p73 Isoforms Can Induce T-Cell Factor–Dependent Transcription in Gastrointestinal Cells

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Abstract
A new p53 family member, p73, and its isoform ΔNp73 are increasingly recognized in cancer research as important players in tumorigenesis, as well as in chemotherapeutic drug sensitivity. Despite substantial structural similarities to p53, accumulating evidence suggests that p53 and p73 may play different roles in human tumorigenesis. In this study, we have investigated the role of p73 and ΔNp73 in upper gastrointestinal tumorigenesis. Our results indicate that p73 and ΔNp73 are frequently overexpressed in >60% of primary adenocarcinomas of the stomach and esophagus. We have demonstrated that this overexpression can lead to the suppression of p73 transcriptional and apoptotic activity in gastrointestinal cells. Moreover, it induces β-catenin up-regulation and T-cell factor/lymphocyte enhancement factor–dependent transcription. Wild-type p53, but not mutant p53, can inhibit this effect. Our results demonstrate a novel mechanism for activation of β-catenin in gastrointestinal tumors and support the concept that overexpression of p73 isoforms can play an important role in tumorigenesis.

Introduction
Upper gastrointestinal carcinomas (UGC), adenocarcinomas of the stomach and the esophagus, are the second most common cause of cancer death worldwide, with 1,288,000 new cases reported in the year 2000. Moreover, proximal tumors such as gastroesophageal junction and esophageal adenocarcinomas have the most rapidly rising incidence of all visceral malignancies in the United States and industrialized Western world, for reasons that are unclear (1).

A number of defects in regulatory molecules and cell signaling pathways have been demonstrated to occur in upper gastrointestinal tumors. β-Catenin, a pivotal constituent of the Wnt signaling pathway, is considered to be one such critical molecule. Under normal conditions, β-catenin is a structural component of cell-cell adhesive interactions, and it can also function as a transcription factor. On transcriptional activation, β-catenin translocates to the nucleus, where, together with T-cell factor (TCF)/lymphocyte enhancement factor transcriptional cofactors, it induces the expression of a number of target genes involved in the regulation of cellular proliferation. In cancer cells, β-catenin is typically activated through mutations in APC (a regulator of β-catenin protein stability) or in β-catenin itself. Although several studies have reported abnormal expression and localization of β-catenin in UGCs, for review, see ref. 2), mutations in the β-catenin and APC genes are relatively rare in these tumors, suggesting that there are other mechanisms of β-catenin activation (2).

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The p73 transcription factor was recently identified as a member of the p53 tumor suppressor family. Ectopically overexpressed p73 largely mimics p53 activities, including transactivation of an overlapping set of target genes, induction of cell cycle arrest, and apoptosis. In contrast to p53, the p73 gene gives rise to multiple proteins with functionally different properties. The properties of p73α and p73β, the most studied of all p73 isoforms, are similar to those of p53 when overexpressed. On the other hand, a recently discovered oncogenic isoform of p73, termed ΔNp73, can interfere with p53 function (3, 4).

The role of the p73 gene in human tumorigenesis is not well understood. Here we show that p73 and ΔNp73 are specifically overexpressed in UGCs, which leads to suppression of p73 transcriptional and apoptotic activity, as well as increased β-catenin protein levels and activation of TCF-dependent transcription.

Materials and Methods
Cell Culture, Vectors, and Transfections. The gastric cancer cell lines AGS and Kato III (American Type Culture Collection, Manassas, VA) were maintained in Ham’s F-12 and RPMI 1640 media (Invitrogen, Carlsbad, CA), respectively, supplemented with 10% fetal bovine serum. Expression plasmids for human p73α, p73β, and ΔNp73α have been described previously (3). PG13-luc, a p53 (p73) reporter plasmid, and a dominant-negative TCF construct were kindly provided by Dr. Bert Vogelstein at the Johns Hopkins University School of Medicine, Baltimore, MD. The luciferase reporters pTOP and pPOP were purchased from Upstate Biotechnology (Waltham, MA). Cells were transfected using LipofectAMINE 2000 (Invitrogen) or FuGENE 6 (Roche, Indianapolis, IN) reagents.

Tissue Preparation and Real-Time Reverse Transcription-Polymerase Chain Reaction Analysis. For quantitative real-time real-time reverse transcription-polymerase chain reaction (RT-PCR), 24 primary gastric, gastroesophageal junctional, and esophageal adenocarcinomas and 10 normal gastric epithelial samples were collected from patients at the University of Virginia according to a protocol approved by the Institutional Review Board. All tumors were dissected from frozen tissue specimens and had at least 75% tumor cell content, with the least possible amount of contaminating necrotic, inflammatory, and stromal cells. The normal gastric mucosal epithelial tissues were examined carefully, and they were devoid of any inflammatory or necrotic contaminating cells. Real-time RT-PCR analysis was conducted as described previously (5), with the following specific primers for p73, GGGCAC-CACGTTCGAGGCAACC (sense) and GATTGAACCTGGGCCATGACATG (antisense); and for ΔNp73, GTTAGCTGGTGACCCGACC (sense) and TCAGCTTGACCAAGGAGATGACA (antisense). Results were normalized to β-amyloid precursor protein, which had minimal variation in all normal and neoplastic UGC samples.

Luciferase and Apoptosis Assays. Luciferase activity was determined using a Dual-Luciferase Reporter Assay kit (Promega, Madison, WI). Results were normalized for Renilla luciferase activity, and cell death was measured as described previously (3). Briefly, AGS cells were seeded into 8-well chamber slides and transfected with 600 ng of the indicated expression plasmids per well using FuGENE 6 reagent. After 24 hours, apoptosis was measured using the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) method with the In Situ Cell Death Detection Kit (Roche). Expression was determined by indirect immunofluorescence with a p73-specific antibody in duplicate wells. Transfection efficiency was reproducibly ~40% of...
cells, similar among all constructs, and evenly distributed throughout the wells. TUNEL-positive cells (15 random fields, >500 cells) were counted, and the percentage of apoptosis in transfected cells was determined.

Cell Lysis, Antibodies, and Western Blotting. AGS cells were transfected with 1 µg of p73 expression plasmid together with 2 µg of either ΔNp73α expression plasmid or empty vector. Total cell lysates were prepared 24 hours after transfection and subjected to immunoblot analysis. Gel loading was normalized for equal loading with a monoclonal antibody against actin (C-2; Santa Cruz Biotechnology, Santa Cruz, CA). p73 antibodies were mouse monoclonal ER15 and GC15 (Oncogene Research Products, San Diego, CA) or rabbit polyclonal anti-ΔNp73 (Imgenex, San Diego, CA). Antibodies against HDM2 (IF2) and p21/Waf1 were obtained from Oncogene Research Products (Devon, United Kingdom).

Results

Frequent Overexpression of p73 Isoforms in Upper Gastrointestinal Adenocarcinomas. The p73 gene has two promoters (P1 and P2), which regulate the expression of p73 and ΔNp73, respectively (Fig. 1A). ΔNp73 has been shown to lack the transactivation domain at the NH2 terminus and has dominant-negative properties (3, 6). To examine the expression of p73 and ΔNp73 mRNA, we used real-time RT-PCR with sequence-specific primers that specifically amplify either full-length p73 or all known transcripts encoding ΔNp73.

Our analysis of p73 gene expression demonstrated frequent overexpression of p73 and ΔNp73 transcripts in analyzed UGCs compared with levels in the normal gastric mucosa (Fig. 1B and C). Indeed, in 18 of the 24 cancers (75%), p73 was specifically up-regulated between 5- and 176-fold (Fig. 1B), whereas ΔNp73 was overexpressed between 5- and 239-fold in 15 of these 24 analyzed cases (62%; Fig. 1C). We defined the overexpression as an arbitrary cutoff, delineating tumors with 5-fold or higher mRNA up-regulation compared with the average normal level. This cutoff is illustrated as dashed lines in Fig. 1B and C.

As a control, we examined the expression level of 10 normal mucosal biopsies from the esophagus and stomach. In normal tissues, the expression levels of p73 and ΔNp73 were low and did not vary significantly between samples. Notably, in the majority of UGC cases in which overexpression of p73 was observed, ΔNp73 was also found to be up-regulated in 15 of 18 UGC cases (83%). The relatively small number of tumor cases did not provide the necessary statistical power to evaluate the prognostic significance of the new ΔNp73 expression.

ΔNp73 Suppresses Transcriptional and Apoptotic Activities of p73 Isoforms in Gastrointestinal Cells. We used the p73 luciferase reporter construct PG13-luc, which contains 13 p53 (p73)-binding sites within its promoter, to demonstrate that ΔNp73 affects p73 transcriptional activity in UGCs. AGS (a gastric cancer line harboring wild-type p73 and p73) cells were transfected with p73α and p73β plasmids alone or together with ΔNp73α, along with this reporter. Our results demonstrated strong inhibition of p73 transcriptional activity by ΔNp73 (Fig. 2A). In addition, ΔNp73 suppressed the induction of the p73 (p53)-inducible proteins p21/Waf1 and HDM2 by the p73α and p73β isoforms in AGS cells (Fig. 2B, compare Lanes 2 and 3–5). Transfection of AGS cells with ΔNp73α alone did not affect the expression of these target genes (data not shown).

Moreover, ΔNp73 inhibited apoptosis induced by p73α and p73β isoforms. AGS cells were transfected with empty vector, p73α or p73β alone, or cotransfected with ΔNp73. Cells expressing p73β and, to a lesser extent, p73α underwent marked apoptosis 24 hours after transfection (72% and 32%, respectively). By contrast, cells cotransfected with ΔNp73 were protected from p73-induced apoptosis, reducing it to levels that were comparable with those of the vector control (Fig. 2C). Thus, ΔNp73 inhibits the transcriptional activity and apoptotic function of p73α and p73β in gastric cancer cells.

Activation of T-Cell Factor–Dependent Transcription by p73 Isoforms in Gastrointestinal Cell Lines. To investigate additional biological effects of p73 and ΔNp73 coexpression, we performed a reporter analysis using the TCF-responsive luciferase construct pTOP in the Kato III and AGS cell lines. p73α and p73β were both able to activate the TCF reporter. Surprisingly, in contrast to the inhibition of p73 by ΔNp73 observed with the p53 (p73) reporter PG13-luc (Fig. 2A), cotransfection of ΔNp73α with p73α or p73β resulted in even higher activation of the pTOP reporter than p73 alone in Kato III cells (Fig. 2A). Furthermore, the level of TCF reporter up-regulation induced by p73/ΔNp73 cotransfection was comparable with β-catenin/TCF4–induced activation, which we used as a positive control. A similar TCF response was also seen in AGS cells (data not shown).
To determine whether direct interaction of p73 with the reporter is necessary for its activation, we used point mutants of p73/H9251 and p73/H9252, which carry a mutation (R292H) in the DNA-binding domain (7). These mutants were unable to activate the TCF reporter, suggesting that an intact p73 DNA-binding domain is important for the activation of TCF reporter (Fig. 3A).

Despite the substantial structural homology between the p73 and p53 tumor suppressors, p53 fails to activate the TCF reporter. Moreover, when p73 was cotransfected with p53, activation of the TCF reporter was inhibited. In contrast, a DNA-binding domain mutant of p53 (R175H) enhanced the p73 effect, suggesting that transcriptional activity of p53 may be essential for this inhibition (Fig. 3A).

To further evaluate the specificity of this interaction, we used the pFOP luciferase reporter, which has mutated TCF binding sites. Neither p73/H9251 nor p73/H9252 was able to increase the activity of this reporter, implying that the activation mechanism is dependent on binding of these isoforms to the TCF elements in the reporter (Fig. 3B).

It has been demonstrated previously (8) that a dominant-negative form of TCF4, which lacks the β-catenin–binding domain, can suppress pTOP reporter activation induced by β-catenin. As shown in Fig. 4A, dominant-negative TCF4 also inhibits pTOP reporter activation induced by p73 isoforms.

Subsequently, we determined the effects of p73α, p73β, and ΔNp73α on endogenous β-catenin protein in AGS cells. β-Catenin protein level was increased as a result of cotransfection of p73 isoforms (Fig. 4B). p73α and ΔNp73α cotransfection resulted in a greater increase in β-catenin than p73β and ΔNp73α. One possible explanation for the difference may be the different levels of cellular stress induced by p73α and p73β. In particular, increased stress has previously been demonstrated to inhibit β-catenin up-regulation (9). The demonstration that p73β induces greater apoptosis than p73α in AGS cells (Fig. 2C) supports this hypothesis.

Discussion

In this study, we have demonstrated that p73 and ΔNp73 mRNAs are frequently up-regulated in UGCs. Notably, p73 and ΔNp73 were co–up-regulated in 83% of the studied cases. It has been shown previously (4) that the p73 gene is not mutated in gastric and esophageal carcinomas, indicating that in these tumors p73 is overexpressed without mutations. Using p73 reporter analysis, Western blotting, and TUNEL assay, we have demonstrated that ΔNp73 strongly inhibits “p53-like” transcriptional and apoptotic activity of p73 in gastrointestinal cells. Moreover, our data revealed a novel effect of the p73–ΔNp73 interaction that can lead to TCF-dependent transcriptional activation through the up-regulation of β-catenin in gastrointestinal cells. However, we think additional mechanisms underlying the TCF transcriptional activation may be involved, and indeed, two of our experiments suggest this possibility. Firstly, a mutation in the p73
Fig. 4. p73 in cooperation with ΔNp73 can increase the endogenous level of the β-catenin protein in the AGS gastric cancer cell line. A, a dominant-negative TCF4 mutant can inhibit the TCF luciferase reporter activation induced by p73 isoforms in AGS cells. B, Western blotting for β-catenin protein after transfection with p73 isoforms in AGS cells. Actin served as a loading control.

DNA-binding direct binding to DNA. Secondly, dominant-negative TCF4, a potent inhibitor of β-catenin, does not completely suppress TCF activation induced by p73. Additional studies are required to further elucidate these mechanisms.

The effect of ΔNp73α alone on β-catenin and TCF activity was minimal (Figs. 3A and 4B). By contrast, the ΔNp73 homologue ΔNp63 (an NH2-terminally truncated p63 isoform) can up-regulate β-catenin alone (10). The reason for this difference is not completely clear; as demonstrated in this study, ΔNp73α rather cooperates with full-length p73 isoforms in this regard. The data obtained in this study suggest that under normal circumstances, p73 is capable of both inducing apoptosis and activating TCF activity. However, in the presence of ΔNp73, the apoptotic effects of p73 are inhibited, whereas the TCF activities are enhanced (compare Figs. 2 and 4). The presumption would be that p73 cannot reveal TCF-stimulating properties without suppression of its apoptotic (“p53-like”) activity. This notion is consistent with our observation that mutant p53 has a similar effect on TCF activation as ΔNp73 (Fig. 3A). It has been demonstrated previously that, analogous to ΔNp73, tumor-derived p53 mutants can bind to and inactivate p73 apoptotic activity (11). On the other hand, wild-type p53 counteracts the effect of p73 on TCF-dependent transcription.

p73 has considerable structural similarity to the p53 tumor suppressor. However, the role of p73 in tumorigenesis apparently differs from that of p53. In contrast to p53, which is frequently mutated, only 9 mutations of p73 have been found in a series of 1,400 studied tumors (4). Moreover, p73 expression correlates with poor clinical outcome in hepatocellular carcinomas, colorectal carcinoma, and breast cancer [for review, see Benard et al. (4)]. One of the key differences between p53 and p73 is the presence of multiple p73 isoforms derived from alternative promoter usage and splicing. The discovery of the NH2-terminally truncated p73 isoform ΔNp73 and its essential role in embryonic development of the nervous system as an inhibitor of p53, led to the development of the ΔNp73 promoter. Mol Cell Biol 2002;22:2575–85.


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