

Chapter 2

Alterations of p63 and p73 in Human Cancers

Kazushi Inoue and Elizabeth A. Fry

Abstract *p53* and its related genes, *p63* and *p73* constitute the *p53* gene family. While *p53* is the most frequently mutated gene in human tumors, *p63* and *p73* are rarely mutated or deleted in cancers. Many studies have reported p63/p73 overexpression in human cancers while others showed that a loss of p63/p73 is associated with tumor progression and metastasis. Thus, whether p63 or p73 is a tumor suppressor gene or an oncogene has been a matter of debate. This controversy has been attributed to the existence of multiple splicing isoforms with distinct functions; the full-length TA isoform of p63 has structural and functional similarity to wild-type *p53*, whereas the Δ Np63 acts primarily in dominant-negative fashion against all family members of *p53*. Differential activities of TA and Δ N isoforms have been shown *in vivo* by creating isoform-specific gene knockout mice. All *p53*, p63, p73 proteins bind to and activate target genes with *p53*-response elements; p63 also binds to distinct p63-response elements and regulate expression of specific target genes involved in skin, limb, and craniofacial development. Interestingly, several studies have shown that both p63 and p73 are involved in cellular response to cancer therapy and others have indicated that both of these molecules are required for *p53*-induced apoptosis, suggesting functional interplay among *p53* family proteins. Consistent with these findings, aberrant splicing that result in Δ Np63 or Δ Np73 overexpression are frequently found in human cancers, and is associated with poor clinical outcomes of patients in the latter. Thus immunohistochemical staining of tumor specimen with Δ Np73-specific antibody might have diagnostic values in cancer clinics.

Keywords p63 • p73 • Splicing • Alteration • Overexpression • Knockout • Mouse • Cancer

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Introduction

The p53 tumor suppressor protein integrates endogenous and exogenous signals to modulate cell fate to stress and cellular environments [1–3]. Upon DNA damage or other cellular stresses, such as oxidative stress, hypoxia, carcinogen exposure, and oncogene overexpression, p53 becomes activated with increased levels. Then, p53 directs a variety of responses, including DNA repair, cell cycle arrest/senescence, apoptosis, and autophagy depending on the input signal and severity of the damage [4, 5]. The specific response depends on whether the damage can be repaired or is too serious that death of the cell is required to maintain tissue integrity. The genomic locus for *p53* (*TP53*) is very frequently (~50 %) mutated in human cancers, which is associated with therapy resistance and poor prognosis of patients [6, 7]. Since p53 protects humans from damaged and life-threatening cells that may predispose to tumor development, recent research efforts have been made on reconstituting p53 function to effectively treat cancer patients [8, 9].

In the late 1990s, two other p53 family members, p73 and p63 were discovered [10, 11]. These three proteins, encoded by the *TP53*, *TP63*, and *TP73* genes (*Trp53*, *Trp63*, and *Trp73* in mice, respectively), are transcription factors that bind directly to DNA as tetramers, interact with other transcription factors and the transcription machinery, and together control the expression of genes involved in all aspects of life. It has now become clear that both p63 and p73 are involved in a broad spectrum of biological activities, such as cell proliferation, apoptosis, development, differentiation, senescence, and aging. In particular, p63 has emerged as a critical player in embryonic development, epithelial stem cell maintenance, and differentiation. Both p63 and p73 express as a variety of protein isoforms that originate from two different promoters and extensive gene splicing at the N- and C-termini [12, 13]. Moreover, the *p63* and *p73* genes encode a sterile alpha motif (SAM) domain at the C-terminus that is not found in p53. This domain is responsible for protein-protein interactions and is found in a diverse range of proteins that are involved in developmental regulation. In this chapter, we discuss the structure, splicing isoforms of p63 and p73 in normal and their distinct functions in tumor suppression/proliferation. We also explain their possible interaction with Mdm2 and MdmX. Whether these molecules are tumor suppressors or oncoproteins have been a hot topic of debate. Gene knockout studies will tell us the answer; since both of the genes have multiple splicing isoforms, we have put special interest on the phenotypes of splicing isoform – specific gene knockout mouse models. Finally, we summarize the mechanisms and frequencies for alterations of these genes in human cancers and their prognostic significance.

Structure of the p63 and p73 loci

Both *p63* and *p73* loci (*TP63*, *TP73*) generate mRNAs that produce multiple protein products resulting from use of distinct promoters and alternative mRNA splicing (Fig. 2.1) [10, 11]. Transcription of *p63* and *p73* occurs from two

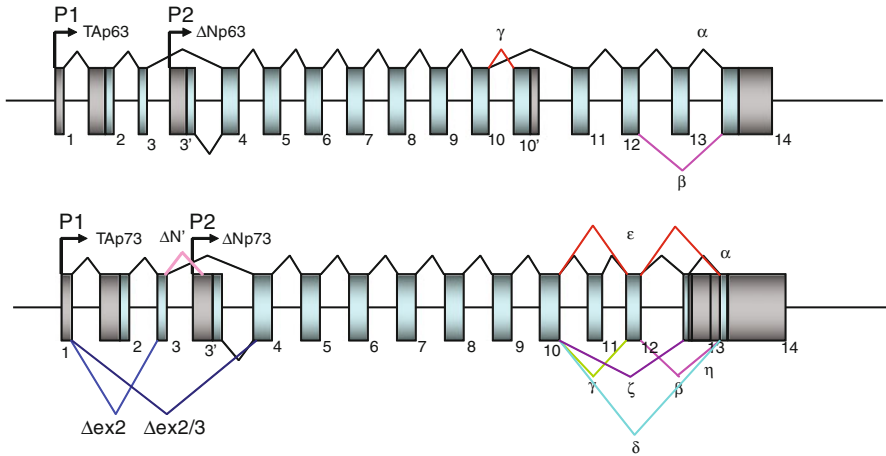


Fig. 2.1 Genomic structure of the *p63* and *p73* loci. Genomic structures of human *p63* and *p73* loci. Numbered boxes indicate exons, black shading denotes untranslated sequences, and light blue shading denotes coding regions. Distinct transcription start sites (P1 and P2) are indicated by arrows. N-terminal alternative splicing for p63 and p73 are indicated by blue and light pink lines, and C-terminal splicing events for these proteins are indicated by different colored lines

promoters: one upstream of exon 1 (P1) and the other located within intron 3 (P2). In both proteins, splicing isoforms transcribed from the P1 promoter have an N-terminal transactivation (TA) domain (i.e., TAp63 and TAp73), which is highly homologous to the TA domain of p53, whereas transcripts generated from the P2 promoter lack the N-terminal TA domain (39 amino acids; called Δ Np63 and Δ Np73, respectively; Fig. 2.1) [10, 11]. The unique structural differences for p63 and p73 are explained below.

p63

The structure of the genomic locus for p63 is shown in Fig. 2.1, upper panel [11]. Both mouse and human *p63* genes consist of 15 exons spanning around 210 kb and 270 kb, respectively, on the genome. The human version has been mapped to chromosome 3q27. The structures for the TAp53 protein, representative p63 isoform proteins are shown in Fig. 2.2 [12]. Wild-type TAp53 has an N-terminal transactivation domain (TA) for recruitment of core transcriptional factors, a central DNA-binding domain (DBD) for recognition of promoter sequences, an oligomerization domain (OD) for tetramerization, and a short basic stretch of 30 amino acids for regulation of transcriptional activity (Fig. 2.2, top panel). The *p63* gene encodes two alternatively spliced isoforms (TA, Δ N) with different ATG at the N-terminus with three alternatively-spliced C-terminal isoforms (α , β , γ), generating 6 different splicing isoforms,

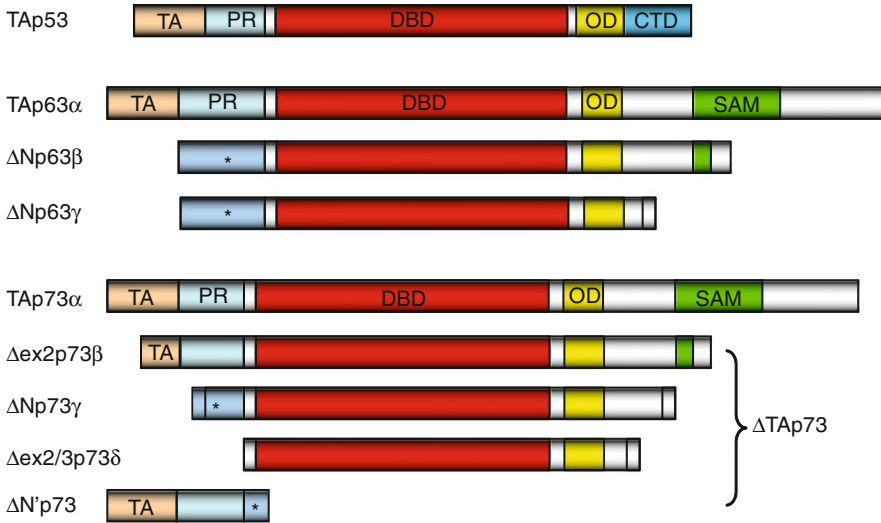


Fig. 2.2 Structure of the p53 family proteins. Protein domains of p53 family members. The transactivation (TA) domains shared by p53, TAp63, and TAp73 isoforms are shown in gold. The proline-rich domain (PR: light blue), DNA-binding domain (DBD: red), oligomerization domain (OD: yellow), carboxyl-terminal regulatory domain (CTD: blue), and sterile alpha motif (SAM: green) are shown in colors. The alpha isoforms of p63 and p73 possess a C-terminal SAM domain followed by a transactivational inhibitory domain (TID: silver). TAp63γ/TAp73γ isoforms most closely look like p53. N-terminally truncated ΔN isoforms for p63 possess the unique N-terminal sequence. p73 has four different isoforms at the N terminus (ΔN, ΔN', Δex2, Δex2/3) dependent on the usage of two different promoters and alternative splicing including exons 2 and 3. ΔN'p73 encodes a small protein having a unique sequence at the C- terminal end, but lacks the DNA-binding domain. * denotes the unique region encoded by exon 3' [12]

i.e., TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β, ΔNp63γ (Figs. 2.1 and 2.2, middle panels). The p63α transcript has all 14 exons while the β transcript lacks exon 13. The γ transcript lacks exons 11–14 by splicing into a unique exon 10' (Fig. 2.1, top panel). The full-length TA isoform of p63 has structural and functional similarity to wild-type p53, whereas the ΔNp63 acts primarily in dominant-negative fashion against all family members of p53: p53, TAp63 and TAp73. Thus, it is generally assumed that *TAp63* is a tumor suppressor gene while *ΔNp63* is an oncogene. In addition, the C-terminus of p63 (and also p73) contains a sterile alpha motif (SAM) domain and a transcriptional inhibitory domain (TID) (Fig. 2.2). The SAM domains are small protein–protein interaction modules that are found in a wide variety of proteins, ranging from kinases and transcriptional regulators to cell surface receptors [14, 15]. The TID, an unstructured region C-terminal to the SAM domain, was shown to inhibit the transcriptional activity of p63 by interacting with the TA domain [16]. These two domains are not found in p53 (Fig. 2.2), suggesting unique functions for p63 and p73.

p73

Both mouse and human *p73* genes consist of 15 exons spanning around 80 kb on the genome. The structure of the genomic locus for *p73* is shown in Fig. 2.1, lower panel. The human version has been mapped to chromosome 1p36.33. The *p73* gene encodes 4 alternatively spliced isoforms (TA, $\Delta\text{ex}2$, $\Delta\text{ex}2/3$, ΔN) with distinct ATG at the N-terminus and 7 alternatively spliced isoforms at C-terminus (α , β , γ , δ , ϵ , ζ , and η ; Fig. 2.1) [17, 18]. In addition, splicing-associated frameshifts yield unique C-terminal sequences for some p63 and p73 isoforms [17, 19]. This alternative splicing can generate 28 plus one ($\Delta\text{N}'$; total 29) different splicing isoforms for p73. Of note, both ΔN and $\Delta\text{N}'$ isoforms have unique amino acids at exon 3' (Fig. 2.1). The difference in the N-terminal region contributes to different protein-protein interactions dependent on the isoform. The C-terminus of p73 has at least 7 splicing variants as shown in Fig. 2.1. The p73 α transcript has all exons 1–14 while the β transcript lacks exon 13. The γ transcript lacks exon 11, the δ transcript lacks exons 11–13 (Fig. 2.1, lower panel). The ϵ isoform lacks 11 and 13, ζ lacks exons 11 and 12; η is close to α , but is different at exon 14 (Fig. 2.1). The TAp63 γ and TAp73 γ isoforms most closely resemble the full-length wild-type TAp53 (Fig. 2.2). In over-expression studies, TAp63 γ has been shown to be as potent as p53 in transactivating target gene expression and apoptosis, whereas the most potent transcriptionally active p73 isoform reported is TAp73 β [10, 11]. Since the $\Delta\text{Np}73$ acts primarily in dominant-negative fashion against all family members of p53, it is generally accepted that *TAp73* is a tumor suppressor gene while *$\Delta\text{Np}73$* is an oncogene.

Unique C-terminal Domains and Transcriptional Targets for p63 and p73

Both p63 α and p73 α isoforms also contain a protein–protein interaction domain known as sterile alpha motif (SAM) (Fig. 2.2). This is a globular domain composed of four α -helices and a small 310 helix. Although this motif is often found to mediate homodimerization with developmentally regulated proteins, the SAM domain does not contribute to homodimerization in p63 and p73 [20]. The SAM domains also appear to possess the ability to bind RNA. The post-SAM region known as the transactivational inhibitory domain (TID) has been identified in p63 α and p73 α isoforms [16]; Fig. 2.2). This region consisting of ~70 amino acids, which is absent in p53, has been proposed to inhibit transcription of both TAp63 α and TAp73 α through inter- or intra-molecular association with the TA domain [16]. Indeed, both of these proteins show decreased potency in transactivation and apoptosis induction as compared to other TA isoforms, and deletion of this region restored transactivating potential for both TAp73 α and TAp63 α [16, 21].

Since both p63 and p73 share strong structural, biochemical and biological homologies to p53, they bind specifically to conventional p53 response elements

(p53RE: RRRCWWGYYY) and transactivate target genes such as *p21^{Cip1}*, *MDM2*, and *BAX*. In spite of their structural similarities between p53 and p63, the latter functions are greatly different from those of p53. The most striking difference is the apparent involvement of p63 in skin and limb development [22] (the details of phenotypes will be explained later). Global *p63* knockout mice that lack all splicing isoforms exhibit skin and limb defects as well as craniofacial abnormalities, but are not tumor prone. This is in contrast to *p53* knockout mice that develop normally, but are prone to develop various cancers from an early age, esp. thymic lymphomas and hemangiosarcomas [23, 24]. In humans, germ line mutations of *p53* cause Li-Fraumeni syndrome, in which affected individuals are very prone to cancer development [25–27]. These differences may be due to the differential regulation of target genes by p53 and p63. The p53 and p63 proteins can bind to two or more tandem repeats of RRRCWWGYYY (p53RE) or some other motifs and subsequently activate target gene expression. By using oligonucleotide expression microarray analysis and analyzing the promoters of p63-induced genes, Osada et al. [28] identified novel p63-specific response elements (p63REs) in the promoter regions of *EVPL* and *SMARCD3*. These p63REs exhibit characteristic differences from the canonical p53RE (RRRCWWGYYY) in both the core-binding element (CWWG) as well as the RRR and/or YYY sequences [28]. Their data indicate that p53 preferentially activates and binds to the RRRCATGYYY sequence, whereas p63 preferentially activates RRRCGTGYYY. Whereas EVPL protein is highly expressed in epithelial cells of the skin and pharynx in the *p63^{+/-}* mouse, it is undetectable in these tissues in the *p63^{-/-}* mouse. Thus p63 can regulate expression of specific target genes such as those involved in skin, limb, and craniofacial development by preferentially activating distinct p63-specific response elements [28]. Until now, a number of genes have been reported to be targets of p63 and p73, such as *REDD1* (regulation of reactive oxygen species), *JAG1/JAG2* (Notch signaling), *IL4R*, Δ *Np73*, and *AQP3* (glycerol and water transporter) [28–30]. Among these, Δ *Np73* is a splicing variant from the *p73* locus, suggesting its autoregulation [31]. In Notch signaling, Sasaki et al. found that the genes encoding ligands for the Notch receptors (*JAG1/2*), are up-regulated by p63 and p73 but not by p53 [30]. They identified a p63-binding site in the second intron of the *JAG1* gene, which could directly interact with p63 *in vivo* as demonstrated by chromatin immunoprecipitation. They also found a target of Notch signaling; HES-1 was up-regulated in Jurkat cells with high expression of Notch1 when co-cultured with p63-transfected cells, suggesting that p63 can trigger the Notch signaling pathway in neighboring cells. This suggests a potential molecular mechanism for the involvement of p63 in normal development [30]. Recently it was reported that BRCA1 activates the Notch pathway in breast cells by transcriptional upregulation of Notch ligands and receptors [32]. They demonstrated that BRCA1 was localized to an intronic enhancer within the *JAG1* gene, an event requiring Δ *Np63*. This BRCA1/ Δ *Np63*-mediated induction of *JAG1* must play important roles in the regulation of breast stem/precursor cells since knockdown of these proteins resulted in increased tumorsphere growth and increased activity of stem cell markers [32]. Thus, BRCA1/ Δ *Np63*-mediated transactivation of Notch signaling is a key event in the normal differentiation process in breast tissue.

Regulation of p63 and p73 by Mdm2

The interactions of p53 with Mdm2 and Mdmx, mediated via the TA domain of p53 have been well-documented [33–36]. The physiological importance of the regulation of p53 by the Mdm2 and Mdmx ubiquitin ligases as well as the role of its aberrant regulation in tumors has also been reported [37–41]. Unlike p53, which protects genomic stability, the two homologous proteins of the same family, p63 and p73, regulate developmental processes as described in this chapter.

Since all three p53 family proteins have homologous TA domains, it was speculated that p63 and p73 may be regulated by Mdm in a similar manner as has been reported for p53. The ability of Mdm2 and Mdmx to bind to p73 has been well-documented [42] and Zdzalik et al. [43] provided a detailed kinetic characterization of this interaction. The interaction of Mdm with p63 has also been studied previously, but the results were controversial due to lower affinity for such an interaction [44–47, 92]. In fact, Zdzalik et al. [43] showed that both Mdm2 and Mdmx form complexes with the p63 TA domain, however the interactions were weaker than those determined for p53 or p73. The interaction of the p63 TA domain is specific and mechanistically similar to that of the p53 TA domain since the p63(Ala) mutant peptide showed no activity in the assays performed. Although the interactions of p73 with Mdm2 and Mdmx have also been studied previously, only the affinity of p73 for Mdm2 has been reported [48, 49]. The interaction between p63 and Mdm2 is one order of magnitude weaker than those of Mdm with p53 and p73. Conversely, the affinities of both Mdm2 and Mdmx for p73 are of the same order of magnitude as those for p53, which justifies the conclusion that these proteins truly interact in cells, as has previously been suggested in other studies [42, 46, 50, 92]. The weaker interactions of both Mdm2 and Mdmx with p63 explain the inconsistent results reported by different groups on the interactions of those proteins. Clearly, at sufficiently high concentrations, these proteins will form a stable complex, but whether such concentrations are ever encountered under physiological conditions in cells remains a very intriguing question for future studies [43]. It is also noteworthy that although the affinities of p53 for Mdm2 and Mdmx are similar, both p63 and p73 interact more strongly with Mdmx. Therefore, Mdmx, but not Mdm2, may have a stronger impact on the regulation of intracellular p63 and p73.

Constitutional, All Splicing Isoforms' Knockout Mice for p63, p73

Mills et al. [22] reported the phenotypes of *p63*-deficient mice (all splicing isoforms). *p63*-null mice are born alive but have striking developmental defects. Their limbs are absent or truncated, defects that are caused by a failure of the apical ectodermal ridge differentiation. The skin of *p63*-null mice did not progress through an early developmental stage lacking stratification with no differentiation markers.

Hair follicles, teeth and mammary glands were absent in *p63*-deficient mice. Thus, in contrast to *p53*, *p63* is essential for several aspects of ectodermal differentiation during embryogenesis. Keyes et al. [51] studied spontaneous and chemically-induced tumor development using *p63*^{+/-} mice since *p63*^{-/-} mice had serious developmental defects, and thus were not suitable for *in vivo* tumor development studies. They found that *p63*^{+/-} mice were not tumor prone and mice heterozygous for both *p63* and *p53* had fewer tumors than *p53*^{+/-} mice. Furthermore, *p63* expression was maintained in carcinomas. These findings demonstrate that *p63* plays a markedly different biological role in cancer than *p53*.

Mice deficient for all *p73* splicing isoforms also exhibited profound developmental and immunological defects, including hippocampal dysgenesis, hydrocephalus, chronic infections, and inflammation, as well as abnormalities in pheromone sensory pathways [52]. It should be noted that mice lacking *p73* showed no increased susceptibility to spontaneous tumorigenesis, in contrast to *p53*-deficient mice [23, 24]. They speculated that potentially dominant-negative, *p73* variants were the predominant expression products of this gene in developing and adult tissues, explaining the mechanistic basis of the hippocampal dysgenesis and the loss of pheromone responses in *p73*-null mice. Thus *p73* plays unique roles in neurogenesis, sensory pathways, and homeostatic control [52].

Flores et al. [53] explored the combined role of *p63* and *p73* in DNA damage-induced apoptosis. The combined absence of *p63* and *p73* severely impaired the induction of *p53*-dependent apoptosis in response to DNA damage in E1A-expressing cells and in the developing central nervous system in mice although the *p53* locus remained intact. This was explained by the inability of *p53* to bind the promoters of apoptosis-associated target genes and to upregulate their transcription in *p63*^{-/-}; *p73*^{-/-}; E1A(+) cells and the developing central nervous system [53, 54].

Splicing Isoform-Specific Knockout Mouse Models for p63, p73

p63

The roles of *p63* in tumor suppression have been a hot topic of debate. The most intriguing question is whether *p63* is a tumor suppressor gene or an oncogene. Many studies have shown *p63* overexpression in human cancers [55]; discussed later in this chapter), while others demonstrate that a loss of *p63* is associated with tumor progression and metastasis [56]. This controversy has been attributed to the existence of multiple splicing isoforms with distinct functions; the full-length TA isoform of *p63* has structural and functional similarity to *p53* (Fig. 2.2), whereas the Δ N*p63* protein acts primarily in dominant-negative fashion against all family members of *p53*. To study splicing isoform-specific differences of *p63* functions *in vivo*, Su et al. [57] developed a *TAp63* conditional knockout mouse and used it to delete *TAp63* in the germline (*TAp63*^{-/-}; using *Zp3-cre* or *Protamine-cre*) or in K14-expressing cells in the basal layer of the epidermis (*TAp63*^{fl/fl}; using *K14cre*⁺).

TAp63^{-/-} mice aged prematurely and developed blisters, skin ulcerations, senescence of hair follicle-associated dermal and epidermal cells, and decreased hair morphogenesis, indicating that TAp63 serves to maintain adult skin stem cells by causing cellular senescence and genomic stability, thereby preventing premature tissue aging [57]. The same group followed spontaneous tumor development in *TAp63*^{-/-}, *TAp63*^{+/-} and wild-type mice for 2.5 years and found that both *TAp63*^{+/-} mice and *TAp63*^{-/-} mice developed carcinomas and sarcomas with significantly shorter lifespan than the wild-type cohort. Consistent with this finding, tumors from *TAp63*^{+/-} mice retained the wild-type allele of *TAp63* suggesting that *TAp63* is haplo-insufficient tumor suppression. Both *TAp63*^{+/-} and *TAp63*^{-/-} mice developed highly metastatic tumors, and 10 % of these metastases were found in the brain, a rare finding in endogenous mouse tumor models. Although equivalent numbers of carcinomas metastasized in the *TAp63*^{-/-} and *TAp63*^{+/-} mice, a greater number of sarcomas metastasized in *TAp63*^{+/-} mice than in *TAp63*^{-/-} mice, indicating that heterozygous loss for *TAp63* rather than homozygous loss results in a more severe phenotype.

Keyes et al. [58] observed that Δ Np63 α overexpression in mouse embryonic fibroblasts (MEFs) bypassed Ras-mediated senescence and drove tumorigenesis *in vivo*. They identified chromatin-remodeling protein Leeh as a novel target for Δ N63 α that is an essential mediator of senescence bypass. This bypass of senescence by Δ Np63 α promoted stem cell-like proliferation and maintained the survival of keratin 15-positive cells. Thus, Δ Np63 α is a novel oncogene that cooperates with Ras to promote tumor development by initiating stem cell proliferation. By contrast, overexpression of TAp63 forms in *p53*^{-/-} MEFs increased senescence and reduced tumor development *in vivo*, consistent with a p53-independent effect of TAp63 [59].

The TAp63 and Δ Np63 isoforms have special effects in epidermal tissue differentiation [60]. In murine embryonic stem cells, Δ Np63, but not TAp63, is highly expressed in epidermis and is critical for the expression of the cytokeratins K5 and K14, two markers of keratinocyte differentiation, indicating that only Δ Np63 is required for the commitment of ectodermal into epidermal cells [61, 62]. In summary, p63 and its splicing variants play specific roles in epidermal commitment, cell proliferation, and senescence bypass; alterations of this intricate balance contribute to tumor development.

p73

Mice with a complete deficiency of *p73* exhibited severe neurological and immunological defects due to the absence of all *TAp73* and Δ Np73 isoforms as described in the previous section. To study mice deficient for specific p73 protein isoforms, Tak Mak's group created *p73* isoform-specific knockout mice [63, 64]. Tomasini et al. [63] created mice in which exons encoding the *TAp73* isoforms were specifically deleted at exons 2/3 to establish a *TAp73*-deficient (*TAp73*^{-/-}) mice. Mice specifically lacking in *TAp73* isoforms showed a phenotype intermediate between the phenotypes of *p73*^{-/-} and *p53*^{-/-} mice with respect to the incidence of spontaneous

and carcinogen-induced tumors, infertility, and aging, as well as hippocampal dysgenesis. In addition, cells from *TAp73*^{-/-} mice showed genomic instability associated with enhanced aneuploidy, which could account for the increased incidence of spontaneous tumors in these animals. Hence, *TAp73* isoforms exert tumor-suppressive functions indicating an emerging role for *Trp73* in the maintenance of genomic stability. Wilhlem et al. [64] generated mice that were selectively deficient for the $\Delta Np73$ isoform by depleting ΔN form-specific exon 3' ($\Delta Np73$ ^{-/-}). These mice were viable and fertile, but showed signs of neurodegeneration. Cells from $\Delta Np73$ ^{-/-} mice were sensitive to DNA-damaging agents and showed an increase in p53-dependent apoptosis. They found that the $\Delta Np73$ protein localized directly to the site of DNA damage, interacted with the DNA damage sensor protein 53BP1, and inhibited ATM activation and subsequent p53 phosphorylation. This finding may explain why human tumors with high levels of $\Delta Np73$ expression showed resistance to chemotherapy.

In summary, these studies show that TAp63 and TAp73 proteins have specific roles in preventing tumor development *in vivo*. Conversely the ΔN forms act as oncogenes by preventing senescence and maintaining progenitor cell status. When overexpressed, both TAp63 and TAp73 proteins transactivate subsets of known p53 target genes involved in cell-cycle arrest and apoptosis, such as *p21*^{Cip1} and *Bbc3* [65–68]. Of note, both TAp63 and TAp73 also regulate distinct sets of genes that are not transcriptional targets for p53 through unique p63RE as described in the previous section. In contrast, $\Delta Np63$ and $\Delta Np73$ proteins have been shown to function in part as dominant-negative inhibitors of the p53 family, leading to the hypothesis that these isoforms may exhibit proto-oncogenic function. ΔN isoforms inhibit the function of TA forms through (1) direct competition for DNA-binding sites and (2) formation hetero-oligomeric complexes with TAp63/TAp73, and less strongly with p53 [11, 20, 69–72]. Interestingly, expression of the $\Delta Np73$ is strongly up-regulated by TAp73 and p53, thus creating a feedback loop that tightly regulates the function of TAp73 and more importantly, of p53 [70].

Aberrant Expression, Altered Splicing, and Mutations of p63 and p73 in Human Cancer

Alterations of p63 Isoforms in Human Cancers

Both p63 and p73 were initially hypothesized to function as tumor suppressors based on their homology to p53. However, accumulating evidence shows that mutation of either of these genes in human cancer is quite rare [73, 74], indicating that they are not classical tumor suppressor genes like *p53* or *RB* that meet the Knudson's two-hit hypothesis [75]. Although there have been numerous studies on p63 expression in human cancers, loss of heterozygosity (LOH) of the *p63* locus has not been studied extensively in human malignancies [76], possibly because the genomic

locus 3q27-28 is not the site of frequent gene deletion in cancer. Conversely, decreased p63 expression is a common feature of high-grade invasive urothelial carcinomas and associates with reduced β -catenin. Both Δ Np63 and TAp63 are frequently downregulated in bladder cancer and this reduction correlates with a poor prognosis [77]. The majority of prostate cancers show loss of p63, but it is overexpressed in some poorly differentiated tumors and correlates with a poor prognosis [78]. In addition, loss of p63 results in enhanced metastasis in prostate cancer [79]. Koga et al. [80] studied the expression of p63, β -catenin, and uroplakin III by immunohistochemistry in high-grade invasive bladder carcinomas. Lower p63 expression was significantly associated with higher TNM stage, lymph-node metastasis, and reduced β -catenin expression. Importantly, lower p63 expression was significantly associated with a poor prognosis. Impaired p63 expression was associated with biological aggressiveness of high-grade invasive urothelial carcinomas. Moreover, loss of p63 expression was a pre-requisite for uroplakin III expression. Their data suggested that p63 plays critical roles in tumor progression and biochemical terminal differentiation of urothelial neoplasms [80].

Oral lichen planus (OLP) is a relatively common chronic disease of the oral mucosa for which the etiology or pathogenesis is not fully understood. Sniezek et al. [81, 82] showed decreased expression of p63 in OLP compared to normal mucosa, a decrease they suggested could explain the hyper-differentiation, or pro-differentiation, seen in this disorder OLP. Consistent with these findings, another group reported downregulation of p63 in this disorder [83].

The *p63* gene maps to chromosome 3q27-28, a region frequently amplified in squamous cell carcinomas [55, 76, 84–86]. Most squamous cell carcinomas retain p63 expression, where it is often overexpressed [55, 87, 88]. Although some controversy exists as to whether p63 is the targeted gene driving amplification of this locus, several groups have reported increased *p63* mRNA levels that correlate with an increase in *p63* gene copy number in squamous cell carcinomas of the lung, head, and neck (HNSCCs) [84, 89, 90]. In other cases, overexpression of p63 appears to be independent of genomic DNA amplification of the locus [91]. In esophageal carcinomas, amplification of the *p63* gene was reported in ~20 % of squamous cell carcinomas and 10 % of adenocarcinomas [76]. Given that the total frequency of tumors in which p63 is upregulated is much higher (>50 %), gene amplification is unlikely to be the main mechanism underlying the increased levels of p63. Rather, transcriptional or post-transcriptional changes are involved. Multiple studies have shown that p63 overexpression occurs in up to 80 % of primary HNSCCs and also in other squamous cell carcinomas, including those in the lung, nasopharynx, and cervix [55, 92–94]. By the use of isoform-specific antibodies, Nylander et al. [95] mapped expression of the different p63 isoforms within normal oral mucosa and HNSCCs, showing increased expression of p63, mainly the Δ Np63 isoforms, in tumors compared to normal mucosa. They indicated specific roles for the individual isoforms in cell differentiation and neoplasia [95]. In invasive breast cancer, the frequency of p63 expression varies, ranging from 0 to 30 % [96–98]. It is now considered that p63 is expressed in at least a subset of breast tumors that are known to have a basal epithelial phenotype [99].

TAp63 vs. ΔNp63 in Cancer

In esophageal carcinomas, p63 isoforms are upregulated not only in carcinomas, but also in squamous dysplasias [76]. Although early studies for the detection of p63 did not differentiate among different isoforms, recent studies used isoform-specific RT-PCR coupled with Western blot analysis to quantitatively demonstrate that ΔNp63α is the predominant p63 isoform expressed in squamous cell carcinomas. Using such an approach, it has been reported that tumor-suppressive TAp63 overexpression is rare in HNSCC, and that ΔNp63 mRNA expression was at least 100-fold more abundant than TAp63 mRNA in all cases [87, 100]. These findings are consistent with the inability of many investigators to detect TAp63 protein isoforms by Western blot analysis in either primary keratinocytes or HNSCC cells. ΔNp63 is the predominant variant that is found in HNSCCs; however, in Barrett's esophagus, a disorder in which the stratified epithelium is replaced by a simple columnar epithelium that consists of mucosecretory cells, the *p63* gene expression is not highly prominent [76].

Tumors often have simultaneous transcriptional upregulation of both TAp63 and ΔNp63 isoforms, with ΔNp63 being predominant at protein levels [55, 87]. This would represent the anti-apoptotic and proliferative effects of ΔNp63 as described in the previous section. Moreover, it was reported that ΔNp63α expression directly correlates with a poor response to cisplatin in HNSCC [101]. In pancreatic cancer, Danilov et al. [102] showed that ΔNp63α enhanced the oncogenic potential of tumor cells through trans-activation of *EGFR* and *14-3-3σ*. Leong et al. [103] reported that the p63/p73 network mediates chemosensitivity to cisplatin in a subset of primary breast cancers. Thus, p63 is involved in chemosensitivity of multiple types of tumors. In HNSCC, DNA damage by chemotherapy caused a decrease in ΔNp63-mediated transcriptional repression by blocking p63-responsive elements or sequestering TAp63 in less active hetero-tetramers, together with increased expression of p73, thus allowing TAp73-mediated cell death [100]. Together, these reports indicate that it is not only the levels of individual p53 family members, but rather the ratio between TA (transcriptionally active, having tumor-suppressor functions) and ΔN (acting as dominant-negative over the TA isoforms, showing oncogenic properties) isoforms that determines the biological outcome.

In lung cancer, amplification of chromosomal region 3q26-3qter is frequently found in tumors. Massion et al. [55] analyzed *p63* gene copy number and expression by immunohistochemistry in tissue microarrays of >200 non-small cell lung cancers (NSCLCs) and correlated them with survival. The *p63* genomic locus was amplified in 88 % of squamous cell carcinomas, but only in 11 % of adenocarcinomas and 2 % of large cell carcinomas of the lung, indicating clear association of gene amplification with squamous cell lung cancer. The major splicing variant of p63 expressed was ΔNp63α. Furthermore, *p63* genomic amplification and protein staining was associated with better survival. They found a significant increase in *p63* copy number in pre-invasive lesions graded severe dysplasia or higher. Thus, there is early and frequent genomic amplification of *p63* in the development of squamous carcinoma of the lung and patients with NSCLC showing amplification

and overexpression of p63 had prolonged survival [55]. However, two other groups have failed to demonstrate the favorable prognostic value of p63 in lung cancer. Iwata et al. [104] reported a lack of prognostic significance regarding Δ Np63 immunoreactivity in lung cancer. Uramoto et al. [105] showed that the expressions of Δ Np63 in lung cancer did not significantly affect survival while patients with a positive Δ Np73 expression had a poorer prognosis in comparison to the negative group. The differential prognostic values of p63 in these, Massion's and two other studies, can be attributed to the fact that the former study focused on gene copy number of *p63* and immunohistochemical staining of p63 (all splicing isoforms) in squamous cell lung cancer while the latter two groups studied the expression of the Δ Np63 protein and survival of non-small cell lung cancer (in Uramoto's study; squamous cell carcinoma only in Iwata's study).

Δ Np63 α can act as a transcriptional repressor, but the link between the transcriptional functions of p63 and its biological role is still unclear. Barbieri et al. [106] depleted endogenous p63 by shRNA to investigate the transcriptional programs controlled by p63. Disruption of p63 in squamous cell carcinoma cell lines resulted in down-regulation of transcripts specifically expressed in squamous tissues and a significant alteration of keratinocyte differentiation. They found that depletion of p63 led to up-regulation of markers of non-epithelial tissues (mesenchyme and neural tissue) in squamous cell carcinomas, which were associated with increased capacity for invasion and metastasis in tumors. Furthermore, loss of p63 expression was accompanied by a shift toward mesenchymal morphology and an increase in motility in primary keratinocytes and squamous cell lines [106]. Thus, loss of endogenous p63 results in up-regulation of genes associated with invasion and metastasis, and predisposes to a loss of epithelial markers and acquisition of mesenchymal characteristics. Although the squamous cell carcinoma cell lines they analyzed expressed predominantly Δ Np63, the interpretation of their experimental results is controversial since their shRNA depleted both *Tap63* and *ΔNp63* at the same time. *p63* isoform-specific shRNA should be used to define the roles of each isoform in cell growth, differentiation, invasion and metastasis.

Regulation of Gene Expression by Δ Np63

Although Δ Np63 lacks the amino-terminal transactivation domain consisting of 39 amino acids that is present in *Tap63*, Δ Np63 still activates a group of genes that includes, but is not restricted to genes regulated by p53 [66]. Helton et al. showed that all NH2-terminally deleted p63 isoforms still retain a potential in transactivation and growth suppression [107]. Interestingly, they showed that Δ Np63 β possessed a remarkable ability to suppress cell proliferation and transactivate target genes, which is consistently higher than that seen with Δ Np63 α . They showed that an intact DNA-binding domain is required for Δ Np63 function. In addition, they found that the novel transactivation domain for the Δ Np63 variant was composed of the 14 unique Δ N residues along with the adjacent region, including a PXXP motif [107]. They also showed that a PPXY motif shared by Δ Np63 α and Δ Np63 β was

required for optimal transactivation of target gene promoters [107]. Very recently, Ceraldo et al. [108] identified a novel p63 transcriptional target, caspase-1. Caspase-1 is pro-inflammatory caspase, which functions in tumor suppression. They showed that both p63 isoforms (TAp63, Δ Np63) increased caspase-1 expression through physical binding to its promoter. Consistently they also identified a direct correlation between p63 and caspase-1 expression in human cancer data sets. Functional interaction between p63 and caspase-1 represented a predictor of longer survival in human cancers. Together, in addition to dominant-negative effects of Δ Np63 on TA isoforms of p53 family proteins, regulation of gene expression by Δ Np63 variants should be re-evaluated from the viewpoint of tumor suppression.

Alterations of p73 Isoforms in Human Cancers

The *p73* gene has been speculated to be classical tumor suppressor genes like *p53* when the cDNAs were cloned [10]. In gastrointestinal tumors, LOH for *p73* has been reported in 10–40 % of the cases [76] although LOH for *p63* has not been reported in cancers. Despite these expectations, subsequent studies have demonstrated that the *TP73* locus was not the hot spot of gene deletion in cancers. Rather, studies of multiple tumor types have shown that *p73* splicing variants are overexpressed, but not mutated or deleted in human malignancies [17].

To investigate the role of the *p73* gene in human carcinogenesis, Han et al. [109] studied genetic alterations of this gene by analyzing the entire coding exons as well as their surrounding exon-intron boundaries by PCR-CCSP and direct sequencing with primary samples from breast, colorectal, gastric cancers, neuroblastomas, and also with lung and pancreatic cancer cell lines since they are known to have frequent LOH in the 1p region. However, of the 185 cases, somatic missense mutation of glutamine from arginine at codon 269 was found in only one breast cancer. Monoallelic expression of *p73* was observed in pancreatic cancer cell lines. Nomoto et al. [110] analyzed 61 primary lung cancer samples of the *p73* locus at 1p36.33 by PCR-SSCP and Southern blotting. Although allelic loss at the 1p36.33 locus was observed in 42 % of cases, somatic mutations of the *p73* gene were not observed in their samples, suggesting the presence of an as yet to be determined tumor suppressor gene at the locus. In summary, inactivation of the *p73* gene is very rare even in cancers involving chromosome 1p [109].

TAp73 vs. Δ TAp73 in Human Cancers

Overexpression of *p73* mRNA and/or protein relative to neighbor normal tissues has been reported in a variety of tumors, such as neuroblastoma, glioma, ependymoma, breast, lung, colon, stomach, liver, ovarian, bladder, cholangiocellular carcinomas, and myelogenous leukemias [17]. Concin et al. [111] studied the expression profile of all N-terminal isoforms, distinguishing between TAp73 and Δ TAp73 (Δ Np73, Δ N'p73, Δ ex2p73, and Δ ex2/3p73) (Fig. 2.2). Ovarian cancers almost universally

overexpressed $\Delta N'p73$ compared with normal tissues (95 % of cancers). About one-third of tumors also exhibited concomitant up-regulation of TAp73, whereas only a small subgroup of tumors overexpressed $\Delta Np73$ [111]. Thus, deregulation of the E2F-responsive P1 promoter, rather than the P2 promoter, is mainly responsible for the production of $\Delta TAp73$ in ovarian cancer. A trend was found for better overall survival in patients with low expression of $\Delta N'p73/\Delta Np73$, compared with those with high expression. Cancers with wild-type p53 showed significantly higher deregulation of $\Delta Np73$, $\Delta N'p73$, and $\Delta ex2/3p73$ (transdominant p73) than p53 mutant cancers. Thus, overexpression of transdominant p73 isoforms can function as epigenetic inhibitors of p53 *in vivo*, thereby alleviating selection pressure for p53 mutations in ovarian cancer [111].

Dominguez et al. [112] analyzed 113 colon and 60 breast cancer patients' primary samples and reported the association of $\Delta TAp73$ variants and advanced pathologic stage, lymph node metastasis, vascular invasion, presence of polyps, and tumor localization. Overexpression of TP73 variants in tumor tissues indicates that they may be involved in carcinogenesis. The association between upregulation of $\Delta TAp73$ isoforms and poor prognosis suggests that they may be of practical clinical prognostic value. Faridoni-Laurens et al. [113] analyzed the expression of TAp73 and $\Delta TAp73$ in HNSCC and compared them to the p53 status. They found that all of the p73 isoforms were upregulated in comparison to those in normal adjacent tissue. Although p73 belongs to the gene family of p53, p53 mutations and p73 transcript alterations were not mutually exclusive. All of the HNSCC specimens studied had at least one p53 mutation and/or one $\Delta TAp73$ transcript alteration. Although both the $\Delta Np73$ and the TAp73 transcripts were upregulated in HNSCC, the predominant protein in the cancers expressed was $\Delta Np73$. Furthermore, a trend was found for better overall survival in patients with a low expression of $\Delta Np73$. Thus deregulation of both the p53 and the p73 pathways plays an important role in inducing HNSCC [113].

By using specific polyclonal $\Delta Np73$ antiserum against the exon 3'-specific peptide for p73, $\Delta Np73$ and $\Delta N'p73$ expressions were studied in paraffin-embedded tumor samples from 132 lung cancer samples [105, 114]. The $\Delta N/\Delta N'p73$ protein was detected mainly in the cytoplasm of tumor cells in 77 of 132 patients (58.3 %) with lung cancer. Importantly, lung cancer patients with positive $\Delta N/\Delta N'p73$ expression had a poorer clinical outcomes than those with negative expression. In addition, multivariate analysis of the clinicopathological characteristics of lung cancer indicated that positive expression of $\Delta N/\Delta N'p73$ was a significant independent factor for predicting poor prognosis ($P < 0.0001$, risk ratio = 3.39). Thus, expression of $\Delta N/\Delta N'p73$ will be a useful marker for predicting poor prognosis of patients who undergo resection of lung cancer. Consistent with these findings, overexpression of the N-terminal splice variants ($\Delta ex2p73$, $\Delta ex2-3p73$), but not TAp73, was shown to be associated with a poor prognosis in low-grade gliomas [115], which should be helpful in decision-making in clinics.

The truncated oncogenic isoform $\Delta ex2p73$ is expressed in hepatocellular carcinomas (HCC); however, the underlying mechanisms regulating this process are unknown. Castillo et al. [116] used human normal and diseased liver tissue samples

to examine the association between activation of epidermal growth factor receptor (EGFR) by its ligand amphiregulin (AR) and the alternative splicing of *p73* pre-mRNA into the tumorigenic isoform $\Delta\text{ex}2\text{p}73$, via c-Jun N-terminal-kinase-1-mediated signaling. $\Delta\text{ex}2\text{p}73$ was expressed in a subset of premalignant cirrhotic livers and in otherwise healthy livers that harbored a primary tumor, as well as in HCC tissues. $\Delta\text{ex}2\text{p}73$ expression was correlated with that of the EGFR ligand AR, previously shown to have a role in hepatocarcinogenesis. Autocrine activation of the EGFR by AR triggered c-Jun N-terminal kinase-1 activity and inhibited the expression of the splicing regulator Slu7, leading to the accumulation of $\Delta\text{ex}2\text{p}73$ transcripts in HCC cells. Their study provided a mechanism for the generation of pro-tumorigenic $\Delta\text{ex}2\text{p}73$ during liver tumorigenesis via activation of EGFR signaling by AR and c-Jun N-terminal kinase-1 activity, leading to inhibition of the splicing regulator Slu7 [116]. This is a unique report that showed the specific role of a particular splicing factor in aberrant *p73* splicing.

The molecular mechanisms underlying overexpression of $\Delta\text{Np}63$ or $\Delta\text{Np}73$ in cancers in comparison to normal tissues need further investigation. Methylation-mediated silencing of the P1 promoter for *TAp73* was reported in lymphoblastic leukemias and Burkitt's lymphomas [117, 118]. These findings indicate that either $\Delta\text{Np}63$ or $\Delta\text{Np}73$ overexpression or *TAp73* promoter silencing is required to inactivate the tumor-suppressive activity of *TAp73*. Although *TAp73* isoforms were paradoxically overexpressed (18–30 folds) in HNSCC tumor cells in comparison to non-transformed keratinocytes, $\Delta\text{Np}63\alpha$ was also overexpressed in these tumors and was physically associated with *TAp73*, thereby inhibiting *p73*-dependent pro-apoptotic activity [87, 100]. *BRCA1*-deficient tumor cells exhibit increased sensitivity to cisplatin, and patients with *BRCA1*-associated ovarian carcinomas had better outcomes with platinum-based chemotherapy compared with sporadic cases. Ibrahim et al. [119] reported that *BRCA1*-deficient ovarian carcinoma cells exhibited hypermethylation within the P1 promoter for *p73*, which included the binding site for the *p73* transcriptional repressor ZEB1, leading to the abrogation of ZEB1-binding and increased expression of transactivating *p73* isoforms (*TAp73*), explaining increased cisplatin sensitivity of *BRCA1*-deficient ovarian carcinomas. Thus, *TAp73* might represent a response predictor and potential therapeutic target for enhancing chemosensitivity in ovarian cancer.

Although promoter methylation is the major mechanism of *p73* inactivation in hematopoietic malignancies [120], the situation is different in epithelial tumors – carcinomas. Daskalos et al. [121] studied the DNA methylation status of both P1 and P2 promoters as a means of epigenetic transcriptional control of their corresponding isoforms in 102 primary NSCLCs and reported that the P2 hypomethylation-associated overexpression of $\Delta\text{Np}73$ mRNA is a frequent event, particularly among squamous cell carcinomas. P2 hypomethylation strongly correlated with long interspersed nuclear element-1 element hypomethylation, indicating that $\Delta\text{Np}73$ overexpression may be a consequence of global DNA hypomethylation. Guan and Chen analyzed *p73* in prostate cancer and found that $\Delta\text{Np}73$ was significantly increased in 20 of 33 prostate carcinomas [122]. However, none of the specimens expressed $\Delta\text{N}^{\text{p}}73$. The positive expression of $\Delta\text{Np}73$ correlated with the Gleason

score in prostate cancer. Interestingly, prostate cancer samples with wild-type p53 had significantly higher expression of $\Delta Np73$ than p53 mutant cancers. These data suggested a potential role for $\Delta Np73$ in prostate cancer progression.

Diaz et al. [123] conducted a translational study to evaluate whether 1,25(OH)(2) vitamin D(3) downregulates TP73 variants in colon and breast cancers [123]. They reported that ectopic survivin expression led to an increase in all of the TAp73, $\Delta Np73$, $\Delta Ex2p73$, and $\Delta Ex2-3p73$ transcripts. In these cancers, direct correlations were observed between TP73 variants and survivin levels. Interestingly, 1,25(OH)(2) vitamin D(3) negatively regulated survivin and TP73 variants in these tumors. Thus positive regulation of TP73 isoforms by survivin may exist, which raised the possibility that the downregulation of TP73 isoforms may be possible with 1,25(OH)(2)D(3) through survivin.

In summary, although somatic point mutations are rarely found in *p73* in human cancers, aberrant splicing that result in $\Delta TAp73$ overexpression are very frequently found. Since these proteins have transdominant activity on all p53 family proteins, it is speculated that this abnormal splicing contributes to human carcinogenesis, esp. in ovarian, breast, lung, and prostate cancers, HNSCCs, and hematological malignancies. Published results indicate that $\Delta TAp73$ overexpression is associated poor clinical outcomes at least in lung cancer and HNSCCs. Of note, it may be possible to correct aberrant expression of p73 isoforms in cancer through the use of 1,25(OH)(2)D(3).

Conclusive Remarks

Judging from the very low frequency of mutations for *p63* and *p73* in human cancers, these are not classical tumor suppressor genes, but the possibility remains that these are haplo-insufficient tumor suppressors, just like *p27^{Kip1}*, *PTEN*, or *DMP1* [124–132]. Detailed analyses with specific primers are required to determine whether these are true tumor suppressors. Accumulating pieces of evidence suggest that TA- and ΔN - isoforms play distinct roles in cell cycle progression, apoptosis, and tumor development/prevention. Detection of each isoform by Western blotting or immunohistochemistry with specific antibodies or real-time PCR-mediated quantification of each splicing isoform will be needed to determine the prognostic value of each splicing isoform in cancer. Of note, both $\Delta Np73$ and $\Delta N'p73$ have unique amino acid sequences generated from the exon 3' that is absent in TAp73. This has made it possible to generate $\Delta N/\Delta N'p73$ -specific antibodies that can be used in diagnostic immunohistochemistry.

Not many studies have been done to elucidate the mechanisms of overexpression of ΔN isoforms of p63 and p73 in human cancers. Identification of critical splicing factors and characterization of signaling pathways that contribute to this process will be critical to correct the errors for splicing for these genes in human cancers. Finally, specific targeting of ΔN - isoforms with antisense DNA, stabilized RNA, shRNA may have therapeutic values in treating human cancer overexpressing these splicing isoforms with oncogenic activity.

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