

p53 family proteins in thyroid cancer

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Abstract

At variance with other human malignancies, p53 mutations are not frequent in thyroid cancer and are believed to be responsible mainly for cancer progression to poorly differentiated and aggressive phenotype. p63 and p73, two proteins with a high degree of homology with p53, are overexpressed in thyroid cancer, but their role in cancer initiation or progression is controversial. Regulation of p53 family protein function depends on: (1) the balance between the expression of transcriptionally active (p53, TAp63, and TAp73) and inactive isoforms (Δ Np63 and Δ Np73); (2) their interaction and competition at DNA-responsive elements; (3) their interaction with regulatory proteins, either inhibitory or activating. In thyroid cancer, therefore, although mutations of the p53 oncosuppressor protein family are rare, other mechanisms are present, including aberrant expression of p53 family dominant negative isoforms, up-regulation of inhibitory proteins, and functional inhibition of activating proteins. The overall result is a defective oncosuppressor activity. These inactivating mechanisms may be present in the early stages of thyroid cancer and in different cancer histotypes. A better understanding of this complex network may not only ameliorate our comprehension of cancer biology, but also open the possibility of innovative diagnostic procedures and the development of targeted therapies.

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p53 in thyroid cancer

Oncogene gain of function is the most frequent molecular alteration described in thyroid cancer. It mainly includes the aberrant activation of the RAS/RAF/MEK/ERK pathway (Kroll 2004, Hunt 2005). These alterations regard the rearrangements of Ret/PTC and Trk tyrosine kinase receptors and point mutations of *RAS* or *BRAF* genes (Kroll 2004, Hunt 2005). Recently, mutations in the *PI3KCA* gene, resulting from the constitutive activation of the PI-3K pathway, have also been described in anaplastic thyroid carcinomas (Garcia-Rostan *et al.* 2005). Loss of function of tumor suppressor proteins may also occur in thyroid cancer and includes PAX-8/PPAR γ rearrangement, PTEN down-regulation, β -catenin, and p53 mutations (Kroll 2004, Hunt 2005).

While inactivating mutations of the *p53* gene is very frequent in human cancers (50% of all human malignancies), they have been found in only 10% of thyroid carcinomas (Olivier *et al.* 2002; Fig. 1A) and mainly in poorly differentiated and aggressive histotypes. These observations and the indolent progression of most thyroid carcinomas have brought

to the paradigm that p53 role is minor in thyroid cancer initiation and early stages, while it is important only in the rare case of progression to aggressive phenotypes (Harvey *et al.* 1995). However, the role of p53 functional inactivation in thyroid cancer has never been carefully studied and remains controversial.

The p53 protein has three major functional domains, all conserved in the p53 homologs p63 and p73: the N-terminal transactivation domain (TAD), the C-terminal oligomerization domain (OD), and the core domain, which has DNA-binding activity (DNA-binding domain, DBD; Fig. 2A) (Murray-Zmijewski *et al.* 2006). The vast majority of p53 mutations occurs within the DBD domain (Olivier *et al.* 2002), thus impairing p53 binding to DNA (Fig. 1B). p53 is an important tumor suppressor, as it integrates multiple stress signals and regulates cell response to DNA damage by the induction of a series of target genes, which regulate cell cycle arrest. This allows DNA damage repair or apoptosis when cells are severely damaged (Murray-Zmijewski *et al.* 2006). These biological effects are elicited by p53 binding to responsive promoters which, in turn, activate the transcription of genes like *p21* (G1 cell growth arrest),

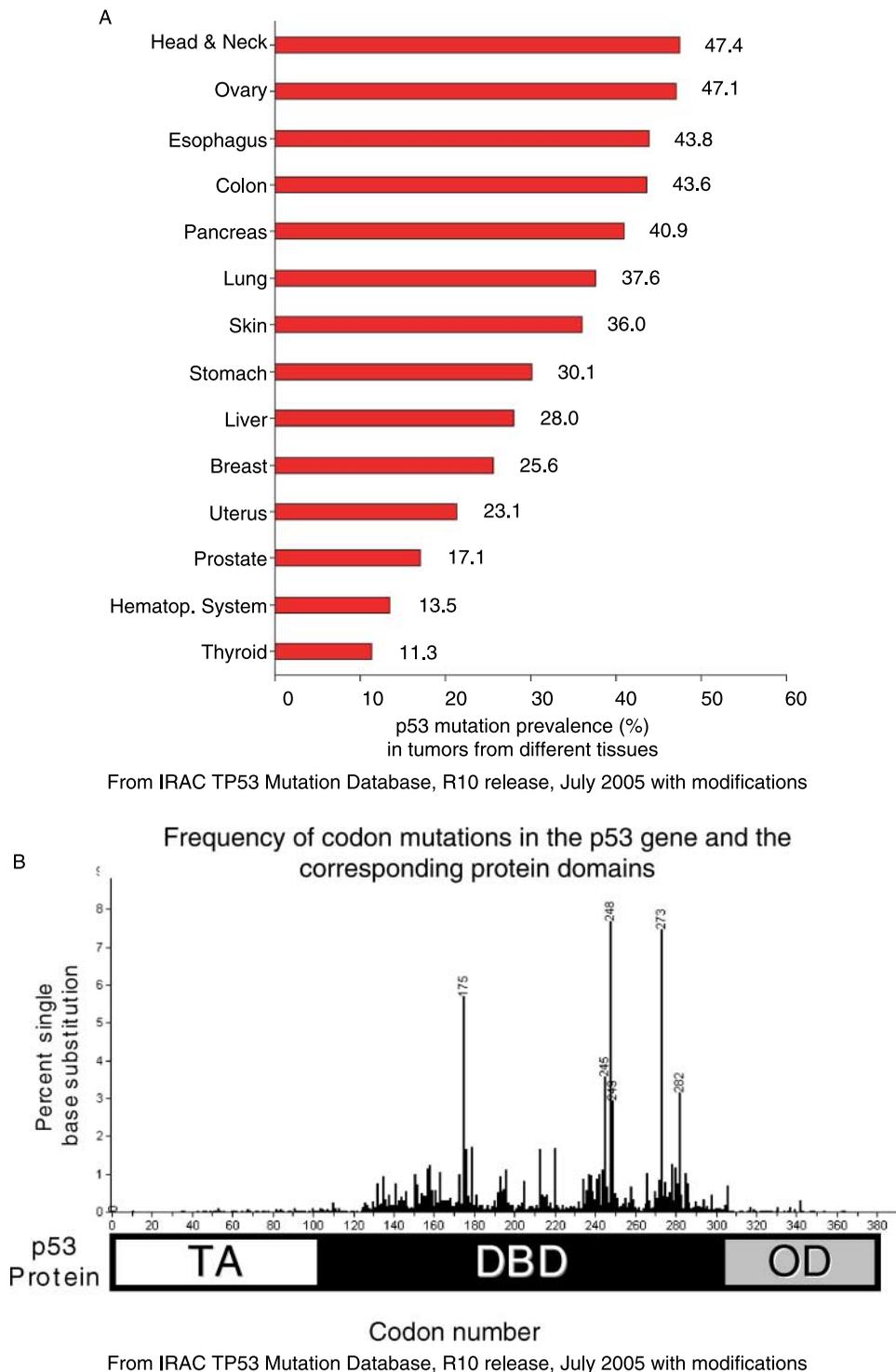


Figure 1 p53 inactivating mutations. (A) Frequency of p53 mutations among tumors from different human tissues. Mutations most frequently occur in the DNA-binding domain. (B) Frequency of mutations among the different codons of the *p53* gene. Data are obtained from the indicated reference (Olivier et al. 2002).

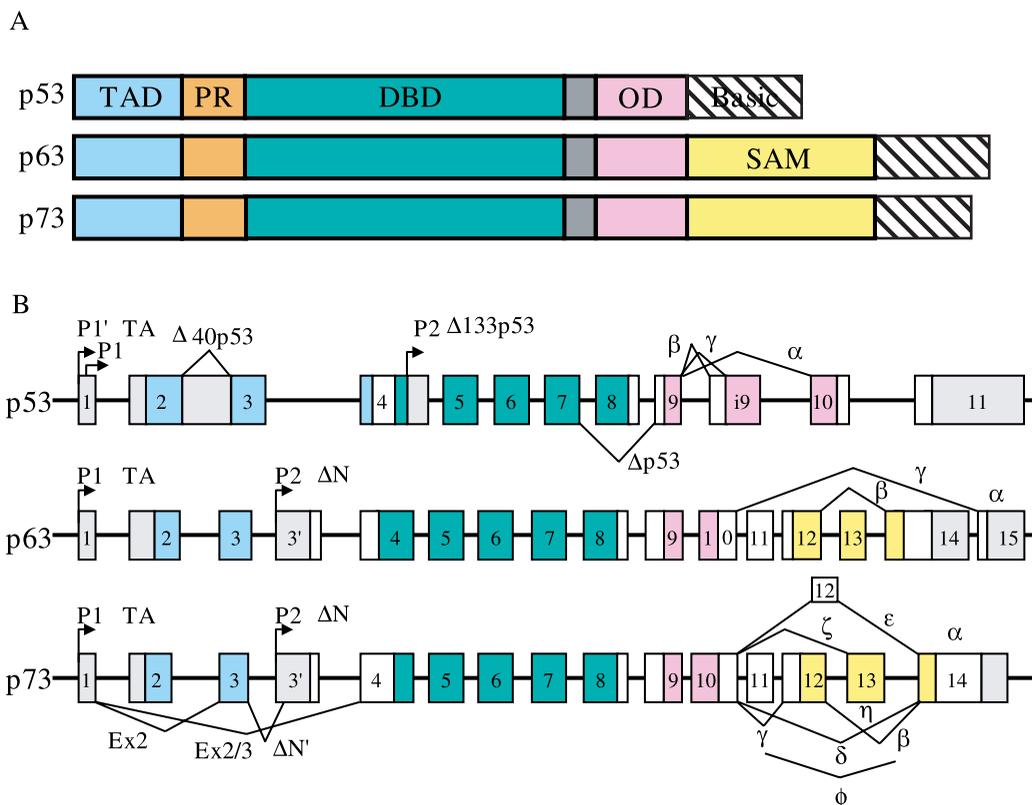


Figure 2 p53 family members and isoforms. (A) Functional domains of p53 family member genes. Blue, transactivation domain (TAD); orange, proline-rich region (PR); green, DNA-binding domain (DBD); pink, oligomerization domain (OD); yellow, sterile α motif (SAM); hatched, post-SAM basic domain. (B) Intron/exon structure. Introns are depicted in gray, while exons are colored according to the functional domains. All three genes express multiple splicing variants and contain different internal promoters. The p53 isoforms include: TAp53, $\Delta 40$ p53 (generated by either alternative splicing of intron 2 or alternative initial translation site in exon 4 at codon 40), Δ p53 (produced by alternative splicing of exons 7/9 with a deletion of 66 amino acids within DBD), and $\Delta 133$ p53 (generated using an internal promoter in intron 4). The alternative splicing of intron 9 gives rise to α , β and γ isoforms. Altogether, p53 gene can transcribe ten different variants: p53 (α , β , γ), $\Delta 40$ p53 (α , β , γ), $\Delta 133$ p53 (α , β , γ) and Δ p53 (Courtois *et al.* 2004, Bourdon *et al.* 2005, Mills 2005, Rohaly *et al.* 2005, Mills 2006). In the case of p63 and p73, the proximal promoter P1 yields the TA isoforms, while the distal promoter P2, in intron 3, gives rise to Δ Np63/p73-truncated variants. In addition, the COOH-terminal splicing leads to p73 α , β , γ , δ , ϵ , ζ , η , ϕ and p63 α , β , γ for both TA and Δ N forms. Moreover, p73 can use an additional NH₂-terminal splicing site, within exon 2, that produces Δ N like proteins Ex2p73, Ex2/3p73, and Δ N'p73 (Moll & Slade 2004).

Bax, and *PUMA* (apoptosis; Murray-Zmijewski *et al.* 2006). Another p53 major target gene is *Mdm2*, encoding for an ubiquitin ligase that binds to the N-terminus of the p53 protein and causes p53 inactivation, nuclear export, and degradation. *Mdm2* induction in response to p53 is the major negative feedback loop aimed at restraining p53 proapoptotic function and thus allowing cell repair. p53 is also a major regulator of cell senescence. Indeed, telomere shortening caused by cell replications triggers p53 activation, thereby blocking cell cycle and favoring cell entry into senescence. As a consequence, p53 inactivation may contribute to the increased number of cell replications and, eventually, to the accumulation of further genetic abnormalities and the acquisition of the immortalized phenotype in cultured cells (Stewart & Weinberg 2006). Finally, the p53 gene can use different

internal promoters and/or internal translation start sites to produce a variety of N-terminally truncated isoforms, with a variable degree of dominant negative activity. As depicted in Fig. 2B, the p53 gene contains two upstream promoters (P1 and P1') that transcribe $\Delta 40$ p53 and Δ p53. Furthermore, the use of an internal promoter in intron 4 leads to an NH₂-truncated p53 protein termed $\Delta 133$ p53 (Courtois *et al.* 2004, Bourdon *et al.* 2005, Mills 2005, Rohaly *et al.* 2005, Mills 2006).

While in anaplastic carcinomas (Salvatore *et al.* 1996, Quiros *et al.* 2005), mutations of both *RAS/BRAF* and p53 genes may be observed, in well-differentiated thyroid cancer, *BRAF* and *RAS* mutations, but not p53 mutations, are frequent. This suggests the possibility that p53 mutations may occur as a secondary event, concomitant with the loss of cell differentiation.

Factors favoring p53 mutations in thyroid cancer are not fully understood. *In vitro* experiments have shown that exposure of immortalized human thyroid cells to either α -particles or γ -rays causes p53 mutations and the acquisition of cell ability to be tumorigenic in nude mice (Gamble et al. 1999). *In vivo*, however, the relationship between exposure to radiation and p53 mutation is less clear. Studies performed in the Belarus population after the Chernobyl accident did not prove a definitive correlation between radiation exposure and p53 mutations in thyroid cancer (Nikiforov et al. 1996, Smida et al. 1997, Suchy et al. 1998, Pisarchik et al. 2000). Recent evidences suggest that p53 mutations in thyroid cancer may be favored by the genomic instability occurring during the tumor progression process (Shahedian et al. 2001).

Other studies on p53 protein expression in a large series of thyroid tumor specimens suggest that, although not mutated, p53 activity may be inhibited in thyroid cancer by other mechanisms. Indeed, increased p53 protein levels were observed by immunohistochemistry not only in anaplastic and poorly differentiated thyroid cancer, where p53 mutations are frequent, but also in well-differentiated cancers, in the absence of any p53 mutation (Soares et al. 1994, Pollina et al. 1996, Park et al. 1998). Nonfunctioning p53 cannot induce Mdm2, its major degrading protein, and consequently accumulates in the cell nucleus. A strong p53 staining in paraffin-embedded specimens, therefore, is considered indirect evidence of nonfunctioning p53. Nuclear accumulation of wild-type p53 protein and reduced p53 tumor suppressor function in some differentiated thyroid cancers is also suggested by the observation of a correlation between elevated p53 protein content and poor clinical outcome (Dobashi et al. 1993, Gerasimov et al. 1995, Nishida et al. 1996, Ruter et al. 1996, Hosal et al. 1997, Godballe et al. 1998, Chen et al. 1999). Wild-type p53 inactivation is also suggested by *in vitro* studies in thyroid cancer cells: H-RAS-transformed rat thyrocytes display wild-type p53 protein accumulation (Burns et al. 1992) and isolated rat thyroid cancer cells in culture display both wild-type p53 overexpression and a defect in G1 arrest in response to DNA damage (Wyllie et al. 1995). Several independent mechanisms may be hypothesized to explain the wild-type p53 inactivation in thyroid carcinomas, including p53 cytoplasmic retention (Zedenius et al. 1996) and Mdm2 overexpression (Jennings et al. 1995, Zou et al. 1995). Indeed, in a large series of differentiated thyroid cancer specimens, immunohistochemistry indicated that Mdm2 is overexpressed and its expression level directly correlates with a poor clinical

outcome (Jennings et al. 1995, Zou et al. 1995, Czyz et al. 2001, Horie et al. 2001).

In addition to its role in tumor progression in poorly differentiated thyroid cancer, p53 inactivation has also been implicated in chemoresistance (Blagosklonny et al. 1998, Ceraline et al. 2003, Hassan et al. 2006). Several reports indicate that wild-type p53 gene delivery into anaplastic thyroid cancer cells induces a partial differentiation, with the re-expression of thyroid specific-genes, and makes cells more vulnerable to the effect of chemotherapy (Fagin et al. 1996, Moretti et al. 1997, Blagosklonny et al. 1998). This effect may be increased by the concomitant use of histone deacetylase inhibitors, which stimulate p53 acetylation and functional activation (Imanishi et al. 2002). More recently, in anaplastic thyroid cancer, p53 mutations were used as a target for oncolytic viruses. For instance, the *E1B* gene-defective adenovirus (ONYX-15), which is able to replicate only in cells that are p53 defective, was used to kill anaplastic thyroid cancer cells both *in vitro* and *in vivo* (Portella et al. 2003, 2002). These approaches of gene therapy, although novel and intriguing, are unlikely to be available for clinical use in the next future because of some concerns about cell-killing efficiency.

In conclusion, although p53 is rarely mutated in thyroid cancer and mainly in undifferentiated tumors, the paradigm that it is only involved in advanced cancer progression is today contradicted by a series of evidences indicating that it may also play a role in the early stages of thyroid cancer. In these tumors, wild-type p53 activity may be inhibited by several inactivating mechanisms acting on the protein and/or its signaling pathway.

Other members in p53 family: p63 and p73

Two novel genes, named *p63* and *p73*, have recently been discovered as members of the p53 tumor suppressor family because of their remarkable similarity with p53 structure and functional domains (Lohrum & Vousden 2000). Structural homology between these three proteins suggests that they also share similar tumor suppressor functions. However, recent studies have demonstrated that the functions of these proteins are not entirely redundant since each of them can behave as a p53 agonist or antagonist, and can also play its own unique biological tasks (Courtois et al. 2004, Moll & Slade 2004).

Like p53, p63 and p73 also contain three major domains: the TAD, the DBD, and the (OD) (Moll et al. 2001). Similarly, p63 and p73 also are expressed as multiple isoforms, the products of alternative splicing, and the use of two different promoters. P1 promoter,

upstream of exon 1, yields full-length proteins (TAp63 and TAp73, containing the TA domain), while P2, spanning intron 3, produces NH2-terminally truncated forms Δ Np63 and Δ Np73 (missing TAD). The latter isoforms may play a dominant negative function (Moll & Slade 2004) by both competition for DNA binding and oligomerization with isoforms containing the TA domain. Moreover, p73 can undergo alternative splicing of exon 2, producing additional isoforms lacking the TAD (Ex2p73, Ex2/3p73, and Δ N'p73).

Additional complexity to this network of protein isoforms arises from multiple splicing of the COOH terminus, skipping one or several exons (Moll & Slade 2004). Thus far, several COOH-terminus transcripts have been identified for p53 family members: α , β , γ for p53 (Fig. 2B); α , β , γ for p63; and α , β , γ , δ , ϵ , ζ , η , ϕ for p73. Interestingly, the α isoforms of p73 and p63 (but not of p53) contain a sterile α motif (SAM; Fig. 2A) that mediates specific protein–protein interactions (Ghioni *et al.* 2002, Blandino & Dobbelsstein 2004). With respect to p63 and p73 activity, it depends on many factors, including their expression pattern and the functional crosstalk between full-length protein and other isoforms.

Several reports have indicated that, whereas p53 protein is ubiquitously expressed, p63 and p73 expression is more restricted and dependent on cell differentiation and development stage (Blandino & Dobbelsstein 2004). For instance, p63 is expressed in proliferating basal cells of the epidermis and squamous epithelia (Mills *et al.* 1999) and p63-deficient mice exhibit defects in the epithelia as well as craniofacial malformations. In humans, p63 germ-line missense mutations cause Ectrodactyly, Ectodermal dysplasia, facial Clefts (EEC) and Ankyloblepharon, Ectodermal dysplasia, Clefting (AEC) syndromes (Celli *et al.* 1999, Mills *et al.* 1999, Fomenkov *et al.* 2003).

Like p63, p73 also has its own distinct development functions (Yang *et al.* 2000). p73 knockout mice show site-specific development defects in hippocampus, immune system, and behavior (Yang *et al.* 2000, Benard *et al.* 2003). Moreover, Δ Np73 has been shown to inhibit neuronal apoptosis by blocking p53 pro-apoptotic function (Pozniak *et al.* 2000, Casciano *et al.* 2002). These observations suggest that p73 and Δ Np73 play an important role in development and differentiation, by protecting neuronal precursors from apoptotic death (Pozniak *et al.* 2000).

Different isoforms of p53 family members can play opposite roles, depending on various conditions. Ectopic TAp63 and TAp73 are able to bind to p53-responsive promoters and cause p53-like functions (tumor suppressor) in human cells, whereas the

N-terminally truncated Δ Np63 and Δ Np73 isoforms may have an oncogenic role by antagonizing full-length TAp63, TAp73, and TAp53 (Benard *et al.* 2003). Thus, the tumor suppressor function of these proteins depends on the balance between the different isoforms expressed in different cells and tissues.

Recent findings suggest that TAp63 and TAp73, isoforms that have potential tumor suppressor activity, under certain conditions may also be involved in the acquisition and maintenance of the transformed phenotype.

TAp63 and TAp73 are very rarely mutated in cancer cells and often overexpressed. These observations are in concert with a possible role of these proteins in tumorigenesis (Ikawa *et al.* 1999, Yang *et al.* 2000, Moll 2003, Flores *et al.* 2005). Interestingly, in some tumors, this overexpression is concomitant with that of the dominant negative isoforms Δ Np63 and Δ Np73, which may override the effects of the transcriptionally active isoforms explaining why, as a final effect, p63 and p73 may function as oncogenes (Moll 2003). A possible role of p63 and p73 in cancer progression might also be explained by the ability of each protein isoform to affect the functions of the other members of the family (Lohrum & Vousden 2000). For instance, p53 mutants can oligomerize with p63 and p73 transcriptionally active isoforms via the DBD and inhibit their transcriptional activity. On the other hand, p63 and p73 can antagonize wild-type p53 binding to the DNA-responsive promoters (Strano *et al.* 2001). In this way, p63 and p73 isoforms may differentially interfere with the biological properties of wild-type p53, inhibiting its tumor suppressor activity and thereby conferring a selective survival advantage to cancer cells (Benard *et al.* 2003). These observations provide a possible explanation for the negative role of p63 and p73 overexpression in many human cancers, (Hibi *et al.* 2000, Benard *et al.* 2003, Mills 2006).

In conclusion, the discovery of p53 homologs, p63 and p73, has clarified that p53 exerts its tumor suppressor function in association with its homologs, within a complex network of physical and functional interactions, where the specific activities of TAp63 and TAp73 are still less understood than those of p53, because their expression level and the function of different isoforms and their regulation have not yet been studied. Undoubtedly, understanding the relationship between these three proteins and their isoforms with either overlapping or opposite roles will provide intriguing insights into new and exciting therapeutic approaches for cancer.

p63 and thyroid cancer

A possible role of p63 in the malignant transformation of thyroid follicular cells is supported by two lines of evidence: (1) p63 is involved in epithelial cell differentiation (Yang *et al.* 1998, Reis-Filho *et al.* 2003, Reis-Filho & Schmitt 2002); and (2) a number of human epithelial malignancies express a high levels of p63 isoforms (Hibi *et al.* 2000, Marin & Kaelin 2000, Moll *et al.* 2001, Massion *et al.* 2003, Moll & Slade 2004). How p63 isoform expression pattern may influence tumor progression, however, remains unclear.

A specific role of p63 in thyroid cancer was first denied by studies performed in paraffin-embedded specimens, which did not detect p63 in either normal or malignant thyroid tissues (Di Como *et al.* 2002). Subsequent studies, however, found p63 immunostaining in a small subset of papillary and anaplastic thyroid carcinomas (Preto *et al.* 2002, Reis-Filho *et al.* 2003, Reis-Filho & Schmitt 2002), but not in normal thyroid tissue, nodular goiter, and oncocytic follicular adenomas (Unger *et al.* 2003, Burstein *et al.* 2004).

The latter results were confirmed by the immunohistochemistry experiments performed in frozen thyroid tissues, where the TAp63 α protein was found in a high percentage of thyroid carcinomas, but, again, not in normal thyroid cells and benign adenomas (Malaguarnera *et al.* 2005). In the same study, RT-PCR, western blot and immunohistochemistry indicated that TAp63 α is the predominant isoform expressed in both thyroid cancer tissues and malignant thyroid cells in permanent culture (Malaguarnera *et al.* 2005). One possible explanation for the discrepancy between these studies and the previous ones is that the paraffin-embedding process could damage the p63 epitopes, thereby preventing its detection by immunohistochemistry (Malaguarnera *et al.* 2005).

Although the high prevalence of TAp63 α expression in thyroid cancer suggests its possible role in thyroid carcinogenesis, no clue is available on the specific function of TAp63 α in thyroid cancer initiation and/or progression. TAp63 α , in fact, may display bi-functional properties (either tumorigenic or tumor suppressor) depending on the cellular context (Mills 2006), its binding to different target gene promoters, and the interaction with specific proteins. When functional studies on p63 were performed in order to evaluate p63 effects on target genes, at variance with other human cell types where p63 exerts some p53-like functions (Zhu *et al.* 1998, Sasaki *et al.* 2001), in thyroid cancer cell lines, no effect was observed in terms of transactivation of p53-responsive promoters p21, bax, and Mdm2 (Malaguarnera *et al.* 2005). In

thyroid cancer cells, therefore, the tumor suppressor activity of endogenous TAp63 α is absent. Moreover, ectopic TAp63 α also failed to induce p21, bax, and Mdm2 genes, thereby suggesting the existence of an inhibiting mechanism, not due to the TAp63 α molecule but due intrinsically to thyroid cancer cells (Malaguarnera *et al.* 2005).

A defect downstream of TAp63 α was ruled out because the ectopic expression of p53 (which activates similar signaling pathways) was still able to transactivate target genes and to exert tumor suppressor activity in these cells (Malaguarnera *et al.* 2005). Since the inhibition of target gene expression was restricted to p63, a specific p63 inhibitor, i.e. Δ Np63 α , which is often up-regulated in cancers (Yang *et al.* 1998, Crook *et al.* 2000, Hibi *et al.* 2000, Park *et al.* 2000, Yamaguchi *et al.* 2000, Massion *et al.* 2003) was evaluated, but was not detected in thyroid cancer tissues and cell lines by RT-PCR (Malaguarnera *et al.* 2005).

Finally, co-immunoprecipitation experiments excluded that the inhibitory mechanism was the direct interaction between the core domains of TAp63 α and p53 mutants (Gaiddon *et al.* 2001, Strano *et al.* 2001), with the formation of complexes that impair TAp63 α transcriptional activity (Malaguarnera *et al.* 2005), a mechanism that is anyway unlikely in well-differentiated thyroid carcinomas, which seldom harbor p53 mutations.

Interestingly, thyroid cancer cell transfection with p63 isoforms different from TAp63 α (i.e. TAp63 β and TAp63 γ) was able to elicit the activation of p63 target genes (Malaguarnera *et al.* 2005). Recently, a C-terminal inhibitory domain was identified in TAp63 α , but not in TAp63 β and TAp63 γ isoforms. This domain is both necessary and sufficient for TAp63 α transcriptional activity inhibition, since it binds to the TAD and masks the residues that are important for the protein transactivation activity (Moll *et al.* 2001, Serber *et al.* 2002). The TAp63 α C-terminal domain also includes a SAM domain, which is a protein-protein interaction sequence (Thanos & Bowie 1999, Ghioni *et al.* 2002). It is possible to speculate, therefore, that one or more co-repressor protein(s) are present in thyroid cancer cells and may interact with this domain, present in the TAp63 α isoform but not in TAp63 β and TAp63 γ isoforms, and, consequently, specifically inhibit only TAp63 α transcriptional activity (Malaguarnera *et al.* 2005). This possibility is under investigation in our laboratory.

As an additional mechanism for TAp63 α tumorigenic activity in thyroid cancer, we have to consider that TAp63 α , devoid of oncosuppressor activity, may bind DNA at the same sites than p53 (Yang *et al.* 1998) and, therefore, antagonize p53 oncosuppressor activity

by competition, preventing the binding of more transcriptionally active p53 family members (Malaguarnera *et al.* 2005). Indeed, in thyroid cancer cells, TAp63 α prevents the effect of p53 on thyroid cancer cell viability and foci formation at an extent comparable with that of Δ Np63 α (Fig. 3). This antagonistic role of TAp63 α against p53 in thyroid cancer is confirmed by gene silencing experiments: suppression of p63 expression by siRNA causes an improvement of p53 transcriptional function and oncosuppressor activity (Malaguarnera *et al.* 2005).

These data indicate that TAp63 α has an oncogenic rather than oncosuppressor role in thyroid cancer by a variety of mechanisms, including counteraction of p53 tumor suppressor function. Recent evidence, indicating that in mice TAp63 overexpression is able to accelerate skin tumorigenesis by up-regulating pro-tumorigenic proteins, confirms the potential tumorigenic role of TAp63 α (Koster *et al.* 2006).

The mechanisms underlying this aberrant p63 expression in malignant thyroid cells are not clear. One possible explanation is that p63 expression is a direct consequence of cancer cell origin. A subset of papillary thyroid carcinomas may originate from pluripotent, p63-positive embryonic remnants, more biologically labile than mature thyrocytes and thereby more susceptible to undergo oncogenic changes leading to papillary thyroid carcinomas (Burstein *et al.* 2004). In concert with this possibility is the observation of a similar p63 immunostaining in solid cell nests of the normal thyroid (remnants of the ultimo-brachial body) and in some papillary thyroid cancers (Reis-Filho *et al.* 2003, Burstein *et al.* 2004).

Since in thyroid cancer cells alterations of p53 signaling and abnormal p63 expression may be present at the same time, it is not possible to attribute to p63 expression a causative role in thyroid tumor development (Hibi *et al.* 2000, Di Como *et al.* 2002, Westfall

& Pietenpol 2004). On the other hand, there is no evidence that the pro-oncogenic role of p63 can be attributed only to p53 tumor suppressor activity inhibition. As already mentioned, the possibility that p63 may confer oncogenic properties to pluripotent stem cells cannot be excluded.

In conclusion, we know that p63 may have an oncogenic rather than oncosuppressor function in thyroid cancer, but mechanisms and specific role in the p53 family protein network require a better understanding.

p73 and thyroid cancer

Although a large amount of data is available on p73 expression in human malignancies, data on human thyroid cancer are scanty. A quantitative RT-PCR analysis indicates that both TAp73 and Δ Np73 transcripts are present in a consistent number of human thyroid carcinomas, although no correlation was found with the tumor clinical and pathological characteristics (Ferru *et al.* 2006). At immunohistochemistry, the presence of p73 and Δ Np73 staining was observed in human thyroid cancer specimens, predominantly at nuclear level (Frasca *et al.* 2003, Ito *et al.* 2006), and RT-PCR analysis confirmed that both TAp73 α and Δ Np73 α are expressed in malignant, but not in normal, thyroid tissues.

Data in tissues were confirmed by *in vitro* studies: RT-PCR and Western blot detected TAp73 α and Δ Np73 α isoforms in the large majority of thyroid cancer cell lines from different histotypes (papillary, follicular, and anaplastic), but not in normal cultured thyrocytes. These data suggest that p73 expression in cancer cells is related to thyroid malignant transformation (Frasca *et al.* 2003, Vella *et al.* 2003, Ito *et al.* 2006). Similarly to TAp63 α , the presence of TAp73 α , a protein with potential tumor suppressor activity, is also counter-intuitive in malignant thyroid cells.

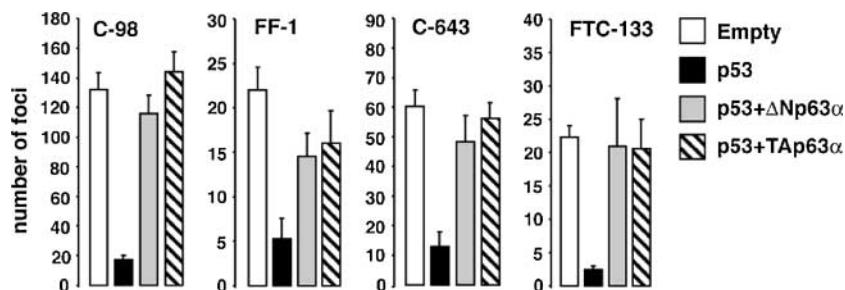


Figure 3 In thyroid cancer cells, TAp63 α prevents p53 tumor suppressor function. The indicated thyroid cancer cell lines were transfected with either empty vector (white bars), p53 (black bars), p53 plus Δ Np63 α (gray bars), or p53 plus TAp63 α (hatched bars). Transfected cells were then subjected to antibiotic selection with G418. Compared with empty vector, p53 dramatically inhibited the number of foci formation, whereas co-transfection with the dominant negative p63 isoform Δ Np63 α prevented the p53 suppression on colony formation, as expected. Surprisingly, also TAp63 α co-transfection inhibited p53-driven foci suppression.

To clarify this issue, functional studies were carried out and indicated that, in thyroid cancer cells, TAp73 α is not functional in response to the DNA-damaging agent doxorubicin and fails to cause G1 arrest and/or apoptosis (Frasca et al. 2003, Vella et al. 2003).

TAp73 mutations, which rarely occur in human tumors (including central nervous system, lung, and breast; Melino et al. 2002), were never found in thyroid cancer cells by p73 mRNA sequence analysis. Moreover, ectopic TAp73 α activity was also blunted in thyroid cancer cells, suggesting that inactivating mechanisms other than mutations are present (Melino et al. 2002).

Previous studies had shown that the apoptotic function of p73 is regulated by the c-Abl tyrosine kinase (Agami et al. 1999, Gong et al. 1999). Differences in c-Abl expression and activity were not found in either normal or malignant thyroid cells (Vella et al. 2003). In the latter, however, indirect immunofluorescence staining and nuclear/cytoplasmic fraction separation revealed a predominant cytoplasmic localization of c-Abl and a reduced nuclear import, suggesting that the subcellular segregation of c-Abl and p73 could contribute to the defective activation of p73 tumor suppressor function. To explore this hypothesis, we used an Abl construct with constitutive nuclear localization (AblNuk; Fig. 4) and bound to a FK506-binding protein (FKBP) sequence, to allow dimerization and conditioned activation (McWhirter et al. 1993, McWhirter & Wang 1997, Klemm et al. 1998, Yang et al. 1998, Smith & Van Etten 2001). When the nuclear AblNuk-FKBP was activated by the presence of the dimerizer AP20187 (Fig. 4), apoptosis occurred in p73-positive, but not in p73-negative thyroid cancer cells (Fig. 4), indicating that when c-Abl can reach p73 in the nucleus, the oncosuppressor function will follow. Co-expression of either p73DD (which interferes with p73 oligomerization) or the baculovirus p35 protein, a potent inhibitor of caspases (Clem 2001), reduced the apoptotic response. These results indicate that in thyroid cancer cells, the restoration of c-Abl nuclear import can induce a p73- and caspase-dependent apoptosis (Fig. 4).

Thyroid cancer cell lines used in these studies are derived from poorly differentiated cancer and harbor p53 mutations (Frasca et al. 2003). Previous reports demonstrated that p53 mutants may interact with TAp73 and inhibit its function (Gaiddon et al. 2001, Frasca et al. 2003). Indeed, co-immunoprecipitation experiments performed in these thyroid cancer cells showed that, at variance with TAp63 α , TAp73 α interacts with p53 mutants. This is an additional p73 inactivating mechanism, which may occur in poorly differentiated thyroid cells where p53 is frequently mutated. In

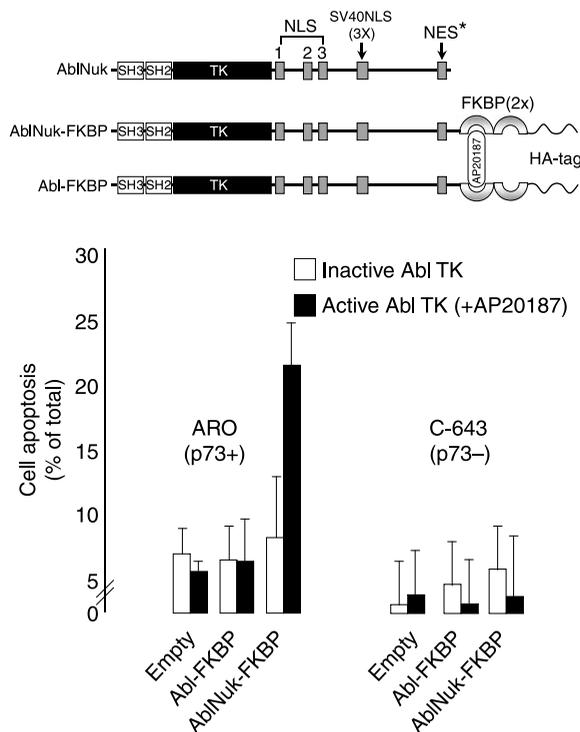


Figure 4 Restoration of c-Abl nuclear import activates p73 tumor suppressor function. Diagram of AblNuk, AblNuk-FKBP, and Abl-FKBP. To render Abl constitutively nuclear, its nuclear export sequence was inactivated with a single point mutation (NES*), whereas three copies of SV40 nuclear localization signals (SV40NLS(3X)) were inserted in-frame downstream of the native NLSs of c-Abl (AblNuk). To conditionally activate c-Abl tyrosine kinase through dimerization, two copies of FKBP were fused to the C terminus of c-Abl and AP20187 was employed as a dimerizer (Abl-FKBP). ARO (p73-positive) and C-643 (p73-negative) thyroid cancer cells were transfected with either AblNuk-FKBP or Abl-FKBP and treated (black bars), with 50 nM AP20187 (dimerizer) to activate Abl tyrosine kinase (Abl TK). Restoration of c-Abl nuclear import by AblNuk-FKBP transfection induced apoptosis in p73-positive thyroid cancer cells (ARO, left), but not in p73-negative cancer cells (C-643, right).

addition, previous studies suggested that in ovarian cancer and leukemia cells, TAp73 α antagonizes p53 transcriptional activity (Vikhanskaya et al. 2000, 2001, Freebern et al. 2003): this is another possibility that cannot be excluded in thyroid cancer cells. Finally, co-immunoprecipitation experiments indicated that TAp73 α may also interact with its dominant negative isoform Δ Np73 α , which, at variance with Δ Np63 α , is highly expressed in thyroid cancer cells.

Overall, these data indicate that the presence of either p53 mutants or Δ Np73 α contributes to the functional inactivation of TAp73 α in thyroid cancer cell lines. In many human tumors including thyroid cancer, in fact, TAp73 α is co-expressed with the dominant negative isoform Δ Np73 α (Frasca et al. 2003), as a consequence of the fact that TAp73 α is a

major inducer of $\Delta Np73\alpha$ (Nakagawa *et al.* 2003). In some thyroid cancer, $\Delta Np73\alpha$ is expressed at a higher level than TAp73 α (resulting in an increased ΔN /TAp73 expression ratio), a condition that in other tumors (i.e. ovarian cancer) is related to an increased aggressiveness and chemoresistance (Casciano *et al.* 2002, Zaika *et al.* 2002, Concin *et al.* 2004, 2005, Ugur *et al.* 2004, Guan & Chen 2005, Muller *et al.* 2005, Dominguez *et al.* 2006). $\Delta Np73\alpha$ is also expressed in well-differentiated thyroid cancers that have a wild-type p53. In these well-differentiated tumors, $\Delta Np73\alpha$ expression may represent an additional mechanism for inactivating the tumor suppressor function of p53. In these cases, TAp73 α expression may be considered indirectly responsible for wild-type p53 inactivation via the induction of $\Delta Np73\alpha$ expression (Fagin *et al.* 1993, Frasca *et al.* 2003).

In summary, (a) p73 expression is a marker of thyroid cell malignant transformation, (b) overall, the biological role of TAp73 α in malignant thyroid cells may be ambivalent and depends on many different factors, and (c) the interactions of the various p73 isoforms within the molecular network of p53 family members are complex and await additional investigation.

All p53 family members are inhibited by HMGA1 up-regulation in thyroid cancer cells

The high mobility group A factors (HMGA1a, HMGA1b, and HMGA2) are non-histone proteins, with several different functions, including gene transcription, malignant transformation promotion, and metastatic progression (Reeves 2001, Sgarra *et al.* 2004). All members of the HMGA family contain multiple copies of a DNA-binding site called the ‘AT-hook’, which binds to the narrow minor groove of stretches of AT-rich DNA sequences. In addition, the HMGA proteins participate in specific protein–DNA and protein–protein interactions that induce both structural changes in chromatin and formation of stereospecific complexes called ‘enhanceosomes’ on the promoter/enhancer regions of genes. The expression level of *HMGA* genes is maximal during the embryonic development, while it is very low in well-differentiated and adult cells (Zhou *et al.* 1995, Chiappetta *et al.* 1996, Hirning-Folz *et al.* 1998). HMGA proteins are rapidly induced by growth factor stimulation and are involved in the control of cell proliferation (Ayoubi *et al.* 1999). Consistent with such a growth regulatory role, homozygous mutations in the *Hmga2* gene in mice result in the *pygmy* or ‘mini-mouse’ phenotype (Zhou *et al.* 1995).

Increased expression of HMGA proteins may promote a transformed phenotype in cell lines (Reeves 2001, Wood *et al.* 2000a, 2000b, Treff *et al.* 2004a, 2004b) and transgenic mice overexpressing HMGA proteins develop tumors (Fedele *et al.* 2002, 2005, Xu *et al.* 2004). In thyroid cancer, as in several human cancers (including colorectal, prostate, breast, cervical, lung), the HMGA1 protein level is high and correlates with the increasing degree of malignancy and metastatic potential (Sgarra *et al.* 2004). Interestingly, the use of HMGA2 antisense vector results in the suppression of both HMGA1 and HMGA2 synthesis and in the prevention of the retrovirus-mediated neoplastic transformation of rat thyroid cells (Berlingieri *et al.* 1995). The requirement for HMGA1 expression during rat thyroid cell transformation is further confirmed by antisense techniques specific for HMGA1 (Berlingieri *et al.* 2002). Moreover, suppression of HMGA1 protein synthesis by adenoviruses causes apoptosis in anaplastic human thyroid cancer cell lines leading to a drastic decrease in tumor growth *in vivo* (Scala *et al.* 2000). Despite these numerous lines of evidence regarding the pro-tumorigenic role of HMGA, the mechanism(s) underlying this effect are still poorly understood.

In thyroid cancer cells, all the three p53 family members are present with an expression pattern that is complex and different in different tumors (Ruter *et al.* 1996, Frasca *et al.* 2003, Vella *et al.* 2003, Malaguarnera *et al.* 2005). In general, the tumor suppressor activity of these proteins is kept latent by several mechanisms, including interaction with p53 mutants, dominant negative isoform overexpression, and impaired activation mechanisms. Since HMGA overexpression is very common in thyroid cancer, the possibility of an interference of HMGA proteins on p53 family oncosuppressor function was investigated. In several thyroid cancer cells of different histotypes, *HMGA1* gene silencing indicated that HMGA1 protein has an inhibitory effect on both ectopic and endogenous p53 family member activity (Fig. 5A). Moreover, fluorescence activated cell sorting (FACS) analysis indicated that HMGA1 is able to inhibit p53 activity on G1 cell cycle arrest and apoptosis, both under basal conditions and after exposure to the DNA-damaging agent doxorubicin (Fig. 5B). Co-immunoprecipitation experiments showed that HMGA1 protein directly interacts with all members of the p53 family. Further experiments with deletion mutants demonstrated that the C-terminal oligomerization domain of the p53 family members is required for the direct interaction with the HMGA1 protein (Frasca *et al.*

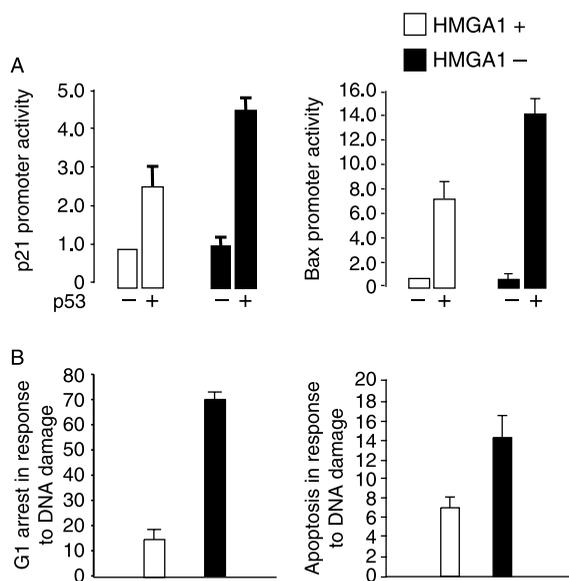


Figure 5 HMGA1 inhibits the function of p53, TAp63 α , and TAp73 α in thyroid cancer cells. TPC-1 thyroid cancer cells were silenced for HMGA1 by siRNA. In these cells, ectopic p53 transcriptional activity was increased by HMGA1 depletion (compare black with white bars in A). Depletion of HMGA1 resulted in increased p53-mediated G1 arrest and apoptosis in response to DNA damage (compare black with white bars in B).

2006). Indeed, HMGA1 silencing resulted in increased p53 oligomerization in response to doxorubicin in thyroid cancer cells. Moreover, electrophoretic mobility shift assays indicated that the interaction of HMGA1 with p53 attenuates p53 DNA-binding activity (Frasca *et al.* 2006).

These data indicate that HMGA1 overexpression is another mechanism by which p53 family member function is kept latent in thyroid cancer cells and that HMGA1 directly interacts with the oligomerization domain of p53 family transcription factors, thereby preventing proper oligomerization, DNA binding, and, as a consequence, transcriptional and tumor suppressor activity (Frasca *et al.* 2006). Since HMGA1 overexpression is very common in thyroid cancer and occurs also in well-differentiated histotypes, it is reasonable to suppose that the p53-blunted function due to overexpressed HMGA1 is also a very common mechanism and may explain the positive correlation between HMGA1 expression and poor prognosis in some thyroid carcinomas. These observations also suggest that p53 network inactivation may be an important prerequisite for oncogene-driven thyroid cancer progression.

The specific mechanism, leading to HMGA1 up-regulation during thyroid malignant transformation, remains to be elucidated.

Clinical applications of p53 family member expression in thyroid cancer

Possible role of p53 family proteins as diagnostic markers

Since p53 family protein expression is mostly restricted to thyroid malignant tissue, it may represent a hallmark of thyroid cell malignant transformation (Dobashi *et al.* 1993, Nishida *et al.* 1996, Ruter *et al.* 1996, Zedenius *et al.* 1996, Preto *et al.* 2002, Frasca *et al.* 2003, Unger *et al.* 2003, Vella *et al.* 2003, Hunt *et al.* 2004, Malaguarnera *et al.* 2005).

In the last 10 years, several attempts have been made to identify by immunohistochemistry protein markers (single or in combination) that are able to differentiate benign from malignant thyroid nodules. These marker proteins have included Galectin-3 (Orlandi *et al.* 1998), CD44v6 (Bartolazzi *et al.* 2001), HBME-1 (Miettinen & Karkkainen 1996), CK19 (Schelfhout *et al.* 1989), hTERT (Wood *et al.* 2000a), and S100A4 (Ito *et al.* 2004). The recent availability of microarray techniques has allowed a different approach, through the identification of clusters of genes, whose expression is specific of some types of thyroid tumors and malignancies (Lubitz *et al.* 2005, Musholt *et al.* 2006). Since a consistent series of thyroid malignant tissues display a positive immunostaining for at least one of the p53 family members (Table 1; Frasca *et al.* 2003, Malaguarnera *et al.* 2005, unpublished results), it is reasonable to hypothesize a possible use of these proteins in thyroid nodule differential diagnosis by immunostaining in both tissue specimens and cytoaspirates. Commercially available anti-p53 family protein antibodies have not yet been extensively tested and their possible use for this purpose requires further investigations.

Possible role of p53 family proteins in thyroid cancer chemoresistance

In several cancer types, chemoresistance has been attributed to the presence of p53 mutants (Koike *et al.* 2004). Thyroid carcinomas are very resistant to chemotherapy agents, including doxorubicin and cisplatin (Asakawa *et al.* 1997), even when p53 mutations are not present in these tumors. One possible explanation is that p53 family members with dominant negative function, that are often expressed in thyroid cancer, may inhibit p53 activity and account for chemoresistance (Frasca *et al.* 2003, Vella *et al.* 2003, Malaguarnera *et al.* 2005). Indeed, in other tumors (i.e. ovary cancer), TAp73 α is believed to be responsible for chemoresistance by antagonizing p53 activities (Vikhanskaya *et al.* 2001), a similar effect has

Table 1 Immunostaining of p53 family members in frozen human thyroid samples

	p53	p63	Δ Np73	p73
<i>Normal (n=8)</i>				
74 N	–	–	–	–
81 N	–	–	–	–
89 N	–	–	–	–
143 N	–	–	–	–
212 N	–	–	–	–
390 N	–	–	–	–
560 N	–	–	–	–
361 N	–	–	–	–
<i>Adenomas (n=7)</i>				
196 A	++	+	–	–
206 A	–	–	–	+
212 A	–	–	–	–
218 A	–	–	–	++
232 A	–	–	–	–
387 A	–	–	–	–
392 A	–	–	–	+++
<i>Papillary (n=9)</i>				
201 P	++	+	NE	NE
321 P	++	+++	+++	+++
340 P	+	+	+++	++++
354 P	+++	++	++	++
360 P	++	++	+++	++
380 P	++	++	++	++
383 P	+	++	NE	NE
386 P	++	++	++	+
395 P	+++	++	+++	++
<i>Follicular (n=7)</i>				
97 P	++	++	++++	++
146 P	+	+	NE	NE
191 P	++	+++	++	+
260 P	+	+	NE	NE
267 P	++	+++	+++	+++
283 P	++	+++	+++	+++
371 P	+++	++	+++	+++
<i>Anaplastic (n=11)</i>				
6 P	++	–	++++	++++
17 P	+	+	++	+
32 P	+++	++	NE	NE
96 P	++++	++	+++	++
103 P	++	++	+++	+++
161 P	+++	+++	+++	+++
164 P	++++	+++	NE	NE
219 P	+++	+++	+++	+
238 P	++++	+++	+++	+++
241 P	++++	–	++++	++
333 P	+++	+++	+++	+++

–, No expression; +, low expression, ++, moderate expression; +++, high expression; +++++, very high expression; NE, not evaluated.

been reported for Δ Np73 α in several cancer types (Concin *et al.* 2005). At this regard, we have observed that cells transfected with an ecdysone-inducible Δ Np73 α are more resistant to doxorubicin-induced apoptosis (unpublished results). In the light of these results, studies on the relationship between the

expression pattern of p53 family members in thyroid cancer cells and tumor chemoresistance should be carried out for future clinical application.

Possible role of p53 family proteins in gene therapy

The high frequency of p53 mutations in the anaplastic histotype of thyroid cancer encouraged several attempts to restore wild-type p53 status by gene delivery, in order to make these cells more differentiated and sensitive to chemotherapy (Moretti *et al.* 1997, Blagosklonny *et al.* 1998, Narimatsu *et al.* 1998, Nagayama *et al.* 2000, Imanishi *et al.* 2002). Results obtained with ectopic expression of wild-type p53, however, have been disappointing. In the light of complexity of the p53 family protein network, the failure of ectopic p53 overexpression to cure thyroid cancer can be explained by many reasons. The ectopic wild-type p53 activity may be neutralized by a variety of mechanisms, including the interference of TAp63 α , Δ Np73 α , and HMGA1 proteins. Moreover, the dominant negative function of p53 mutants expressed in poorly differentiated thyroid cancers and the reduced ectopic wild-type p53 stability, due to the robust Mdm2 induction occurring in infected/transfected cells, may be additional mechanisms of failure of p53 activation (Zeimet & Marth 2003). According to this view it is possible that, to be effective, p53 gene delivery should be accompanied, for instance, by antisense sequences aimed at down-regulating TAp63 α , Δ Np73 α , and HMGA1 in thyroid cancer cells. An alternative approach could be the delivery of a mixture of p53 family members aimed at neutralizing the effect of dominant negative isoforms present in malignant cells. To this end, p53 gene delivery could be accompanied by TAp63 γ gene delivery. TAp63 γ , in fact, is devoid of SAM domain and is able to neutralize the TAp63 α dominant negative effect on p53 by direct oligomerization (Malaguarrera *et al.* 2005). In a similar manner, the addition of TAp73 α to p53 gene delivery may neutralize Δ Np73 α by oligomerization and also increase p53 stability by competing with p53 for Mdm2 binding (Save *et al.* 1998, Balint *et al.* 1999). Finally, the use of a c-Abl analog with constitutive nuclear localization may restore TAp73 function and its tumor suppressor activity (Fig. 6).

Recently, pharmacogenomic approaches have also been proposed, with the use of synthetic compounds able to bind specifically to the DBD of the p53 mutants and to restore their proper protein configuration, and, thus, allow DNA binding (Bykov *et al.* 2002, Tanner & Barberis 2004). These small synthetic compounds

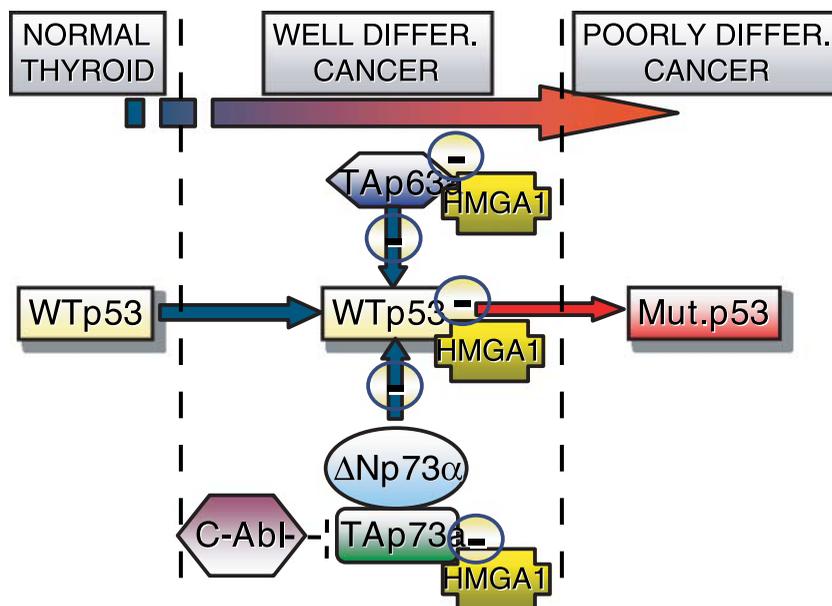


Figure 6 Schematic representation of a possible network among p53 family members in thyroid cancer cells. Normal thyrocytes express a functional wild-type p53, which may exert a tumor suppressor activity in response to DNA damage (left). Well-differentiated thyroid carcinomas express not only wild-type p53, but also other p53 family members with a dominant negative function (middle). As a result of different mechanisms, the oncosuppressor activity may be blunted: (1) TAp63 α , which may be regarded as a de-differentiation marker, may antagonize p53 transcriptional activity by occupying p53-responsive promoters; (2) TAp73 α tumor suppressor activity may be kept latent by c-Abl nuclear exclusion; (3) Δ Np73 α , on the one side, may inactivate TAp73 α by both oligomerization and promoter occupancy and, on the other side, may antagonize p53 by promoter occupancy. In addition, HMGA1 overexpression may inhibit all transcriptionally active p53 family members (p53, TAp63 α , and TAp73 α) by interfering with protein oligomerization. In undifferentiated and anaplastic thyroid cancer, p53 may be inactivated by point mutations (right). p53 mutants not only have lost their tumor suppressor function, but may also inhibit TAp73 α by direct interaction via the core domain.

could be used in combination with traditional chemotherapy to sensitize poorly differentiated thyroid cancer cells to the cytotoxic effect of these agents.

Conclusions

Although p53 mutations are rare in thyroid cancers, p53 functional inactivation by a variety of different mechanisms is apparently very common. The involved mechanisms are based on the very complex network of p53 family member isoforms and include the expression of proteins with dominant negative function (i.e. Δ Np73 α and TAp63 α). Malfunction or overexpression of regulatory proteins is other, independent, inhibitory mechanisms. p53 inactivation by these mechanisms may be an important prerequisite for oncogene-driven thyroid cell transformation (in the early stages of cancer) and in cancer progression and may render thyroid cancer cells resistant to the common chemo- and gene therapy approaches. The unraveling of such a complex network may result helpful not only for better understanding of the important aspects of thyroid tumor progression, but also to design novel multi-

targeted therapies for the poorly differentiated and most aggressive thyroid carcinomas.

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