TAp73α Increases p53 Tumor Suppressor Activity in Thyroid Cancer Cells via the Inhibition of Mdm2-Mediated Degradation

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Abstract
p53 family proteins include p53 tumor suppressor, p63, and p73. Despite the high similarity in structure and function with p53, p63, and p73 function in tumor suppression is still controversial. Here, we show that TAp73α, a transcriptionally active p73 isoform, is able to synergize p53 tumor suppressor function in thyroid cancer cells. Indeed, depletion of p73 by small interfering RNA in thyroid cancer cells resulted in a reduced transcriptional activity of p53. Ectopic coexpression of both p53 and TAp73α in thyroid cancer cells resulted in increased transcription and tumor suppressor function compared with p53 or TAp73α alone, as well as in increased p53 protein levels. The enhancing effect of TAp73α on p53 activity is Mdm2 dependent because it is prevented by Mdm2 depletion by small interfering RNA. At least two mechanisms may explain the interference of TAp73α with p53 function. First, in thyroid cancer cells, TAp73α inhibits the effect of p53 on Mdm2 induction by antagonizing p53 at the Mdm2 promoter level. Second, a TAp73α mutant (G264W), which is devoid of DNA binding capability, is still able to increase p53 protein levels by competing with p53 for Mdm2 protein binding. Taken together, these results indicate that in thyroid cancer cells, TAp73α is able to increase p53 protein level and function by interfering with Mdm2-mediated p53 degradation. These results may be useful for designing gene therapies aimed at restoring a normal p53 function in thyroid cancer cells.

Introduction
p53, p63, and p73 are transcription factors sharing extensive homologies in structure and functions. In particular, p53 is a tumor suppressor activated by DNA damage, which elicits cell cycle arrest and apoptosis, thereby preventing the replication of cells carrying abnormal DNA and formation of tumors (1-4). In vitro experiments have shown that p63 and p73 are also able to transactivate p53-responsive genes, including p21, Bax, and Mdm2, and to induce cell cycle arrest and apoptosis (1-4). p63 and p73 genes undergo multiple COOH-terminal splicing and, by skipping one or more exons, may give rise to six isoforms for p73 (α, β, γ, δ, ε, and θ) and three isoforms for p63 (α, β, and γ). Very recently, p53 variants have also been described (1, 5-9). The use of alternative starting codons adds more complexity to p53, p63, and p73 networks, with the generation of isoforms lacking the transactivation domain and having a dominant negative effect on the corresponding full-length protein (1-4, 6, 7, 10-13).

Regulation of p53 family protein activity is complex and includes posttranslational modifications, protein stabilization, protein-protein interaction, and modulation of subcellular localization (6, 10, 14, 15). One of the most important p53 target genes is Mdm2, which encodes for a protein binding p53 NH2-terminal domain and targeting p53 for proteasome degradation. Although Mdm2 also binds p73 at a similar domain, it does not regulate p73 degradation (16, 17).

Studies with knockout mice revealed that, whereas p53 is mainly involved in tumor suppression, p63 and p73 are rather involved in embryonic development (18-20). These observations are in accordance with previous data showing that p63 and p73 expression is maximal in embryonic life, whereas it is very low in adult, differentiated tissues. The prevalent tumor suppressor function of p53 is also suggested by the observation that p53-inactivating mutations are very common in tumors whereas, in contrast, mutations in p63 and p73 are rarely observed in human malignancies (21-23). p63 and p73, however, may have a role in tumor progression as suggested by the observation that the dominant negative isoforms ΔNp63 and ΔNp73 are frequently up-regulated in cancer (24-32).

Recent evidences suggest that all p53 family members may cooperate in preventing tumor formation. Indeed, both p63- and p73-deficient cells are resistant to p53-induced apoptosis (33).

Moreover, p53+/−/p73+/− double knockout mice are more prone to develop tumors than p53+/− mice (34). Finally, the aberrant expression of TAp63α and TAp73α isoforms may
favor tumor progression and chemoresistance in some cancer cells (35-37). Taken together, these results indicate that, depending on the cell context, cooperation among p53 family members may be either synergistic or antagonistic with respect to tumor suppression. In this view, cancer may result from a decreased signaling output from p53 family interactome due to either p53 mutation and/or unbalanced expression of isoforms with a dominant negative function.

One model of deregulated interaction among p53 family members is represented by thyroid cancer cells, which may express TAp63α, DNp73α and may harbor p53 mutations (37-46). It is noteworthy that the ectopic expression of p53 by gene delivery in thyroid cancer cells harboring p53 mutations is not able to fully restore p53 tumor suppressor function (47-51). It is possible, therefore, that full restoration of tumor suppressor activity in these cells should take into account additional molecular abnormalities involving the other members of the p53 family proteins. Indeed, we have observed that the restoration of p53 function in anaplastic thyroid cancer cells is accompanied by the down-regulation of TAp73α, which may prevent the full effect of p53 tumor suppressor activity (38).

For this aim, we have verified the hypothesis that in anaplastic thyroid cancer, tumor suppressor activity of ectopic p53 may be ameliorated by preventing TAp73α down-regulation. Hence, in the present study, we have compared the tumor suppressor activity of the ectopic expression of both p53 and TAp73α with the expression of only p53. We found that TAp73α coexpression enhances the cell p53 protein level and its tumor suppressor function by inhibiting Mdm2-mediated p53 degradation. These results may provide useful elements to design novel gene therapy strategies aimed at restoring p53 function in thyroid cancer cells.
FIGURE 2. Cooperation of p53 and TAp73α on p21 and Bax promoters in thyroid cancer cells. **A.** TPC-1 cells were transfected with the indicated doses of either an empty vector (white columns), p53 (gray columns), TAp73α (hatched columns), or both (black columns) along with either p21Luc or BaxLuc reporter promoters. Lysates were subjected to luciferase assay as indicated in Materials and Methods. Real-time PCRs for p53 and p73 are also shown. **B.** ARO cells were transfected with the indicated doses of an empty vector (white columns), p53 (gray columns), TAp73α (hatched columns), or both (black columns) along with H2B-GFP and either p21Luc or BaxLuc reporter promoters. Lysates were subjected to luciferase assay as indicated in Materials and Methods. Real-time PCRs for p53 and p73 are also shown. Bottom, Western blot analysis for p21, Bax, and GFP as a transfection control, and β-actin as a loading control. Representative blots are shown. **C.** Human osteosarcoma Saos-2 cells were transfected with the indicated doses of an empty vector (white columns), p53 (gray columns), TAp73α (hatched columns), or both (black columns), and either p21Luc or BaxLuc reporter promoters. Lysates were subjected to luciferase assay as indicated in Materials and Methods. Real-time PCRs for p53 and p73 are also shown. Numbers are expressed as fold increase over empty-transfected cells. Columns, mean of three separate experiments done in triplicate; bars, SE. Bottom, Western blot analysis for p21, Bax, HA, p53, and β-actin. Representative blots are shown.
Results

Cooperation of p53 and TAp73α in Thyroid Cancer Cells

To study the functional interaction between p53 and TAp73α, two thyroid cancer cell lines were used: SW-1736 (anaplastic thyroid cancer cell line), expressing p73 but not p53 (38), and TPC-1 cells (human papillary thyroid cancer cell line), expressing wild-type p53 but not p73 (52).

p53 was transiently transfected in SW-1736 cells, treated or not with p73 small interfering RNA (siRNA) as described in Materials and Methods. The effect of ectopic p53 on target genes p21 and Bax was evaluated by Western blot (Fig. 1, left), which indicated a clear increase of both proteins after transfection. p73 silencing dramatically decreased the effect of p53 on the induction of both p21 and Bax proteins, suggesting that p73 expression is a prerequisite for p53 activity on these genes. To test this hypothesis, the opposite experiment was done in TPC-1 cells, which express p53, but not p73. p73 transfection in TPC-1 cells clearly increased the cell content of p21 and Bax (Fig. 1, right). However, in p53-depleted cells, the effect of ectopic p73 on both p21 and Bax protein content was decreased, suggesting that p73 is cooperated by p53 in the activation of target genes. Real-time PCR experiments shown in Fig. 1B indicated that the both p53 and p73 siRNA used in these experiments were specific for target transcripts as they did not react with the other member of the p53 family.

Because the absence of either p53 or p73 results in severely attenuated activity of either p73 or p53, these results suggest a cooperation between p53 and p73 in thyroid cancer cells.

FIGURE 3. Effect of TAp73α on p53 binding to p21 and Bax promoters in thyroid cancer cells. A, TPC-1 papillary thyroid cancer cells were transfected with either GFP-p53 or HA-TAp73α, or both, as indicated in Materials and Methods. After 24 h, cells were subjected to ChIP assay for p21 and Bax promoters (right) as described in Materials and Methods. Thymidine kinase promoter was also used as a control. Left, Western blot analysis for GFP (ectopic p53), HA (ectopic TAp73α), p21, Bax, and β-actin as a loading control. Representative blots are shown. Bottom, columns, mean of densitometric analyses of three separate ChIP experiments; bars, SE; values are expressed as percent of input. B, TPC-1 papillary thyroid cancer cells stably transfected with ecdysone-inducible HA-TAp73α (TPC-1–TAp73α cells) were incubated in the presence (PonA) or the absence (Untr.) of ponasterone. After 24 h, cells were subjected to ChIP assay for p21 and Bax promoters (right) as described in Materials and Methods. Thymidine kinase promoter was also used as a control. Left, Western blot analysis in TPC-1–TAp73α cells for HA, p53, p21, Bax, and β-actin. Representative blots are shown. Bottom, columns, mean of densitometric analyses of three separate ChIP experiments; bars, SE. Values are expressed as percent of input.
FIGURE 4. Tumor suppressor effect of the combined expression of p53 and TAp73α in thyroid cancer cells. A, TPC-1, SW-1736, and ARO thyroid cancer cells were transfected with the indicated doses of either empty vector (white columns), p53 (gray columns), TAp73α (hatched columns), or both (black columns), together with H2B-GFP to mark the transfected population. Forty-eight hours after transfection, GFP-positive (viable) cells were counted under a fluorescence microscope and expressed as a percentage of empty-transfected cells. Columns, mean of three separate experiments done in triplicate; bars, SE. B, ARO thyroid cancer cells were transfected as above. Twenty-four hours after transfection, cells were subjected to phycoerythrin-Annexin staining and FACS analysis as described in Materials and Methods. Annexin-positive cells were analyzed among GFP-positive population. A representative experiment is shown. Columns, mean of three separate experiments; bars, SE. C, The indicated inducible cell clones were incubated in the presence or absence of ponasterone for 24 h and with doxorubicin for further 12 h. Cells were then stained with propidium iodide and subjected to cell cycle analysis by FACS as indicated in Materials and Methods. Values are expressed as percent of total cells. Columns, mean of three separate experiments; bars, SE. D, Western blot analysis for the indicated proteins in cells treated as above. A representative experiment is shown.
To study the cooperation between p53 and p73 in thyroid cancer cells, luciferase assays were done. TPC-1 cells were transfected with increasing doses of p53, TAp73α, or both, together with p21Luc and BaxLuc promoters (see Materials and Methods; Fig. 2A, top graphs). The mixture of p53 and TAp73α was significantly more effective in activating p21 and Bax promoters (Fig. 2A, top graphs, black columns). These experiments were also done in ARO cells (Fig. 2B, top graphs, black columns), SW-1736, BC-PAP, and KAT-4 thyroid cancer cell lines (not shown). Cooperation between p53 and TAp73α was not observed in the human osteosarcoma cell line Saos-2, suggesting that this phenomenon is specific of thyroid cancer cells (Fig. 2C, top graphs, black columns). Real-time PCR confirmed the proper ratios of transfected transcripts (Fig. 2A-C, bottom graphs), whereas Western blot analysis confirmed the transfection efficiency and the synergistic effect of ectopic p53 family members on target genes (Fig. 2B and C, right panels). These results suggest that in thyroid cancer cells, coexpression of TAp73α enhances p53 transcriptional activity.

**TAp73α Enhances p53 Activity in Thyroid Cancer**

To study the cooperation between p53 and p73 in thyroid cancer cells, luciferase assays were done. TPC-1 cells were transfected with increasing doses of p53, TAp73α, or both, together with p21Luc and BaxLuc promoters (see Materials and Methods; Fig. 2A, top graphs). The mixture of p53 and TAp73α was significantly more effective in activating p21 and Bax promoters (Fig. 2A, top graphs, black columns). These experiments were also done in ARO cells (Fig. 2B, top graphs, black columns), SW-1736, BC-PAP, and KAT-4 thyroid cancer cell lines (not shown). Cooperation between p53 and TAp73α was not observed in the human osteosarcoma cell line Saos-2, suggesting that this phenomenon is specific of thyroid cancer cells (Fig. 2C, top graphs, black columns). Real-time PCR confirmed the proper ratios of transfected transcripts (Fig. 2A-C, bottom graphs), whereas Western blot analysis confirmed the transfection efficiency and the synergistic effect of ectopic p53 family members on target genes (Fig. 2B and C, right panels). These results suggest that in thyroid cancer cells, coexpression of TAp73α enhances p53 transcriptional activity.
contrast, ChIP done in anti-p73 immunoprecipitates did not reveal any effect of p53 cotransfection on p73 promoter binding. To confirm whether this cooperation may also occur with endogenous p53, TPC-1 cells, stably transfected with ecdysone-inducible TAp73α (TPC-1-TAp73α cells), were also used (pIND system, see Materials and Methods). Western blot analysis in TPC-1-TAp73α cells indicated that incubation with ponasterone A induced HA-TAp73α (Fig. 3B, left panels). In accordance with the results shown in Fig. 3A, induction of TAp73α increased the level of endogenous p53 (Fig. 3B, left panels). ChIP assay done in these cells indicated that expression of HA-TAp73α was concomitant with increased p53 binding to p21 and Bax promoters (Fig. 3B, right panels). Moreover, comparison of ChIP done in either anti-p53 or anti-p73 immunoprecipitates revealed that DNA binding activity of p53 was significantly higher than that of TAp73α (Fig. 3B, right panels and graphs), suggesting that p53 is the major player in the positive p73/53 cooperation.

Taken together, these results indicate that TAp73α is able to enhance p53 activity on the target gene promoters p21 and Bax. However, the mechanisms underlying this p73 effect remained to be elucidated as well as its biological relevance in thyroid cancer cells.
**Figure 7.** Effect of TAp73α on p53 binding to Mdm2 protein. A. TPC-1 papillary thyroid cancer cells were transfected with an empty vector, HA-TAp73α, or HA-TAp73α (G264W) as indicated in Materials and Methods. Thyroid kinase promoter was also used as a control. Representative experiments are shown. B. TPC-1 cells were transfected with GFP-p53 along with an empty vector, HA-TAp73α, or HA-TAp73α (G264W) as indicated in Materials and Methods. H2BGFP was also transfected to control transfection efficiency. After 24 h, cells were subjected to Western blot analysis for GFP, HA, and β-actin (as a loading control). Representative blots are shown. C. TPC-1 cells were transfected with an empty vector, HA-TAp73α, or HA-TAp73α (G264W) as indicated in Materials and Methods. Transfection efficiency is indicated in the anti-HA blot (bottom). Endogenous p53 was immunoprecipitated with anti-p53 polyclonal antibody and subjected to Western blot with either anti-ubiquitin or anti-p53 monoclonal antibody. D. TPC-1 cells were transfected as above, immunoprecipitated with either nonimmune serum or anti-Mdm2 antibody, and blotted with anti-p53 antibody. A crude lysate of transfected cells was also loaded (input). A representative experiment is shown. E. TPC-1 cells were transfected with the indicated doses of an empty vector, p53, TAp73α, p53+TAp73α, TAp73α (G264W), p53+TAp73α (G264W), or TAp73α+TAp73α (G264W) along with p21Luc, BaxLuc, or Mdm2Luc reporter promoters. Lysates were subjected to luciferase assay as indicated in Materials and Methods. Numerals are expressed as fold increase over empty-transfected cells. Columns, mean of three separate experiments done in triplicate; bars, SE.

*TAp73α Increases p53 Oncosuppressor Function in Thyroid Cancer Cells*

To study the biological effects of p73/p53 cooperation, TPC-1, SW-1736, and ARO thyroid cancer cells were transfected with p53, TAp73α, or both, along with H2BGFP to mark transfected population. The number of surviving GFP-positive cells was scored under a fluorescence microscope 48 h after transfection (see Materials and Methods). Both p53 and
TAp73α were able to cause a reduction of GFP-positive cells, although at variable degree of potency (Fig. 4A). In accordance with the results shown in Fig. 2, the mixture of p53 and TAp73α was more effective than p53 or TAp73α alone in reducing the number of viable GFP-positive cells (see Fig. 4A, black columns).

The reduction of the number of GFP-positive cells by p53 family members was then further investigated by fluorescence-activated cell sorting (FACS) analysis (see Materials and Methods). To this end, ARO cells were transfected as above and stained with phycoerythrin-Annexin. In accordance with the results obtained by cell scoring, cotransfection of p53 and TAp73α resulted in increased apoptosis compared with transfection with either p53 or TAp73α alone (Fig. 4B, representative experiment on the left and average of three separate experiments on the right). Similar results were obtained with TPC-1 and SW-1736 thyroid cancer cells (not shown). To confirm results obtained by transient transfection, TPC-1 cell clones stably transfected with either empty vector (TPC-1–empty), ecdysone-inducible TAp73 (TPC-1–TAp73α), or ecdysone-inducible p53 (TPC-1–p53) were used (see Materials and Methods). FACS analysis revealed that coexpression of TAp73α and p53 was more effective in determining cell apoptosis and S-phase decrease in response to doxorubicin than expression of p53 alone (Fig. 4C, compare 8 with 12). Western blot analysis confirmed that coexpression of TAp73α and p53 was more effective in eliciting the response of target genes (including p21, Bax, PUMA, NOXA, p53AIP1, and caspase-3 than p53 alone; Fig. 4D).

These results confirm that TAp73α increases p53 tumor suppressor activity. However, the mechanisms underlying this cooperation remained to be elucidated.

**TAp73α Increases p53 Protein Level via the Inhibition of Mdm2-Mediated Degradation**

To explore the mechanisms of the synergistic effect of TAp73α on p53, we first measured in TPC-1 cells the effect of TAp73α on p53 protein levels. To this end, cells were transfected with GFP-p53 along with either an empty vector or HA-TAp73α and then treated with the protein synthesis inhibitor cycloheximide for the indicated time points (Fig. 5A, top panels). Interestingly, Western blot analysis indicated that cotransfection with TAp73α and p53 was more effective in reducing p53 protein level and stability. Cotransfection with H2BGFP excluded that increased GFP levels were due to increased transfection efficiency (Fig. 5A, top panels). Parallel experiments, done with endogenous p53 in TPC-1-TAp73α cells, indicated that induction of TAp73α increased p53 protein stability (Fig. 5A, bottom panels).

The major mechanism of p53 degradation is regulated by Mdm2 protein, which binds to the p53 NH2-terminal domain (53). To explore the involvement of Mdm2 in the p73-induced potentiation of p53, TPC-1 cells were silenced for Mdm2 expression by small interfering RNA technique. Luciferase assays done on Bax-Luc promoter indicated that, in comparison with control cells, the enhancing effect of TAp73α on p53 transcriptional activity was absent in Mdm2-depleted cells (Fig. 5B). We therefore studied the interaction between p53 and Mdm2 under these conditions. To this purpose, TPC-1 thyroid cancer cells were transfected with GFP-p53 along with either an empty vector or TAp73α and subjected to immunoprecipitation with either anti-p53 or anti-Mdm2 antibody (see Materials and Methods). Cotransfection with TAp73α resulted in a decreased level of p53 in anti-Mdm2 immunoprecipitates, indicating a reduced Mdm2-p53 interaction (Fig. 5C, top panels). Similar results were obtained with endogenous p53 in inducible TPC-1 cells (Fig. 5C, bottom panels). Western blot analysis with ubiquitin antibody in anti-p53 immunoprecipitates indicated that, in accordance with a reduced interaction with Mdm2, induction of TAp73α caused a decreased p53 ubiquitination (Fig. 5D).

Taken together, these results indicate the involvement of Mdm2 protein in the p73/p53 cooperation. How TAp73α may regulate p53 protein level and function via Mdm2 regulation remained to be clarified. Two hypotheses were then tested: (a) TAp73α may directly antagonize p53 binding to the Mdm2 promoter; (b) TAp73α may somehow reduce Mdm2 binding to p53 protein.

**TAp73α Inhibits Mdm2 Promoter Binding and Activation by p53**

To study the interference of TAp73α on p53 binding to the Mdm2 promoter, ChIP was done in TPC-1 cells transfected with either GFP-p53 or HA-TAp73α, or both. ChIP assay done in anti-p53 immunoprecipitates revealed that p53 binding to the Mdm2 promoter is decreased when TAp73α is coexpressed (Fig. 6A).

In contrast, such effect was not seen in anti-p73 immunoprecipitates, indicating that p53 does not exert a similar action on p73 promoter binding (not shown). Western blots indicated the successful transfection of ectopic genes (Fig. 6A, right panels) and confirmed the inhibition of the p53 effect on Mdm2 protein induction by TAp73α (Fig. 6A, right panels).

The negative influence of TAp73α on the activation of the Mdm2 promoter by p53 was then studied in TPC-1 cells transfected with increasing doses of p53, TAp73α, or both, along with reporter genes (see Materials and Methods). In accordance with the results obtained by ChIP assay, TAp73α was able to significantly antagonize the ability of p53 to activate the Mdm2 promoter (Fig. 6B). Experiments done in ARO cells (Fig. 6C) and in SW-1736, BC-PAP, and KAT-4 thyroid cancer cell lines confirmed these results (not shown). Again, Western blot analysis showed the antagonistic effect of TAp73α on Mdm2 protein induction by p53 (Fig. 6C, right panels). Similar results were obtained in ecdysone-inducible TPC-1 cells (Fig. 6D).

Taken together, these results indicate that TAp73α antagonizes p53 binding to the Mdm2 promoter. This TAp73α effect results in a reduced Mdm2 promoter activation by p53, a reduced Mdm2 protein synthesis and, as a consequence, a reduced Mdm2-mediated p53 degradation, which may explain the increased p53 protein level.

**TAp73α Stabilizes p53 Level by Competition for Binding Sites on Mdm2 Protein**

Although p73 degradation is Mdm2 independent, Mdm2 also binds p73 (54). We then tested the alternative mechanism; that is, the possible competition of TAp73α with p53 for
binding sites of the Mdm2 protein, which may result in an increased p53 protein stability. To this end, a naturally occurring p73 mutant, TAp73α(G264W), devoid of DNA binding domain capability, was used (55). Indeed, ChIP experiments indicated that, at variance with TAp73α, TAp73α(G264W) was not able to bind the p21 promoter in TPC-1 cells (Fig. 7A).

Interestingly, in TPC-1 cells transfected with GFP-p53 together with either an empty vector or HA-TAp73α or HA-TAp73α(G264W), cotransfection with HA-TAp73α(G264W) resulted in clearly increased GFP-p53 protein levels (Fig. 7B, see anti-GFP blot), suggesting that p73 is able to increase p53 protein level in thyroid cancer cells also by a mechanism independent of promoter binding. Anti-ubiquitin Western blots in these cells indicated that transfection of HATAp73α(G264W) was able to decrease the ubiquitination of endogenous p53 at a level comparable with that of HA-TAp73α (Fig. 7C, anti-ubiquitin blot).

p53-Mdm2 coimmunoprecipitation experiments were consistent with these findings: Again, cotransfection with the p73 mutant TAp73α(G264W) resulted in a decreased p53 level in anti-Mdm2 immunoprecipitates, indicating that TAp73α(G264W), although not binding to DNA, was able to reduce the interaction between p53 and Mdm2 (Fig. 7D).

The effect of TAp73α(G264W) on p53 transcriptional activity was then tested by the luciferase assay. As expected, TAp73α(G264W) was devoid of any promoter activation capacity and displayed a dominant negative effect against TAp73α. Moreover, TAp73α(G264W) was able to enhance p21 and Bax promoter activation by p53 to an extent similar to that of TAp73α (Fig. 7E). Finally, TAp73α(G264W) displayed no antagonistic effect on the p53 activation of the Mdm2 promoter (Fig. 7E, right columns).

These results indicate that the TAp73α protein, independently of its DNA binding capacity, is still able to enhance p53 stability by competing for its binding with the Mdm2 protein. However, whether most TAp73α effect is dependent on Mdm2 promoter binding or on Mdm2 protein interaction is still unclear.

Discussion

The present study indicates that in thyroid cancer cells, p53 and TAp73α synergistically cooperate for activation of target genes and, as a consequence, for potentiation of the oncosuppressor function typical of these proteins. The mechanism of this cooperation is based on the increased p53 protein level and stabilization caused by TAp73α expression, which inhibits Mdm2-mediated p53 protein degradation via two mechanisms. First, TAp73α inhibits p53 binding to the Mdm2 promoter: As a consequence, Mdm2 induction in response to p53 is reduced. Second, TAp73α competes with p53 for binding to the Mdm2 protein.

Our results, obtained in thyroid cancer cells, are in accordance with previous reports indicating a blunted p53-induced apoptosis in p73+/−/− cells (33). The cooperation between p53 and p73 is also supported by studies in knockout models: Mice lacking one allele of both p53 and p73 (p53+/−, p73+/−/− mice) are more prone to develop spontaneous epithelial tumors than mice lacking only one allele of p53 (p53+/− mice; ref. 34). Taken together, these evidences suggest that coexpression of p73 favors p53 tumor suppressor activity in vivo and conversely the lack of p73 may result in blunted p53 function. A p53/p73 cooperation has also been described in other models: ectopic TAp73α on endogenous p53 protein level and activity in SK-N-DZ, MCF-7, and U-2OS cells (56), although the mechanism of this p73 effect was not clarified. In contrast, in mouse T cells and in lymphomas, apparently p73 is not required for p53-induced apoptosis (57).

Discrepancies regarding p53/p73 cooperation may depend on both the cell type and the experimental context. Indeed, TAp73α enhances p53 activity in human thyroid cells but not sarcoma cancer cells. The present study shows that in thyroid cancer cells, TAp73α is a poor activator of the Mdm2 promoter, although it binds to this promoter with high affinity; therefore, it acts as p53 antagonist. This finding is in accordance with previous reports in ovarian cancer and leukemia cells that show an antagonistic effect of TAp73α (35, 58, 59) due to the occupancy of p53-responsive promoters. At variance with those previous reports, however, the present study indicates that in thyroid cancer cells, such antagonistic effect of TAp73α is selective for the Mdm2 promoter. This selectivity, however, may be apparent as the antagonistic effect of TAp73α on other p53-responsive promoters may be hidden by the concomitant increase of p53. Because this antagonism was not observed in Saos-2 osteosarcoma cells (not shown), the effect is likely cell type dependent.

It is also noteworthy that in thyroid cancer cells, TAp73β did not display the antagonistic effect of the α isoform toward p53 on the Mdm2 promoter (not shown). The different behavior of the two TAp73 isoforms may suggest that this effect of TAp73α depends on the SAM domain, which is not present in TAp73β. Because the SAM domain is a site module for protein-protein interaction in both p63 and p73, it is possible that thyroid cancer cells express proteins acting as TAp73α coresspressors (1, 14, 60-62). A similar mechanism has already been described for TAp63α able to antagonize p53 effects in thyroid cancer cells (37).

Although Mdm2 binds to the NH2 terminus of both p53 and p73 and, via this binding, it inhibits their transactivating capabilities, different effects derive from Mdm2 binding to the two proteins: When binding to p73, at variance with p53, Mdm2 does not cause p73 nuclear export and protein degradation (54). Rather, Mdm2 binding to p73 results in p73 nuclear accumulation (63). It is possible, therefore, that p73 bound to Mdm2 accumulated in the nucleus and, although devoid of transactivating capability, maintains its DNA binding capability. Under these circumstances, depending on the Mdm2 promoter (and other promoters) availability and binding affinity, p73 may act as a p53 competitor in a manner similar to that already reported for ΔNp73α (64).

Finally, our study indicates that a DNA binding–independent mechanism is also active in the TAp73α potentiation of p53 activity. Experiments done with the TAp73α(G264W) mutant, which is devoid of DNA binding capability (55), indicate in fact that TAp73α is still able to increase p53 protein level, an effect that may occur because of Mdm2 sequestration and the consequent prevention of its binding to and degradation of p53.

This effect of TAp73α(G264W) mutant on p53 activity is in line with the common observation that mutations of p73...
gene are very rare in human tumors (11, 55). Indeed, it is reasonable to suppose that mutations in the DNA binding
domain of p73 result in a p73 protein devoid of dominant negative effect toward p53 tumor suppressor function, but
rather it may reinforce p53 activities. This hypothesis is supported by the notion that oligomerization domain of p53
family members is highly homophilic and does not allow formation of p53/p73 heterotetramers. Therefore, these p73
mutants may not behave as inhibitors of the other members of the family (65, 66).

We have also investigated the effect of p53 on TAp73α and
found no significant changes in TAp73α protein levels or
promoter binding depending on p53 (not shown). These observations suggest that p53/p73 cooperation is not reciprocal.

Although coexpression of TAp73α and p53 is a common
finding in some thyroid cancers (38), the TAp73α/p53 cooperation is not likely to occur in those tumors because
ΔNp73α is also present and may inhibit any positive p53/p73 cooperation.

The present data provide a better understanding of the complexity of the oncogenic/oncosuppressor balance in tumor
progression. Moreover, these data may contribute to explain the limited success of gene therapies aimed to restore p53 status in
thyroid cancer cells and other tumor types. It is possible that viral vectors, carrying both p53 and TAp73α, may be more
effective than vectors carrying only p53 for increasing cancer cell apoptosis, both spontaneous and after chemotherapy.
TAp73α coexpression, indeed, may neutralize Mdm2 response to p53 reintroduction, which is often very robust in cancer cells carrying p53 mutants (67) and able to (partially) neutralize wild-type p53 reintroduction effect.

Materials and Methods

Cells

Papillary (TPC-1 and BC-PAP) and anaplastic (ARO and
KAT-4) thyroid cancer cells were provided by Drs. A. Fusco
and M. Santoro (Istituto di Biologia e Patologia Cellulare
e Molecolare, University of Naples, Naples, Italy); anaplastic
thyroid cancer cells SW-1736 were provided by Dr. N.E.
Heldin (Department of Genetics and Pathology, Uppsala
University Hospital, Uppsala, Sweden). These cell lines were
grown in complete 10% fetal bovine serum RPMI 1640. The
human osteosarcoma cell line Saos-2 was provided by Dr. J.Y.
Wang and cultured in complete 10% fetal bovine serum
DMEM (Sigma). The TPC-1–TAp73α and TPC-1–p53 cell
lines were constructed by cotransfecting the pIND expression
induction (2.5 g/mL ponasterone A) and selecting cotransfectants in G418–Zeocin–containing
media. Cells were subcloned and screened for TAp73
and p53 protein expression by Western blot after ponasterone A
(2.5 g/mL for 24 h). TPC-1–inducible clones were
maintained in 300 μg of G418 and 200 μg of zeocin per
milliliter.

Plasmids and Transfections

pcDNA3.1-p53-GFP was a gift of Geoffrey Wahl and
Jane Stommel (The Salk Institute, La Jolla, CA). PBOS-
H2BGFP, pcDNA3.1-HA-TAp73α, pcDNA3.1-HA-ΔNp73α,
and pcDNA3.1-p53 were kindly provided by Dr. J. Wang
(University of California at San Diego, La Jolla, CA); p21Luc,
BaxLuc, and Mdm2Luc were donated by Dr. G. Blandino
(Regina Elena Cancer Institute, Rome, Italy). pcDNA3.1-HA-
TAp73α (G264W) mutant was kindly provided by Prof. K.
Hagiwara (Tohoku University, Tokyo, Japan). The pIND-
TAp73α and pIND-p53 expression vectors were constructed
using standard techniques by cloning the TAp73α and p53
cDNA into the KpnI/NotI cloning site of the ecdysone-
ducible expression vector pIND (Invitrogen).

All transfections were done by the Fugene 6 method (Roche Biochemical, Inc.) according to the manufacturer’s instructions, and cells were processed 24 h after transfection.

Immunoprecipitation and Immunoblot Analysis

Cell lysates were prepared in complete radiolabeled
proteinase inhibitor cocktail (Pepe), 1 mg of cell lysate was incubated for 2 h with 2 μg of antibody and
protein A–Sepharose was then added (Amersham Biosciences). Samples were then subjected to SDS-PAGE and
membranes incubated with primary antibodies in 5% milk-
TBST (1 μg/mL). Horseradish peroxidase–conjugated secondary antibodies were used for protein detection by enhanced chemiluminescence (Pierce).

The following antibodies were used for immunoprecipitation:
monoclonal antibody DO-1 against the NH2 terminus of
p53 (Santa Cruz Biotechnology), anti-p53 (C-19) polyclonal
antibody (Santa Cruz Biotechnology), anti-Mdm2 polyclonal
antibody (Santa Cruz Biotechnology) for immunoprecipitation experiments.

The following antibodies were used for Western blotting:
anti-HA monoclonal antibody (CRP); anti-p53 monoclonal
antibody DO-1 (Santa Cruz Biotechnology); anti-p21Cip1
polyclonal antibody (Santa Cruz Biotechnology); a mixture
of anti-p73 monoclonal antibodies (Ab-4; Neomarkers), anti-
β-actin monoclonal antibody (Sigma), anti-GFP monoclonal
antibody (CRP), and a polyclonal anti-Mdm2 antibody (Santa
Cruz Biotechnology); anti–caspase-3 monoclonal antibody
(3G2; Cell Signaling); anti-NOXA (FL-54) polyclonal
antibody (Santa Cruz); rabbit anti-p53AIP1 (H-91) polyclonal
antibody (Santa Cruz Biotechnology); and anti-ubiquitin
(P4D1) monoclonal antibody (Cell Signaling).

Real-time PCR

Total RNA (5 μg) was reverse transcribed by Thermo-
Script RT (Invitrogen) and oligo(dT) primers. Synthesized
cDNA (0.15 μL) was then combined in a PCR reaction using
primers 5'-TGTCCCCCGACGATATTTG-3' (forward),
5'-TGCGATTCTGAGGATCCTG-3' (reverse) and probe 5'-
CAATTGCTCAGAAGCCGAGCTCA-3' specific for
the p53. Tp73 amplification was done using the following
primers: 5'-GCACCGTCTTGGACGACCTTCT-3' (forward) and
5'-GCAGATGACTGCGCCGAT-3' (reverse). Primer Express
software (PE Applied Biosystems) was used to design
appropriate primer pairs and fluorescent probes. Primer pairs
and probes with 5-FAM reporter dye and 3-TAMRA quencher
dye were synthesized by MWG-Biotech. Probe and primers for
endogenous control (actin) were from predeveloped TaqMan
assay reagents (Applied Biosystems). Quantitative real-time PCR was done on ABI Prism 7700 (PE Applied Biosystems) using Sybr Green PCR Master Mix and Taqman Universal PCR Master Mix (PE Applied Biosystems) following manufacturer’s instructions.

**Luciferase Assay**

The p21Luc, BaxLuc, and Mdm2Luc constructs were cotransfected with pcDNA3.1, pcDNA3.0-Myc-TAp73α, pcDNA3.1HATp73α (G264W), and pcDNA3.1-p53 (DNA ratio 1:1). A vector coding for the Renilla luciferase was also cotransfected in all conditions (DNA ratio 1:20). In some experiments, the transfection efficiency was monitored by cotransfection with H2B-GFP. Twenty-four hours after transfection, cells were processed with the Dual Luciferase Assay (Promega Corp.) according to the manufacturer’s instructions. Luciferase activity was normalized for transfection efficiency (Renilla activity).

**Gene Silencing by siRNA**

Cells were plated onto six-well plates (10⁵ per well); maintained in antibiotic-free medium for 24 h; and transfected with a mixture containing Opti-MEM, 8 μL/well Lipofectamine (Lipofectamine 2000, Invitrogen), and either 0.5 μg/well scrambled siRNA or a mixture of 4 Mdm2 siRNA (Dharmacon Research, Inc.) 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 either p53, TAp73α, or both (0.5-1.0 μg/well) along with either p21Luc, BaxLuc, or Mdm2Luc (1.0 μg) and Renilla (0.2 μg), using Fugene6 reagent (Roche). Cells were processed 24 h after transfection.

**FACS Analysis**

For apoptosis evaluation, GFP vector was cotransfected as marker along with either p53 or TAp73α, or both. Apoptosis measurement was done by staining with phycoerythrin-Annexin (BD) and evaluating Annexin-positive cells among the GFP-positive cell population, according to the manufacturer’s protocol. Cells were then evaluated by FACS analysis (GFP = FL1, phycoerythrin = FL2). Annexin-positive cells were considered to be in early apoptosis.

**ChIP Assay**

TPC-1 cells (1 x 10⁶/150 mm PD) were transfected with the indicated constructs by Fugene6 reagent according to the manufacturer’s instructions (Roche). Twenty-four hours after transfection, DNA and proteins were cross-linked by the addition of formaldehyde to a final concentration of 1% for 10 min before harvesting. Cross-linking reaction was stopped by adding glycine at 0.125 mol/L final concentration for 10 min at room temperature. Plates were rinsed twice with ice-cold 1 x PBS and cells were scraped off the plates, resuspended in cell lysis buffer [5 mmol/L PIPES (pH 8.0), 85 mmol/L KCl, 0.5% NP40, and protease inhibitors]. Cells were dounced on ice with a B dounce several times to aid nuclei release. Then, nuclei were spun down and resuspended in nuclei lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.1), and protease inhibitor mixture] and sonicated to generate chromatin to an average length of ~200 to 600 bp. After centrifugation at 14,000 rpm for 10 min at 4°C, samples (2-4 μL of protein extracts) were precleared with 10 μL protein G–Sepharose beads (preblocked with 1 μg/mL bovine serum albumin and 1 μg/μL salmon sperm DNA) for 2 h at 4°C and immunoprecipitated overnight at 4°C with 2 μg anti-p53 monoclonal antibody (DO-1 from Santa Cruz Biotechnology). Two micrograms rabbit IgG were used as a negative control or immunoprecipitation. Immunoprecipitates were washed with radiouimmunoprecipitation assay buffer and immunoprecipitation washing buffer [100 mmol/L Tris-HCl (pH 8.0), 500 mmol/L LiCl, 1% NP40, 1% deoxycholic acid]. Twenty percent of supernatant from the rabbit IgG immunoprecipitation was saved as total input of chromatin and was processed with the eluted immunoprecipitates beginning at the cross-link reversal step. Immunocomplexes were eluted with elution buffer (1% SDS, 50 mmol/L NaHCO₃). Salmon sperm DNA (0.1 μg/μL) and 10 μg of RNase A were added to the pooled eluates and cross-links were reverted by incubation at 65°C for 6 h. Samples were diluted with 125 μL water containing 0.16 μg/μL proteinase K and incubated for 1 h at 50°C. DNA was purified with phenol/chloroform and a fraction (5 μL) was used as PCR template to detect the presence of promoter sequences of p21, Bax, Mdm2, and thymidine kinase using the primers listed below:

- p21 forward: ATGTATAGGACGAAGGTGCAGAC.
- p21 reverse: CCTCCTTTCTGTGCCCTGAAC.
- Bax forward: GGGTTATCTCTTGGGCTCACAA.
- Bax reverse: GAAGCTCTCCACCCAGCGCA.
- Mdm2 forward: GGAATGGGCCCAGGTCATGGG.
- Mdm2 reverse: GGTCTACCTCCAAATCGCCAC.
- TK forward: GGTTGGCTCCTCCCTGGAATT.
- TK reverse: GTCAAGGGATGGTTCTATACCTCCT.

The PCR products were resolved in 2% agarose gels and visualized by ethidium bromide staining.

**Statistical Analysis**

Results were compared by two-way ANOVA. Significance was obtained by t test (*P < 0.05, **P < 0.01, ***P < 0.001). Statistical analysis was carried out with Microsoft Excel Software.

**References**


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Roberta Malaguarnera, Veronica Vella, Giuseppe Pandini, et al.


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