p53 family network and human cancer

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ABSTRACT

Since the 1980s cancer is the leading cause of death in Japan. Most human cancers exhibit inactivation of the p53 network, either through direct mutation of p53, or through disruption of regulatory pathways essential for p53 function. The tumor suppressor gene p53 encodes a transcriptional activator as a nodal point for cellular responses to several stress conditions. p53 is one of the most highly connected nodes in the cell, and an attack on p53 by mutation will disrupt basic cellular functions, particularly responses to DNA damage and tumor-predisposing stresses. p63 and p73 are functionally and structurally related to the tumor suppressor p53. Recent findings from others and us have provided evidence for a broader role for the p53 family than were previously reported. In this review, we provide an overview of the networks controlled by the p53 family as a framework for developing p53 family-based strategies to treat cancer.

Key words : Cancer, p53, p63, p73, Tumor suppressor, Gene therapy

INTRODUCTION

The tumor suppressor gene *p53* encodes a transcriptional activator as a nodal point for cellular responses to DNA damages, oncogenic stress and cellular stress conditions. The majority of cancer-associated mutations in p53 are missense mutations in its DNA-binding domain[1]. These mutations usually lead to the formation of a full-length mutant protein (mutant p53) incapable of transactivating p53 target genes and suppressing tumorigenesis. Besides the loss of wild-type activity, many p53 mutants also function as dominant-negative proteins that inactivate wild-type p53 expressed from the remaining wild-type allele. Some data indicate that mutant p53 proteins not only lose tumor suppressive functions, but also gain new abilities that promote tumorigenesis. Although still controversial, the presence of mutant p53 in cancer cells is associated with more aggressive tumors and correspondingly worse outcomes. The means by which mutant p53 exerts such pro-oncogenic activity are currently under exten-

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sive investigation and different models have been proposed. A subset of tumor-derived p53 mutants is proposed to physically interact with p53 family members, p63 and p73, and negatively regulate their proapoptotic function[2]. As half of human tumors harbor mutant forms of p53 protein, approaches aimed at disrupting the pathological interactions among p53 family members might be clinically useful.

The *p53* gene family consists of three members *p53*, *p63* and *p73* that are homologous in three domains: the N-terminal transcriptional activation domain (TA), the central core sequence-specific DNA-binding domain (DB) and the C-terminal located oligomerization domain (OL) (Fig. 1). p63 and p73, which generally share more homology with each other than with p53, can be expressed as two N-terminal isoforms that either contain (TA) or lack (ΔN) a full TA domain through alternate promoter usage (Figure 1). These two isoforms in turn can each have different C-termini as a result of alternative splicing. In general, the full-length TA versions of p63/p73 can exert p53-like activities, whereas the ΔN versions have an opposite effect[3].

At least three and seven C-terminal isoforms have been identified for p63 and p73, respectively[4, 5]. These distinct C-termini are thought to modulate the ability of the respective TA isoforms to transactivate target gene expression. The TAp63α and TAp73α isoforms are the largest proteins in each family. The TAp63γ and TAp73γ isoforms most closely re-

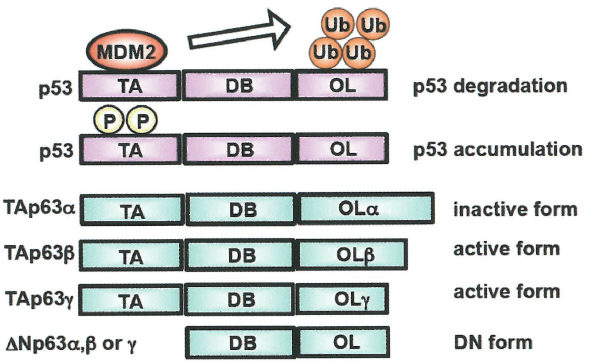
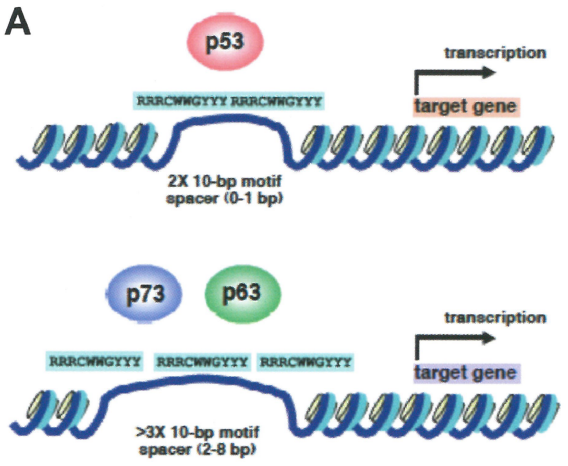


Figure 1 Structures of p53 and p63 major isoforms. The p53 family protein consists of three separable and functionally essential domains: an N-terminal transcriptional activation domain (TA), a central core sequence -specific DNA-binding domain (DB) and a C-terminal located oligomerization domain (OL). The regulation of transcriptional activity is quite different between p53 and p63. The amount of p53 protein in normal cells is determined mainly by the rate at which it is degraded. The degradation proceeds through ubiquitin-mediated proteolysis. The MDM2 protein is one of the major enzymes involved in labeling p53 protein with ubiquitin. As a cellular response to DNA damage, oncogenic stress and cellular stress conditions, p53 is phosphorylated, rendering it unable to bind to MDM2, which in turn results in the stabilization of p53 protein. Phosphorylation of the N-terminus of the p53 protein does not affect its DNA-binding activity, but affects its affinity for MDM2 and its subsequent degradation.
The *p63* gene uses several transcription initiation sites and extensive alternative splicing to generate a bewildering number of different mRNAs. For clarity, several alternative-splicing routes at the 5′ end of the gene are not indicated. Several protein domains can be distinguished; of these, the TA domains, the DB, and the OL domain are highly homologous to the corresponding domains in p53. p63 can be expressed as two N-terminal isoforms that either contain a TA domain or lack an N-terminal domain (ΔN) a full TA domain through alternate promoter usage. These two isoforms in turn can each have multiple C-termini as a result of alternative splicing. The capacity to transactivate gene expression at a p53-responsive target is given for each of the indicated isoforms (inactive form or active form). The ΔN isoforms have dominant negative (DN) effects against p53 and TAp63/p73.

semble full-length p53. In overexpression studies, TAp63γ was shown to be as potent as p53 in inducing apoptosis, whereas TAp73β is the most potent transactivating p73 isoform. However, both TAp63α and TAp73α isoforms cause weaker transactivation and apoptosis than other TA isoforms, and deletion of a C-terminal SAM (sterile alpha motif) domain restores the transactivation potency of both TAp63α and TAp73α (Fig. 1)

It is still unclear whether and to what extent TAp63 and TAp73 play roles in tumor suppression. *p63/p73* mutations are not commonly



B

	transactivation			binding site	reference
	p53	p73	p63		
JAG1	-	+	++	intron 2 RRRCWWGYYY — RRRCWWGYYY — RRRCWWGYYY — RRRCWWGYYY	ref. 9
JAG2	-	++	+	intron 2 RRRCWWGYYY — RRRCWWGYYY — RRRCWWGYYY RRRCWWGYYY	ref. 9, 14
AQP3	-	+	++	promoter RRRCWWGYYY — RRRCWWGYYY — RRRCWWGYYY	Zheng et al FEBS Lett (2001)
IL-4Rα	-	++	-	intron 1 RRRCWWGYYY — RRRCWWGYYY — RRRCWWGYYY RRRCWWGYYY RRRCWWGYYY — RRRCWWGYYY - RRRCWWGYYY RRRCWWGYYY	ref. 8
PEDF	-	+	++	promoter RRRCWWGYYY — RRRCWWGYYY — RRRCWWGYYY	ref. 10
FLOT2	-	++	+	promoter RRRCWWGYYY RRRCWWGYYY — RRRCWWGYYY	ref. 11
APOD	-	++	-	promoter RRRCWWGYYY — RRRCWWGYYY RRRCWWGYYY	ref. 12
TNNI2	-	++	-	promoter RRRCWWGYYY ———— RRRCWWGYYY — RRRCWWGYYY	

Figure 2 Specific target sequence for p53 family members. A. Nearly all p53 response elements previously reported contain two adjacent copies of the 10-bp motif. In contrast, studies in our laboratory and in others suggest that spacing between at least three copies of the 10-bp motif may be important for p73- and p63-specific transcriptional activation. B. List of the specific target genes for p53 family members, experimentally validated by ChIP and reporter assays. Left, the capacity to transactivate gene expression is given for each of the members. Right, schematic representation of the binding site within the target genes.

observed in human tumors. However, several lines of evidence suggest that p63 and p73 also mediate cellular responses to DNA damage agents and possess potential tumor suppression activity *in vivo*. When overexpressed, TAp63/p73 can activate a number of common p53 responsive genes involved in cell cycle arrest and apoptosis. Additionally, in one genetic background, mice heterozygous for *p63* and *p73* developed tumors[6].

2. Target genes of p53 family members

Despite extensive studies on the biological functions of the p53 family, little is known about the specific DNA binding sequences of p63 and p73 or the target genes regulated specifically by p63 and p73 but not by p53 (Fig. 2A). Identifying the specific target genes for each member of the p53 family that plays a role in cell proliferation and/or development is an important step for better understanding the physiological and tumor-repressive roles of p53 family members. So far, several genes have been reported to be specific targets of p63 and p73, such as *AQP3, REDD1, JAG1, JAG2, IL4R, deltaNp73, PEDF, FLOT2,* and *ApoD*. We and other groups have identified several target genes specifically regulated by p63 and p73 and their response elements, as summarized in Fig. 2B.

Nearly all p53 response elements reported previously contain two adjacent copies of the 10

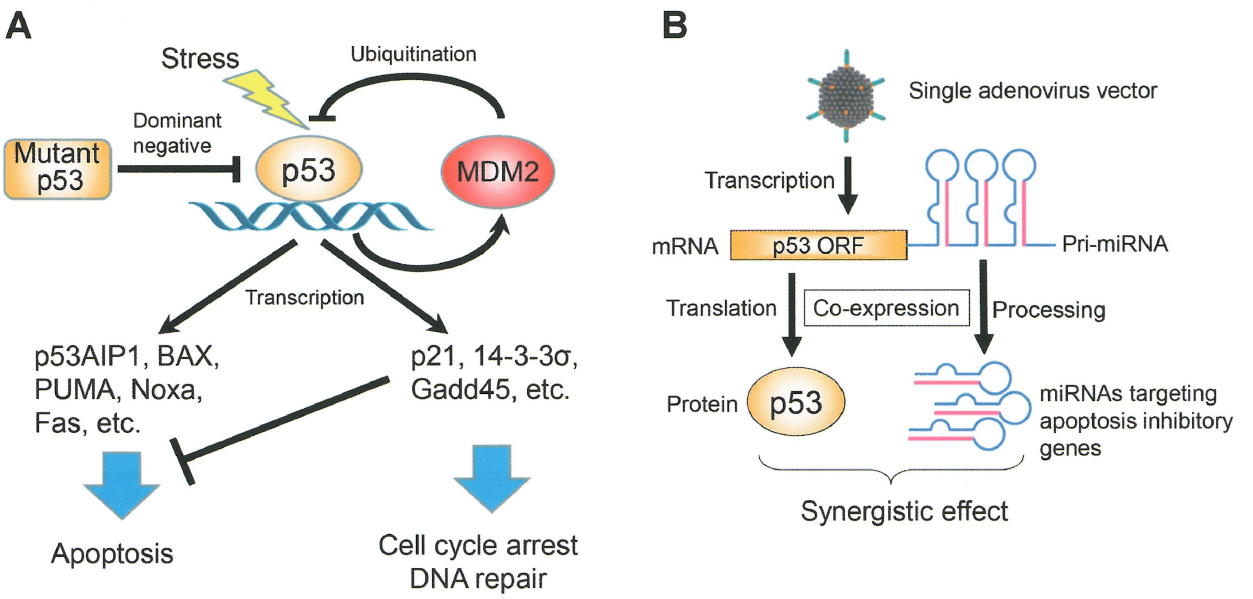


Figure 3 p53 transcriptional targets in apoptosis. A. The activation of p53 is induced by a variety of cell stresses, such as DNA damage, oncogene activation, spindle damage and hypoxia. Activated p53 transactivates a number of genes, many of which are involved in DNA repair, cell cycle arrest and apoptosis. Induction of p53 apoptotic targets can promote apoptosis. On the other hand, the cell cycle arrest mediated by cell cycle inhibitors like p21 can inhibit the apoptotic response. The suppression of these cell cycle inhibitors leads to the preferential induction of apoptosis rather than cell cycle arrest following p53 activation. B. Synergistic effect of p53 and miRNAs on cancer therapy. *p53* gene transfer does not always have a good therapeutic outcome. To explore the potential for a cancer therapy strategy that reintroduces p53 while inhibiting p21, we designed a recombinant adenovirus that includes p53 and microRNAs targeting p21. This vector expresses p53 while knocking-down p21. Introduction of this vector resulted in apoptosis in cancer cells and reduced tumor volume in a xenograft model more successfully than p53 alone. This result demonstrates the synergistic effect of p53 and miRNAs that target apoptosis inhibitory genes, and points the way to a novel approach for overcoming resistance to *p53* gene therapy.

-bp p53-binding motif[7], whereas studies in our laboratory and in others of the response elements for p73 and p63 within the *AQP3, JAG1, JAG2, IL-4Rα, PEDF, FLOT2, APOD,* and *TNNI2* genes and the *p73* gene itself show that these elements consist of three or four copies of the 10-bp consensus p53-binding motif separated by spacer sequences of up to 8-bp (Fig. 2A). We demonstrated that the spacer nucleotides in a response element of the IL-4R alpha *(IL-4Rα)* gene enhance p73-specific transactivation of *IL-4Rα*[8], suggesting that a spacer of at least three copies of the 10-bp motif may be important for p73- and p63-specific transcriptional activation.

(1) JAG1 and JAG2

To investigate how p63 and p73 are involved in tumorigenesis and normal development, we attempted to identify target genes that are specifically regulated by p63 and/or p73 but not by p53. We found that the *Jagged1 (JAG1)* and *Jagged2 (JAG2)* genes, which encode ligands for the Notch receptors, are up-regulated by p63 and p73[9]. In addition, we identified p63-binding sites in the second intron of both *JAG1* and *JAG2* genes, which can directly interact with p63 protein in cells, as assessed by a chromatin immunoprecipitation assay. Our findings show an association between the *p53* family genes and Notch signaling, and suggest a potential molecular mechanism for the involvement of the *p53* family genes in normal development.

(2) IL-4Rα

p73 plays important roles in neurogenesis, sensory pathways, and homeostatic regulation. We found that the IL-4Rα gene is up-regulated by p73 but not significantly by p53 in several human cancer cell lines[8]. In addition, we identified a p73-binding site in the first intron of the *IL-4Rα* gene that directly interacts with the p73 protein in cells. This p73-binding site consists of eight copies of a 10-bp consensus p53-binding motif and is a functional response element that is relatively specific for p73 and p63 among p53 family members. We also found that p73β-transfected tumor cells are sensitive to IL-4-mediated apoptosis. Our data suggest that IL-4Rα could mediate, in part, certain immune responses and p73-dependent cell death.

(3) PEDF

We discovered that expression of pigment epithelium-derived factor (PEDF) was specifically induced by either p63 or p73, but not by p53[10]. We also found that the *PEDF* gene contains a response element specific for p63 and p73 proteins in its promoter region and is a direct target of p63 and p73. Additionally, PEDF acts as a natural angiogenesis inhibitor in two hormone-sensitive organs, prostate and pancreas. Together, p63 and p73 may be involved in cell fate by inducing PEDF expression.

(4) FLOT2

We report here that flotillin-2 (FLOT2) a major hydrophobic protein localized to biomembrane microdomain lipid rafts, is a direct transcriptional target of members of the p53 family[11]. Such rafts have been suggested to play an important role in many cellular processes, including signal transduction, membrane trafficking, cytoskeletal organization, and pathogen entry. We found that the expression of flotillin-2 was specifically up-regulated by either TAp73β or TAp63γ, but not significantly by p53. Furthermore, we identified a conserved p73/p63-binding site located upstream of the *flotillin-2* gene that is responsive to p53 family members. We also found, by assessing IL-6-mediated STAT3 phosphorylation, that ectopic expression of TAp73 as well as TAp63 enhances signal transduction. Thus, in addition to direct transactivation, p53 family member genes enhance a set of cellular processes via lipid rafts.

(5) ApoD

We found that the expression of apoD was specifically up-regulated by either TAp73 or TAp63, but not significantly by p53[12]. In addi-

tion, *apoD* transcription is activated in response to cisplatin in a manner dependent on endogenous p73. We also identified a p73/p63-binding site in the promoter of the *apoD* gene that is responsive to p53 family members. The ectopic expression of TAp73 as well as the addition of recombinant human apoD to culture medium induced the osteoblastic differentiation of the human osteosarcoma cell line Saos-2, as assessed by alkaline phosphatase (ALP) activity. Importantly, apoD knockdown abrogated p73-mediated ALP induction. These results suggest that apoD induction may mediate the activity of p73 in normal development.

3. p53 family gene therapy

Exogenous overexpression of p53 inhibits the growth of cancer cells by inhibiting cell cycle progression and inducing apoptosis. Loss of p53 not only results in the development of tumors, but also in chemoresistance in many types of cancer. Therefore, introducing wild-type p53 back into tumor cells results in the activation of growth inhibitory pathways, which makes tumor cells sensitive to chemotherapeutic drugs. Indeed, *p53*-based gene therapy has been shown to be very effective against several types of cancer. However, *p53*-based gene therapy is not effective under certain conditions. For example, amplification *of MDM2*, mutational inactivation of p14ARF, or presence of the HPV E6 oncogene in HPV positive cervical cancer inactivates the p53 pathway of growth suppression. Although *p53* gene therapy is being tested clinically for the treatment of human cancer, some cancer models (in vivo and in vitro) are resistant to p53. To explore the potential use of two p53 homologues, p63 and p73, in cancer gene therapy, we introduced p53, p63 and p73 into colorectal cancer and osteosarcoma cell

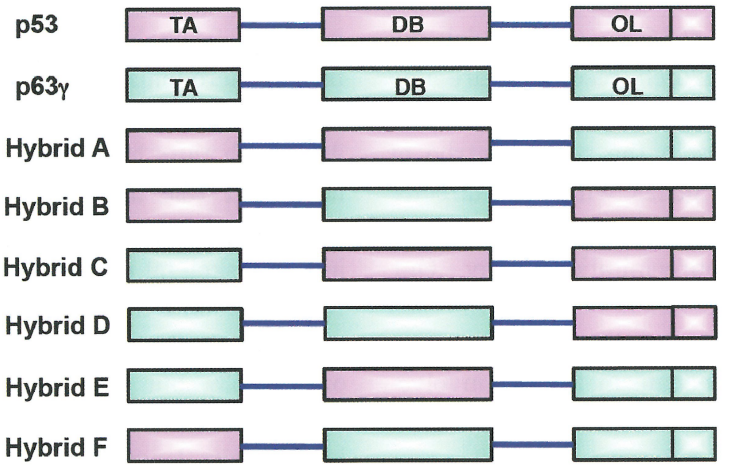


Figure 4 p53/TAp63γ hybrid constructs. We have generated a set of novel hybrid genes by taking advantage of the differential regulation of p53 and p63. The six hybrid proteins consist of three functional domains, each of which is derived from either p53 or p63. The constructs used are shown: for example, 'Hybrid A' is a hybrid protein containing the N-terminal and central domains of p53 fused in-frame to the C-terminal domain of p63; 'Hybrid B' is a hybrid p53 protein whose central domain is replaced with the central domain of p63. First, we expected that 'Hybrids A, D and E' containing the C-terminal domain of p63 would not oligomerize with endogenous mutant p53 in cancer cells. We also expected that 'Hybrids C and E' containing the N-terminal domain of p63 would neither bind to MDM2 nor be degraded in the proteasome pathway, although they still contain the central DNA-binding domain of p53. Finally, we supposed that 'Hybrids B and F' would change the transcriptional activity of p63 because the distinct C-termini (α, β and γ) of TAp63 are thought to modulate the ability of the respective TA isoforms to transactivate gene expression.

lines via adenoviral vectors, and compared their effects on cell growth.

(1) Adenovirus-mediated transfer of *p53* family genes: potential application to gene therapy of colorectal cancer and osteosarcoma

Among 10 cell lines tested, six cell lines displayed a similar response each other following transduction of p53, p63 or p73; two lines underwent cell-cycle arrest; three lines exhibited apoptosis; and one line showed no effect following transduction. The effect on cell-cycle progression varied in the other four cell lines. Interestingly, three cell lines were resistant to p53-mediated apoptosis, including two lines with endogenous wild-type *p53* alleles, but underwent apoptosis after transduction of p63 or p73. Similar to p53, transduction of p63 induced extensive apoptosis when combined with adriamycin or X-radiation in SW480 cells, which are resistant to apoptosis. Transduction of p63 and p73 also reduced the tumorigenicity of two colorectal cancer cells in vivo. These results suggest that adenovirus-mediated p63 and p73 transfer could represent a novel approach for the treatment of human cancers, particularly tumors that are resistant to *p53* gene therapy[13].

Osteosarcoma is one of the most common malignancies of the bone. Although prognosis of osteosarcoma has improved significantly during the past several years due to more intensive chemotherapy and radiotherapy regimens, new therapeutic approaches are needed for recurrent and inoperable cases. Like p53, p63 and p73 induce apoptosis in several cell types. We evaluated the antitumor effects of p63 and p73 on eleven different human osteosarcoma cell lines[14]. In vitro, adenovirus-mediated transduction of p63γ induced apoptosis in osteosarcoma cells that were resistant to p53-mediated apoptosis. Interestingly, the apoptotic effects of p63γ were greater than those of wild-type p53 in osteosarcoma cells with *MDM2*-amplification. We then determined the therapeutic effect in vivo of intratumoral injection of adenovirus-vectors expressing p53 family members into xenografts derived from Saos-2 cells implanted in nude mice, and found that infection with Ad-p63γ suppressed tumor growth more effectively than infection with Ad-p53. In addition, exogenous p73β and p63γ significantly increased the chemosensitivity of osteosarcoma cells to doxorubicin and cisplatin, which are commonly used as chemotherapeutic agents in the treatment of osteosarcoma. Our studies suggest that adenovirus-mediated transduction of p53 family members may be useful in gene therapy of osteosarcoma, particularly when they are combined with chemotherapeutic agents[14].

(2) Histone deacetylase inhibitor FK228 enhances adenovirus-mediated *p53* family gene therapy

Current clinical success rates of adenoviral vector-based gene therapy remain unsatisfactory. A major obstacle to this approach is thought to be the low adenoviral vector transduction efficiency of tumor cells, which has been attributed to the weak expression of the coxsackie-adenovirus receptor (CAR). Therapeutic replacement of the wild-type *p53* gene has been pursued as a potential gene therapy strategy in a variety of cancer types; however, some cancer models are resistant to p53 *in vivo* and in vitro. Therefore, to improve *p53* gene therapy, it is important to overcome the resistance to p53-mediated apoptosis.

Histone deacetylase inhibitors are a novel class of chemotherapeutic agents that reverse the malignant phenotype of transformed cells. A natural histone deacetylase inhibitor, FK228 (also called Romidepsin/Istodax), is a member of a new class of antineoplastic agents that are active in T-cell lymphoma. FK228 has been approved by FDA and is in clinical use for the treatment of cutaneous T-cell lymphomas. In addition, FK228 is currently in phase II clinical trials for the treatment of advanced solid tumors. FK228 has been reported to enhance adenovirus infection due in part to its ability to up-regulate coxsackievirus adenovirus receptor ex-

pression.

We performed preclinical experiments to establish a mechanistic rationale for the use of a combination of adenovirus-mediated *p53* family gene transfer and FK228 pretreatment in future clinical trials[15]. Pretreatment with FK228 enhanced apoptosis in human cancer cells through enhanced transduction of Ad-p53. FK228 also induced hyperacetylation of the p53 protein and specifically enhanced p53-mediated Noxa expression. Additionally, the combination of FK228 and Ad-p53 induced Bax cellular-translocation to mitochondria. The double knockdown of Bax and Noxa expression by small interfering RNA antagonized the synergistic effect of Ad-p53 and FK228 on apoptosis induction. In human cancer xenograft models, FK228 significantly increased the therapeutic effectiveness of p53 as well as that of p63. These results provide a strong rationale for the use of a combination of *p53* gene therapy and HDAC inhibitor pretreatment in cancer therapy.

(3) A single recombinant adenovirus expressing p53 and artificial microRNAs targeting p21

Although gene transfer involving p53 is viewed as a potentially effective cancer therapy, it does not result in an effective therapeutic response in all human cancers. The activation of p53 induces either in cell cycle arrest or apoptosis (Fig. 3A). Cell cycle arrest in response to p53 activation is mediated primarily through the induction of the cyclin-dependent kinase inhibitor p21[16]. Because p21 also has an inhibitory effect on p53-mediated apoptosis, the suppression of p53-induced p21 expression would be expected to result in the increased induction of apoptosis. We developed an adenovirus vector that expresses p53 and suppresses p21 simultaneously to enhance p53-mediated apoptosis[17]. A replication-deficient recombinant adenovirus (Ad-p53/miR-p21) was constructed, enabling co-cistronic expression of both p53 and artificial microRNAs that were designed to specifically knockdown the *p21* gene (Fig. 3B). An evaluation of therapeutic effectiveness of this vector *in vitro* and *in vivo* showed that the levels of p21 were significantly attenuated following infection with Ad-p53/miR-p21. In colorectal and hepatocellular carcinoma cells, infection with Ad-p53/miR-p21 augmented apoptosis as compared with a recombinant adenovirus that expressed p53 alone (Ad-p53/miR-control). Ad-p53/miR-p21 also significantly increased the chemosensitivity of cancer cells to adriamycin. In a xenograft tumor model in nude mice, tumor volume was significantly decreased following the direct injection of Ad-p53/miR-p21 into the tumor, as compared with the injection of an Ad-p53/miR-control. These results suggest that the simultaneous adenovirus-mediated transduction of p53 and microRNAs designed to specifically knockdown p21 may be useful for gene therapy of human cancers[17].

(4) Super Hybrid p53

The p53 family protein consists of three separable and functionally essential domains: an N-terminal transcriptional activation domain (TA), a central core sequence-specific DNA-binding domain (DB) and a C-terminal located oligomerization domain (OL)[18] (see Fig. 1). However, p53 and p63 show distinct patterns of transcriptional and post-transcriptional regulation. p53 is ubiquitously transcribed at a relatively high level, while the amount of p53 protein in normal cells is determined mainly by the rate at which it is degraded. The degradation proceeds through proteolysis mediated essentially by the ubiquitination of p53 by MDM2. This process is subject to a feedback loop. Phosphorylation of the N-terminus of p53 protein does not affect its DNA-binding activity, but affects its affinity for MDM2 and its subsequent degradation[3].

In contrast to p53, however, protein degradation does not mainly regulate the transcriptional activity of the p63. The structure of p63 is more complex than that of p53 due to the existence of several p63 variant isoforms . The *p63*

gene is expressed as multiple isoforms with different N-and C-termini, which results from the use of two different promoters and alternative splicing. p63 isoforms can possess one of two N-termini; one that is transcriptionally active (TA) and the other N-terminally deleted (ΔN). Additionally, these isoforms can have different C-termini (α, β, γ and etc)[5]. The functional differences among p53 family members could be due partly to differential regulation of alternative promoters and splicing, as exemplified by the multiple splice variants of p63. ΔN variants lack the transactivation domain and are transcribed from an internal promoter within exon 3 of the full-length gene[4, 19]. The different isoforms have different activities; the TA isoforms function similarly to p53 in cultured cells, transactivate some p53 target genes but not others, and induce apoptosis. In contrast, the ΔN isoforms are thought to play a role in inhibiting the transactivation of target genes of p53 and TA isoforms of p63. Therefore, ΔNp63 isoforms can exert dominant-negative effects over p53, TA p73, and TAp63. The TA isoforms might function in tumor suppression, while increased expression of the ΔN isoforms might be oncogenic[19].

To enhance the tumor suppressive activity of the p53 family, we generated a set of novel hybrid genes by taking advantage of the differential regulation of p53 and p63 activities. The basic constructs of the hybrid proteins are shown in Fig. 4. The six hybrid proteins consist of three functional domains, each of which is derived from either p53 or p63. The constructs used our recent study (in preparation) are shown in Figure 4: for example, 'Hybrid A' is a hybrid protein containing the N-terminal and central domains of p53 fused in-frame to the C-terminal domain of p63; 'Hybrid B' is a hybrid p53 protein whose central domain is replaced with the central domain of p63. First, we expected that 'Hybrids A, D and E' containing the C-terminal domain of p63 would not oligomerize with endogenous mutant p53 in cancer cells.

We also expected that 'Hybrids C and E' containing the N-terminal domain of p63 would neither bind to MDM2 nor be degraded in the proteasome pathway, although they still contain the central DNA-binding domain of p53. Finally, we supposed that 'Hybrids B and F' would change the transcriptional activity of p63 because the distinct C-termini (α, β and γ) of TA p63 are thought to modulate the ability of the respective TA isoforms to transactivate gene expression. We have tested the effects of each of the six hybrid constructs on tumor suppression activity. Interestingly, significantly higher proapoptotic activity than that of p53 and p63 was obtained only when one specific hybrid was introduced in tumor cells. Thus, this hybrid construct is a promising candidate for the 'Super Hybrid p53' approach to gene therapy of cancer.

4. Conclusions

The *p53* tumor-suppressor gene integrates numerous signals that control cell life and death. As when a highly connected node in the internet breaks down, the disruption of p53 function has severe consequences[20-22]. The fact that the p53 pathway is impaired in the majority of human cancers has encouraged efforts to understand p53 function in normal and neoplastic states. Most human tumors exhibit inactivation of p53, either through direct mutation of p53 itself, or through disruption of regulatory pathways essential for p53 function.

Functionally, therefore, it is clear that p53-targeted therapy could allow tumor-specific intervention. Small molecule strategies have been designed to activate p53 family members such as p73; to manipulate p53 posttranslational modulators with the aim of increasing wild-type p53 protein levels; to develop protein-protein interaction inhibitors such as Nutlin-3 to prevent wild-type p53 binding to MDM2 or viral proteins; and to restore p53 function to mutant p53 by direct modulation of its conformation. Another direct method is to deliver the functional wild-type p53 to tumors via gene therapy.

Recently, gene therapy has shown promise

in preclinical studies as a novel molecular treatment for malignant tumors. Multiple gene transfer strategies that enhance immune responses, or introduce foreign genes that directly kill tumor cells by restoring the function of a defective tumor-suppressor gene or by inducing apoptosis, are presently under investigation. The effective administration of therapeutic genes to tumor cell targets requires an efficient delivery system. While it is accepted that adenoviral vectors are superior for *in vivo* gene transfer, the current efficiency of gene transfer by these vectors may nonetheless be suboptimal for cancer gene therapy. In several clinical trials involving *in vivo* gene delivery, the resistance of target tissues to adenoviral infection has been reported. The most promising strategy has been pursue the gene transfer experiments that originally helped to establish p53 as a tumor suppressor in human cancer patients. Indeed, several organizations took up this approach, including Introgen Therapeutics (Texas), which reported promising data as early as 1996. However, the trials and transfer of the technology to the clinic were slow to complete. Eventually, p53, delivered with the aid of an adenovirus vector, was approved in 2004 for the treatment of head and neck cancer in China. Although we still have to wait for a critical assessment of the results of this trial, it is remarkable that this is the first ever gene therapy protocol approved for routine clinical use in humans.

We previously reported that the p53 family members p63 and p73 have a significant apoptotic effect in cancer cells[13, 14]. The incorporation of *p53* family genes together with multiple, tandem artificial miRNAs targeting negative regulators of these family members in a single recombinant adenovirus could also be effective[17]. Our studies suggest that adenovirus-mediated transduction of p53 family members may have utility in gene therapy of human cancers, particularly in combination with chemotherapeutic agents. We have also shown that a non-cytotoxic dose of HDAC inhibitor FK228 can enhance transgene expression and the therapeutic effect of adenovirus-mediated *p53* family gene transfer[15]. These studies suggest a simple, clinically practical method for increasing the sensitivity of cancer cells to adenoviral gene therapy vectors. By avoiding the need for high adenoviral titers, the likelihood of viral toxicity is reduced. Hence, future study should be considered in planning clinical trials combining adenovirus-mediated *p53* family gene transfer and HDAC inhibitors[15].

In the current study, we designed an adenoviral vector that expressed artificial p21-specific miRNAs[17]. Artificial miRNAs designed against negative regulators of p53 could also be utilized. The *MDM2* oncogene is amplified in several cancers. However, *MDM2* is a p53 target gene that also mediates the ubiquitination and degradation of p53, thereby creating a negative-feedback mechanism for regulating p53 cellular levels. Missense mutations of *p53* are found in many cancers and exert a dominant negative effect on exogenous wild-type p53. Thus, one could design artificial miRNA sequences that target the 3′UTR of the p53 mRNA, which would specifically knock down endogenous mutant p53, while having no effect on exogenous wild-type p53 expressed from an expression vector containing only the coding region of *p53*. One would expect that if additional negative regulators of p53 are knocked down, with artificial miRNAs designed into this vector system, the apoptotic effect of p53 might be enhanced. Vector-mediated gene transfer of *p53* is viewed as a potentially effective cancer therapy. Therefore, many clinical trials of adenovirus-mediated *p53* gene therapy are ongoing. Our ongoing pre-clinical studies suggest that p53 family members and Super Hybrid *p53* gene therapy, as well as combinations of these approaches with other strategies including HDAC inhibitors and RNAi-mediated suppression of anti-apoptotic genes, should be explored as ways of overcoming cellular resistance to *p53* gene therapy in human cancers. Although most

of current pre-clinical gene therapy needs further optimization, the success in proving that gene delivery can reactivate p53 marks the first step in transferring p53 family-based cancer therapy to the clinic.

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