Aberrant expression of ΔNp73 in benign and malignant tumours of the prostate: correlation with Gleason score

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BACKGROUND: The p73 gene is a p53 homologue that induces apoptosis and inhibits cell proliferation. N-terminal truncated isoforms of p73 (ΔNp73) act as dominant-negative inhibitors of wild-type p53 and TAp73 and result in tumour growth in nude mice.

AIMS: To detect ΔNp73 expression in 24 benign prostatic hyperplasia samples, 33 prostate carcinomas, and five normal samples and to evaluate the relation between ΔNp73, TAp73 concentrations, and the clinicopathological characteristics of patients with prostate cancer.

METHODS: TAp73 was determined by real time polymerase chain reaction (PCR); ΔNp73 and ΔN*p73 were assessed using reverse transcription PCR. Western blotting was used to analyse protein expression. p53 mutation was determined by immunohistochemistry.

RESULTS: A significant increase of ΔNp73 was seen in 20 of 33 carcinomas and 17 of 24 benign prostate hyperplasia tissues, but in none of the normal samples. None of the specimens expressed ΔN*p73. No significant relation was found between TAp73 expression and clinical parameters. The incidence of positive expression of ΔNp73 correlated with the Gleason score in prostate carcinomas. Cancer samples with wild-type p53 had significantly higher expression of ΔNp73 than p53 mutant cancers.

CONCLUSION: These data suggest a potential role for ΔNp73 in prostate cancer progression.

MATERIAL AND METHODS

Cell culture

LNCaP, DU145, SP3031, 22Rv1, PC-3, and K-562 cells were obtained from the American Type Culture Collection (Rockville, Maryland, USA). They were cultured in RPMI 1640 (HyClone, Logan, Utah, USA) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO2 atmosphere at 37°C.

Patients and tissues

BPH samples were obtained from 24 patients after transurethral resection. Clinically localised prostate tumours were

Abbreviations: ΔN, N-truncated; BPH, benign prostate hyperplasia; Ct, cycle threshold; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBS, phosphate buffered saline; PCa, prostate carcinoma; PCR, polymerase chain reaction; PSA, prostate specific antigen; RT, reverse transcriptase.
obtained from 33 patients undergoing radical prostatectomy. Staging was assessed after pathological examination of formalin fixed specimens on the basis of the 1997 TNM classification. Clinical and biological data from the patients are provided (table 1). Five normal prostate samples were obtained at necropsy, eight to 10 hours after death, from five men ages 20–38 years without a history of reproductive or endocrine disease.

**Real time PCR and RT-PCR**

Total RNA was extracted from cells and tissues using the RNAeasy minikit (Qiagen, Hilden, Germany). Total RNA (2 μg) was reverse transcribed using Superscript II RT (Invitrogen, Carlsbad, California, USA) according to the manufacturer’s protocol. Real time PCR was performed using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, California, USA) with SYBR green master mix (Applied Biosystems), 5 μM of each primer at a 5 μM concentration, and 1 μl of the previously reverse transcribed cDNA template. The cycling conditions were as follows: 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C followed by one minute at 60°C.

Calculations were performed using values of average cycle threshold (Ct) as the calibrator from normal samples. For each experimental sample, the Ct of the target, normalised to that of the endogenous reference and relative to the calibrator, is given by: $2^{-\Delta \Delta Ct}$.

Routine RT-PCR for detection of ΔNp73 and ΔN'p73 was carried out using HotStarTaq DNA polymerase (Qiagen). The authenticity of the RT-PCR products was verified by sequencing. Tumour samples were defined as ΔNp73 or ΔN'p73 positive if a PCR amplification band was detectable after 35 cycles of amplification. The primers are as follows: TAp73 sense, 5'-GCACCAGGTTGGACACCCTCATC-3' and antisense, 5'-GACA GTAGAAGCTAGGATG-3'; ΔNp73 sense, 5'-CAAACGGCCCGCATGTCC-3' and antisense, 5'-TCGAGCTGGCCCTCCGC-3'; ΔN'p73 sense, 5'-GGGAGGCGCCAAGGATGTCC-3' and antisense, 5'-GGAAGGCGGAGGATGTCC-3'.

**Immunohistochemical detection of mutant p53**

Tissue sections (5 μm thick) of fixed, paraffin wax embedded specimens were cut, mounted on poly-L-lysine slides (Superfrost Plus; VWR, Milan, Italy), and then dewaxed in ethanol solutions. The sections were then immersed in xylene solutions. The sections were then dewaxed in ethanol solutions. Tissue sections (5 μm thick) of fixed, paraffin wax embedded specimens were cut, mounted on poly-L-lysine slides (Superfrost Plus; VWR, Milan, Italy), and then dewaxed in ethanol solutions. Staining was performed in a total volume of 20 μl, including 10 μl SYBR Green PCR master mix (Applied Biosystems), 5 μl of each primer at a 5 μM concentration, and 1 μl of the previously reverse transcribed cDNA template. The cycling conditions were as follows: 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C followed by one minute at 60°C.

Calculations were performed using values of average cycle threshold (Ct) as the calibrator from normal samples. For each experimental sample, the Ct of the target, normalised to that of the endogenous reference and relative to the calibrator, is given by: $2^{-\Delta \Delta Ct}$.
intracellular accumulation. Therefore, from now on we will refer to immunodetectable p53 as mutant p53.

**Western blot analysis**
Specimens were homogenised in Triton X-100 buffer with 500mM HEPES (pH 7.0), 150mM NaCl, 10% glycerol, 1mM EDTA, and 1.2% Triton X-100. The homogenate was centrifuged at 1000g for 15 minutes to remove whole cells, nuclei, and mitochondria. The supernatant was used to measure the protein concentration. An equal amount of protein (30μg) was loaded on to 10% polyacrylamide gels, followed by transfer to a nitrocellulose membrane sheet. The membranes were blocked with 5% skimmed milk in 0.01M PBS, pH 7.4. After incubation with primary antibodies, the membranes were washed with PBS for 10 minutes three times and then incubated with horseradish peroxidase conjugated second antibody. Signals were detected with an ECL system (Amersham Pharmacia Biotech, St Louis, Missouri, USA).

The following antibodies were used for western blot: a monoclonal antibody against p53 (Ab-1; Calbiochem, Cambridge, Massachusetts, USA), a polyclonal antibody against ΔNp73 (sc-23429; Santa Cruz Biotechnology, Santa Cruz, California, USA), and a monoclonal anti-GAPDH antibody (Chemicon)

**Statistical analysis**
The association between ΔNp73 expression, TAp73 expression, and other prognostic factors and the differences in the distribution of the groups of patients were assessed by the χ² test or Fisher’s exact test. All analyses were two tailed and were considered significant when p < 0.05.

**RESULTS**

**Expression of TAp73**
A change of threefold or greater was considered significant for TAp73 expression. The degree of expression was determined as the ratio between the TAp73 gene tested and the reference GAPDH gene to correct for variations in the amounts of RNA. Ratios were then normalised such that the mean ratio of the five normal prostate samples equalled 1.00. Overexpression of TAp73 was seen in 24 of 33 primary carcinomas, in three of five carcinoma cell lines (LNCaP, SP3031, and 22Rv1), and in 19 of 24 BPH specimens. The mean ratio of the TAp73 of the PCa and BPH samples was 9.65 and 11.21 times the mean ratio of the normals, respectively (p < 0.01) (fig 1).

**Expression of ΔNp73**
The normal tissues did not express detectable amounts of ΔNp73 transcripts, as determined by RT-PCR. Most of the tumours and BPH specimens showed increased expression of ΔNp73 (fig 2). A significant increase in ΔNp73 was seen in 20 of 33 tumours, 17 of 24 BPH samples, and two of five carcinoma cell lines (LNCaP and 22Rv1). We were unable to detect ΔNp73 in the samples. Nucleic acid sequencing revealed that the positive band represents the N-terminal truncated isoform ΔNp73. We failed to reveal a relation between TAp73 and ΔNp73 mRNA expression in PCa and BPH samples (tables 2, 3). Table 4 summarises the various clinicopathological characteristics of the patients with cancer in relation to ΔNp73 and TAp73 mRNA expression. Positive ΔNp73 expression correlated with tumour Gleason score (p = 0.037). There was no significant relation between ΔNp73 expression and age at surgery, TNM stage, or PSA serum concentration. There was also no association between TAp73 expression and these clinical parameters.

**Western blotting**
To verify whether our RT-PCR results reflected p73 protein expression, selected samples were analysed by western blotting using an antibody that recognises an epitope in the N-terminus of the truncated TAp73.

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**Table 3** Correlation between TAp73 expression and ΔNp73 in benign prostate hyperplasia tissues

| Table 3 Correlation between TAp73 expression and ΔNp73 in benign prostate hyperplasia tissues |
|---------------------------------|----------|-----------------|-----------------|
| ΔNp73 expression                | Positive | Negative | p Value |
| TAp73 expression                | Positive | Negative | p Value |
| Positive                        | 15       | 4       | 0.126         |
| Negative                        | 2        | 3       |               |

<table>
<thead>
<tr>
<th>Table 4 Relation between ΔNp73, TAp73, and clinicopathological characteristics in 33 patients with prostate carcinoma</th>
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<tr>
<td>Age at diagnosis</td>
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<td></td>
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<tr>
<td>&lt;65</td>
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<tr>
<td>≥65</td>
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<td>TNM</td>
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<td>T1a–T2b</td>
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<td>T3a–T3b</td>
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<td>Gleason score</td>
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<tr>
<td>2–6</td>
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<tr>
<td>7–10</td>
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<tr>
<td>PSA (ng/ml)</td>
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<tr>
<td>&lt;10.0</td>
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<td>≥10.0</td>
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PSA, prostate specific antigen.
N-terminal transactivation domain of p73 and an antibody against ΔNp73, which recognises ΔNp73 only, with no crossreactivity with TAp73 isoforms. Both TAp73 and ΔNp73 expression were found in the tumour and BHP tissues, but not in normal tissues. As shown in fig 3, protein concentrations correlated with the RT-PCR data.

**Immunohistochemistry**

We determined the p53 mutation status in our tumour collection via immunohistochemistry. Ten of 33 tumours and one of 24 BPH specimens expressed mutant p53 (fig 4). Significantly higher expression of the ΔNp73 isoform was found in p53 wild-type tumours, compared with p53 mutant tumours (p = 0.026). In contrast, there was no difference in the prevalence of TAp73 upregulation between wild-type and mutant tumours (table 5).

**DISCUSSION**

We analysed the expression of two N-terminally truncated p73 isoforms in 33 prostate carcinomas and 24 BPHs of all histological types, and correlated the results with clinicopathological parameters. The most striking finding was the high prevalence of upregulation of ΔNp73 in prostate specimens (20 of 33 PCa and 17 of 24 BPH specimens), suggesting that deregulation of ΔNp73 is a crucial event in the pathogenesis of this tumour. ΔNp73 is derived from the P2 promoter. In contrast, none of the specimens showed upregulation of the P1 promoter derived ΔNp73 transcript. We conclude that ΔNp73, rather than ΔNp73, is the main contributor to total ΔNp73 upregulation in prostate cancers.

Increased expression of wild-type p73 in tumour samples compared with normal tissues has been reported frequently,12–15 and in some cases overexpression of p73 correlated with an advanced tumour stage or poor prognostic parameters.16 17 Consistent with our findings, increased TAp73 expression has been reported previously in prostate tumours compared with normal tissue.18 As shown as table 4, no significant differences were detected concerning median age, TNM stage, Gleason score, or PSA serum concentration, indicating that TAp73 expression cannot be considered as a prognostic marker. However, ΔNp73 expression was strongly associated with the Gleason score, suggesting a potential role of ΔNp73 transcripts in prostate carcinoma progression, because the Gleason score is considered one of the most important prognostic indicators for prostate cancer. Our results are also in agreement with two recent ΔNp73 studies,19 20 which both found significantly higher expression of ΔNp73 in advanced tumours.

"ΔNp73 expression was strongly associated with the Gleason score, suggesting a potential role of ΔNp73 transcripts in prostate carcinoma progression"

Most human tumours show deregulation of the E2F1 family of transcription factors through the loss of cyclin dependent kinase inhibitor INK4, overexpression of cyclin D, or loss of retinoblastoma protein. E2F1 directly regulates p73 activity, suggesting a potential role of ΔNp73 transcripts in prostate carcinoma progression, because the Gleason score is considered one of the most important prognostic indicators for prostate cancer. Our results are also in agreement with two recent ΔNp73 studies,19 20 which both found significantly higher expression of ΔNp73 in advanced tumours.

**Table 5**

<table>
<thead>
<tr>
<th>p53 expression</th>
<th>TAp73 expression</th>
<th>p Value</th>
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</thead>
<tbody>
<tr>
<td>Mutant</td>
<td>Positive 6</td>
<td>0.400</td>
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<tr>
<td>Wild-type</td>
<td>Positive 18</td>
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<tr>
<td>ΔNp73 expression</td>
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<tr>
<td></td>
<td>Positive 5</td>
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<tr>
<td>ΔNp73 expression</td>
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<tr>
<td></td>
<td>Positive 17</td>
<td></td>
</tr>
<tr>
<td>ΔNp73 expression</td>
<td>Negative 7</td>
<td></td>
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<td></td>
<td>Positive 6</td>
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**Take home messages**

- Expression of the N-terminally truncated isoform of p73, ΔNp73, was high in 20 of 33 prostate carcinomas and 17 of 24 benign prostate hyperplasia tissue samples, but not in normal samples.
- Positive ΔNp73 expression correlated with the Gleason score in prostate carcinoma.
- These results suggest a potential role for ΔNp73 in prostate cancer progression.
Another surprising result of our study is the strong correlation between p53 status and the upregulation of dominant-negative ΔNp73 in tumours. Considering the fact that oncogene induced upregulation of p73 expression causes apoptosis, sustained overexpression of p73 therefore requires inhibition of its inherent proapoptotic activity. This can be achieved by p53 mutants, which inhibit the putative tumour suppressor action of p73 in a dominant-negative fashion by generating defective hetero-oligomers with wild-type p73. However, reduction of the apoptotic potential of p73 by mutant p53 cannot account for all cases of p73 overexpression. In our study, no correlation between p53 mutations and p73 overexpression could be detected in prostate carcinoma. In this respect, the interaction between p53 and ΔNp73 might contribute to malignancy in the absence of physical damage to the p53 gene. p53 is capable of inducing ΔNp73, a protein that can block p53 action by competing for p53 DNA binding sites. p53 induces ΔNp73 by directly binding to its promoter. ΔNp73 counteracts p53 action on its own promoter, creating an additional feedback loop that finely tunes the entire system. In addition, a similar loop regulates TAp73 action, because ΔNp73 expression is also induced by TAp73, and ΔNp73 also regulates the function of the TA forms. This suggests that ΔNp73 plays an important role in regulating p53 function and that its overexpression could play a role in tumorigenesis by generating a functional block of p53. We reasoned that if ΔNp73 isoforms are indeed oncogenic inhibitors of p53 and TAp73 in vivo, their upregulation should occur preferentially in wild-type p53 tumours, and our results support this notion.

In conclusion, our study identified a novel biological feature of CaA and BPH. Additional laboratory studies are needed to elucidate the genetic mechanism underlying the frequent expression of ΔNp73 in CaA and BPH and its biological consequences on cell survival.

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REFERENCES