAp63α expression may antagonize p53-mediated tumour suppression in thyroid cancer cells, thus establishing an oncogenic role similar to that of ΔNp63α.

Although this is the first report dealing with direct evidence of a dominant negative/pro-tumourigenic role of TAp63α, indirect evidence of this unsuspected TAp63α role is already present in the literature. Indeed, patients with mutations that introduce a premature stop codon in the TAp63α C-terminus show defects in hands and feet similar to those with mutations in the DNA binding domain (DBD) (Celli *et al*. 1999), suggesting that the loss of DNA binding capability by TAp63α (due to mutations in the DBD) has effects similar to those observed with TAp63α inappropriate activation due to the loss of the TI domain (stop codon). Therefore, both DNA binding and weak transactivation activities may be required for TAp63α to allow proper skeletal development, in order to avoid the premature apoptosis in skeletal precursors. In the light of these considerations, TAp63α should be able to antagonize either homologue (TAp63β and TAp63γ) or paralogue (p53, TAp73) p53 family members, and TAp63α expression in thyroid cancer could be regarded as a mechanism aimed at inhibiting p53-mediated apoptosis.

The TI domain of p63α is very similar to that of p73α, but very different from that of p53 (Moll & Slade 2004). Indeed, a similar antagonistic role against p53-mediated onco-suppression has also been reported for TAp73α in ovarian cancer (Vikhanskaya *et al*. 2000) and leukaemia cells (Freebern *et al*. 2003). It is reasonable to suppose, therefore, that TAp63α and TAp73α, which are endowed with weaker transcriptional activity than p53, may acquire antagonistic properties against p53 in the presence of unknown co-repressor(s) present in some cancer types. This could partially explain why the dominant negative effect of TAp63α is not observed in Saos-2 cells, suggesting that this phenomenon may be cell context-dependent. In this situation, well-differentiated thyroid tumours, rarely harbouring p53 mutations, may take advantage of the expression of TAp63α and ΔNp73α (Frasca *et al*. 2003), which may antagonize p53 and, at variance with p53, are resistant to MDM2-mediated degradation (Okada *et al*. 2002).

The complexity of the p53 family protein network must be taken into account when considering gene therapy in thyroid cancer aimed at restoring the wild-type p53 status. More specifically: (a) unlike TAp63α, TAp63β and TAp63γ are still effective in transactivating target genes and providing tumour suppressor functions in poorly differentiated thyroid cancer cells; (b) p63 proteins are strictly homophilic and are refractory to tetramerization with different members of the family (Moll & Slade 2004) and, therefore, at variance with wild-type p53, ectopic TAp63β and TAp63γ are more resistant to the dominant negative effect of p53 mutants. Hence, adenoviral vectors carrying TAp63β and TAp63γ should be tested in the anaplastic thyroid cancer cells harbouring p53 mutations since they may prove useful for designing possible anti-cancer therapies by gene delivery.

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