# Quantitative *TP73* Transcript Analysis in Hepatocellular Carcinomas

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## ABSTRACT

***Purpose:*** **The p53 family member p73 displays significant homology to p53, but data from primary tumors demonstrating increased expression levels of p73 in the absence of any gene mutations argue against a classical tumor suppressor function. A detailed analysis of the p73 protein in tumor tissues has revealed expression of two classes of p73 isoforms. Whereas the proapoptotic, full-length, transactivation-competent p73 protein (TA-p73) has a putative tumor suppressor activity similar to p53, the antiapoptotic, $NH_2$-terminally truncated, transactivation-deficient p73 protein (ΔTA-p73) has been shown to possess oncogenic activity. The oncogenic proteins can be generated by the following two different mechanisms: (*a*) aberrant splicing (p73Δex2, p73Δex2/3, ΔN′-p73) and (*b*) alternative promoter usage of a second intronic promoter (ΔN-p73). The purpose of our study was to elucidate the origin of ΔTA-p73 isoforms in hepatocellular carcinomas.**

***Experimental Design:*** **We analyzed the underlying mechanisms of p73 overexpression in cancer cells by quantification of p73 transcripts from 10 hepatocellular carcinoma patients using isoform-specific real-time reverse transcription-PCR.**

***Results:*** **Our data demonstrate that only aberrantly spliced ΔTA-p73 transcripts from the TA promoter show significantly increased expression levels in the tumor whereas the ΔN-p73 transcript generated from the second promoter is not significantly up-regulated.**

***Conclusions:*** **Although we only analyzed 10 patient samples the results strongly suggest that the elevated activity of the first promoter (TA promoter) accounts for high-level expression of both full-length TA-p73 and aberrantly spliced ΔTA-p73 isoforms in hepatocellular carcinoma tissues.**

## INTRODUCTION

The p53 family comprises the following three members: the prototype tumor suppressor gene p53 and its newer homologues p73 and p63. Although p73 shares substantial homology with p53, its role in human malignancies is still unclear (1). p73 shares the following characteristic features of the p53 protein: an acidic, aminoterminal TA domain, a central core DNA-binding domain, and a COOH-terminal oligomerization domain (2). In experimental systems, p73 showed many of the following p53-like properties: binding to p53 DNA target sites, transactivation of p53-responsive genes, and induction of cell cycle arrest or apoptosis (2). In addition, p73 is activated by several p53-inducing stimuli such as DNA damage and oncogenes like E1A, c-myc, or E2F1 (3–5).

However, despite these similarities, the functions of p53 and p73 in tumorigenesis appear to be fundamentally different. In contrast to p53, p73 is not inactivated by classic viral oncoproteins to allow host cell transformation, indicating that p73 may augment, rather than inhibit, viral and cellular transformation (6). Whereas p53-deficient mice develop spontaneous tumors at high frequencies in various organs, those lacking p73 show no increased susceptibility to spontaneous tumor formation (7). Instead of the expected mutational inactivation of *TP73* in tumors, increased expression levels of wild-type p73 in the tumor compared with expression levels in the normal tissues were reported frequently (1, 8). In some cases, overexpression of p73 could even be correlated with an advanced tumor stage or poor prognostic parameters (8–10). In hepatocellular carcinomas (HCCs), for example, high p73 expression levels were revealed as an independent prognostic marker of poor patient survival prognosis (8).

A detailed analysis of p73 in tumor cells indicated the presence of $NH_2$-terminally truncated p73 proteins (ΔTA-p73) in p73-overexpressing tumor cells (11). Because the aminoterminal transactivation domain is missing, ΔTA-p73 proteins are transactivation deficient and fail to induce cell cycle arrest and apoptosis: functions that account for most of the tumor suppressor activity of p53 (12). Because ΔTA-p73 proteins retain DNA-binding competence, they act as dominant-negative inhibitors of p53 and full-length, transactivation-competent p73 proteins (TA-p73; Ref. 12). In addition, we showed recently that

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ΔTA-p73 isoforms also inactivate the retinoblastoma tumor suppressor RB (13). Comparable with other inhibitors of p53 function such as MDM2 or viral oncoproteins (human papillomavirus E6, adenovirus E1B 55K, SV40 large T antigen), we could recently show that expression of ΔTA-p73 results in malignant transformation of NIH3T3 cells and tumor growth in nude mice (11). Expression of ΔTA-p73 was identified as a strong predictor of unfavorable outcome independently from other prognostic factors in neuroblastoma patients (10), supporting an oncogenic function of ΔTA-p73. The origin of ΔTA-p73 proteins, however, is still unclear. Some ΔTA-p73 transcripts are generated by aberrant splicing (p73Δex2, p73Δex2/3, ΔN′-p73); others are derived from a second intronic promoter (ΔN-p73).

Classic oncogenes like adenovirus E1A and c-myc as well as oncogenic alterations within the RB-p16 pathway leading to an increased activity of E2F transcription factors were shown to activate the E2F-responsive TA promoter (4, 5, 14, 15). Increased p73 levels in cancer tissues might therefore be attributable to increased transactivation of the *TP73* gene by tumor-associated deregulation of E2F by various oncogenes. In contrast, the alternative ΔN promoter has been shown to be p53/p73-responsive resulting in an autoregulatory feedback loop (16–19).

In this study, we further investigated the underlying mechanism of p73 overexpression in cancer cells by quantification of p73 transcripts from 10 hepatocellular carcinoma patients using isoform-specific real-time reverse transcription (RT)-PCR. Interestingly, in these hepatocellular carcinoma (HCC) patients, only aberrantly spliced ΔTA-p73 transcripts show significantly increased expression levels in the tumor whereas the ΔN-p73 transcript generated from the second promoter is not significantly up-regulated. Therefore, elevated activity of the first promoter (TA promoter) accounts for high-level expression of both TA-p73 and ΔTA-p73 in this set of HCC samples.

## MATERIALS AND METHODS

**Preparation of HCC Samples.** Tissues samples from HCC tissue and adjacent normal liver were obtained from patients undergoing partial hepatectomy. Normal and neoplastic liver cells were isolated from liver sections of 10 HCC patients by laser capture microdissection.

**Loss of Heterozygosity Analysis.** The polymorphic region of *TP73* exon 2 was amplified from genomic DNA extracted from microdissected normal and tumor tissues by PCR using the primers 5′-CAGGAGGACAGAGCACGAG-3′ and 5′-CGAAGGTGGCTGAGGCTAG-3′. The PCR product was then digested with *Sty*I. The presence of the AT polymorphism creates a *Sty*I restriction site. The products were resolved by electrophoresis on 3% agarose.

**Real-Time RT-PCR.** Total RNA was isolated with the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol and reverse transcribed with Omniscript RT (Qiagen) using random hexamers (Applied Biosystems). PCR reactions contained 1× Lithos qPCR SYBR Green I Master Mix (Eurogentec), 150 nM of each primer, 2.5 mM $MgCl_2$, and 0.5 mg/ml BSA (Roche) and were carried out in a LightCycler (Roche Diagnostics). Amplification products were verified by melting curves, agarose gel electrophoresis, and direct sequencing. Standard curves for absolute quantification were obtained with plasmids containing the various amplicons. Sequences of the primers shown in Fig. 1 are as follows: TA-p73 sense, 5′-GGCTGCGACGGCTGCAGAGC-3′ and antisense, 5′-GCTCAGCAGATTGAACTGGGCCATG-3′; p73Δex2 sense, 5′-GGCTGCGACGGCTGCAGGGA-3′ and antisense, 5′-CAGGCGCCGGCGACATGG-3′; p73Δex2/3 sense, 5′-GGCTGCGACGGCTGCAGGCC-3′ and antisense: 5′-CAGGCGCCGGCGACATGG-3′; ΔN′-p73 sense, 5′-TCGACCTTCCCCAGTCAAGC-3′ and antisense, 5′-TGGGACGAGGCATGGATCTG-3′; ΔN-p73 sense, 5′-CAAACGGCCCGCATGTTCCC-3′ and antisense, 5′-TGGTCCATGGTGCTGCTCAGC-3′. The housekeeping gene S9 ribosomal protein was quantified with the following primers: 5′-GATGAGAAGGACCCACGGCGTCTGTTCG-3′ and antisense 5′-GAGACAATCCAGCAGCCCAGGAGGGACA-3′.

**Control Plasmids, Standard Curves.** Amplicons were individually cloned into the pCR2.1 vector using "TOPO cloning" technology (Invitrogen). In addition, ΔTA-p73 amplicons were cloned into the *Msc*I site of pCR2.1-TA-p73 to obtain compound plasmids that were used to generate two different standard curves from the same series of dilutions. By using a plasmid containing two isoforms in a 1:1 ratio as a template, equal quantities could be assigned to each dilution point of the two standard curves. Therefore, generating the standard curves for the ΔTA-p73 isoforms in relation to TA-p73 allowed us to reliably compare copy numbers for all individual isoforms.

## RESULTS

Considering the potential relevance of ΔTA-p73 proteins in tumorigenesis, it is important to understand the mechanisms that give rise to ΔTA-p73 expression. ΔTA-p73 proteins are encoded by at least four different transcripts (Fig. 1). One of them (ΔN-p73) is generated from an alternative, cryptic promoter in intron 3 (ΔN promoter). This transcript was first identified as the predominant p73 isoform in developing mice (7). Later, a similar transcript with high-sequence homology to murine ΔN-p73 was identified by several groups in human cells (11, 12, 16, 20, 21). Apart from this "physiological" ΔN-p73 transcript with a distinct regulation via an independent promoter, at least three aberrantly spliced transcripts (p73Δex2, p73Δex2/3, and ΔN′-p73) regulated by the first promoter (TA promoter) are found in human tumor cells (2, 11, 12, 22, 23). Thus, ΔTA-p73 proteins are translated from several different transcripts that are under the control of two distinct promoters.

Analyzing the ΔTA-p73 proteins themselves, however, does not unambiguously reveal their origin. For example, the ΔN-p73 protein, which has a 13 amino acid epitope specific for exon 3B, is not specific for the ΔN promoter because the TA promoter-regulated ΔN′-p73 transcript encodes for the same protein (Fig. 1*D*). To assign ΔTA-p73 production to one promoter, we therefore rely on the analysis of p73 mRNAs, which contain the information concerning their origin in their sequence. The first 78 base pairs of the ΔN-p73 transcript, for example, are unique and not present in the ΔN′-p73 transcripts, whereas the splice junction exon3/exon3B is characteristic for the ΔN′-p73 transcript (11, 12, 21).

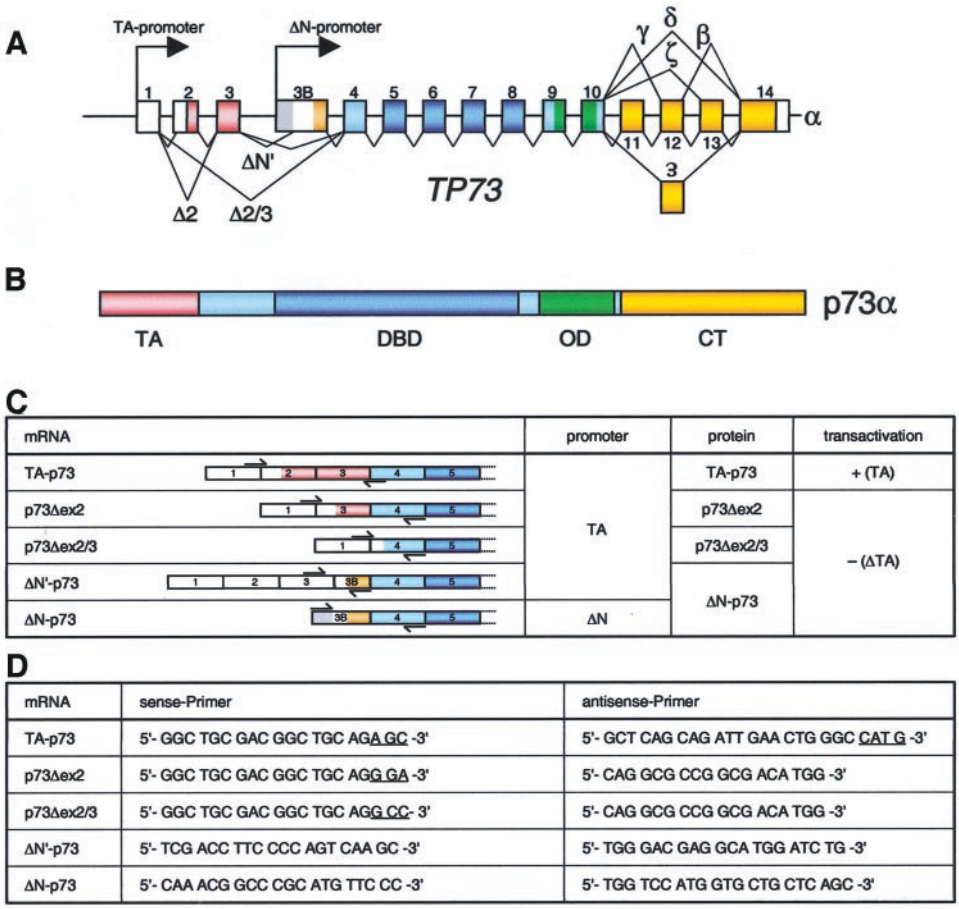


*Fig. 1* Isoform-specific real-time reverse transcription (RT)-PCR for *TP73*. *A*, genomic organization of the *TP73* gene locus. Exons are depicted as boxes and colored according to the following domain structure: *red*, transactivation domain; *orange*, exon 3B-derived coding sequence; *blue*, DNA-binding domain; *green*, oligomerization domain; *yellow*, COOH-terminus. The main COOH-terminal (α-, β-, γ-, δ-, ϵ-, ζ-isoforms) splice variations are shown. The transcriptional start sites of the two promoter regions (TA promoter and ΔN promoter) are indicated by *arrows*. Aberrantly spliced transcripts regulated by the TA promoter are labeled *Δ2* (p73Δex2), *Δ2/3* (p73Δex2/3), and *ΔN′* (ΔN′-p73). The unique 5′-untranslated region of the ΔN-p73 transcript is colored *gray*. *B*, domain structure of the full-length TA-p73α isoform (color coding as in *A*; *TA*, transactivation domain; *DBD*, DNA-binding domain; *OD*, oligomerization domain; *CT*, COOH-terminus). *C*, amino-terminal transcript variations. Indicated is the exon structure of the individual mRNAs (color coding as in *A*), the promoter, from which they are transcribed, the encoded protein and their transactivation potential. Transactivation-competent proteins are named transactivation-competent p73 proteins (TA-p73) and transactivation-deficient $NH_2$-terminally truncated p73 proteins (ΔTA-p73). Note that two differently regulated transcripts (ΔN′-p73 and ΔN-p73) encode for the same ΔTA-p73 protein (ΔN-p73). In addition, the localization of the primer pairs used for real-time PCR assays is marked by *arrows*. *D*, sequences of the sense- and antisense-primers used for isoform-specific amplification of individual p73 isoforms.

To analyze which promoter is responsible for ΔTA-p73 production in cancer cells and to quantify individual *TP73* transcripts, we developed specific real-time RT-PCR assays for each isoform. In general, there are two possible ways to distinguish splice variants directly: (*a*) use of splice junction-specific probes or (*b*) use of splice junction-spanning primers (24). In the case of splice junction-specific hybridization probes, the primer pairs do not discriminate the different isoforms, resulting in a nonspecific amplification of nontarget transcripts. At very low target levels, this coamplification could impair the correct quantification by depletion of primers or other reagents. Using splice junction spanning primers, which amplify only the target sequence, avoids the problem of coamplification. Here, we used primers spanning splice junctions (TA-p73 sense and antisense, p73Δex2 sense, and p73Δex2/3 sense), primers detecting sequences unique for a single transcript (ΔN-p73 sense), or combinations of primers that specifically amplify a single transcript (ΔN′-p73; Fig. 1, *C* and *D*). The primer combinations and corresponding cycling parameters were extensively optimized on the LightCycler system. A representative amplification plot of a standard dilution series, a duplicate measurement of a single patient sample, and a no-template-control along with the corresponding standard curve is shown in Fig. 2*A*. Using hot start technology, we were able to obtain single amplification products that showed a characteristic melting curve and a single band of the calculated size on agarose gels (Fig. 2, *B* and *C*). Amplification specificity was further confirmed by direct sequencing of PCR products. This specificity allowed us to use SYBR Green I reaction chemistry, thus eliminating the

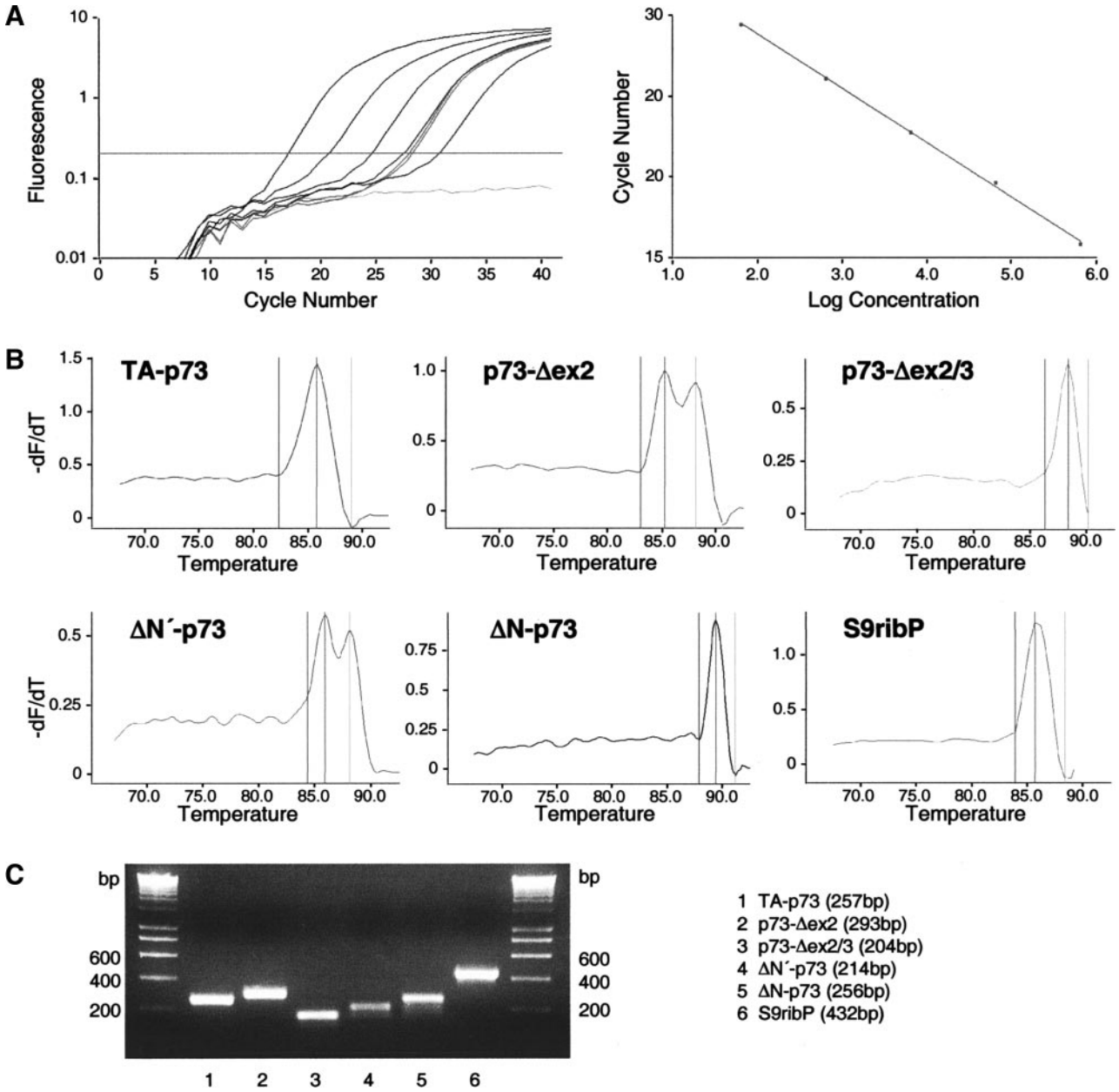


*Fig. 2* Validation of real-time *TP73* reverse transcription (RT)-PCR assays. *A*, representative amplification plot (*left*) for the transactivation-competent p73 protein (TA-p73) isoform showing a standard dilution series (*single curves 1–5*), a duplicate measurement of a patient sample (*duplicate curves between 4 and 5*) and a no-template-control (*lower curve*). *Right*, standard curve for TA-p73. *B*, melting curves for the various real-time RT-PCR amplicons. *C*, The p73-Δex2 and ΔN′-p73 amplicons show a two-step melting behavior but migrate as a single band on agarose gels; agarose gel of the indicated real-time RT-PCR amplicons. The identity of the amplicons was verified by sequencing. -df, -dFluorescence, dT, dTemperature (calculated first derivative); *bp*, base pair(s); *TA*, transmactivation; *S9ribP*, S9 ribosomal protein.

need for relatively expensive probes and increasing the detection sensitivity, because multiple dyes bind to a single molecule. As an example for specificity, our assays could reproducibly discriminate the critical ΔN-p73 and ΔN′-p73 transcripts even in the presence of a 10-fold excess of the competing transcript (Fig. 3*A*).

Few studies using real-time PCR for quantification of splice variants probably reflect the difficulties in creating mutually comparable and therefore reliable standard curves for each variant (24). Knowledge of the copy number ratio between the corresponding dilution points of both standard curves is essential to compare the quantities of one transcript to another. On the basis of the recent report by Vandenbroucke *et al.* (24), we constructed compound plasmids containing both the TA-p73 and one of the ΔTA-p73 amplicons (p73Δex2, p73Δex2/3, ΔN′-p73 and ΔN-p73). Because these plasmids contain TA-p73

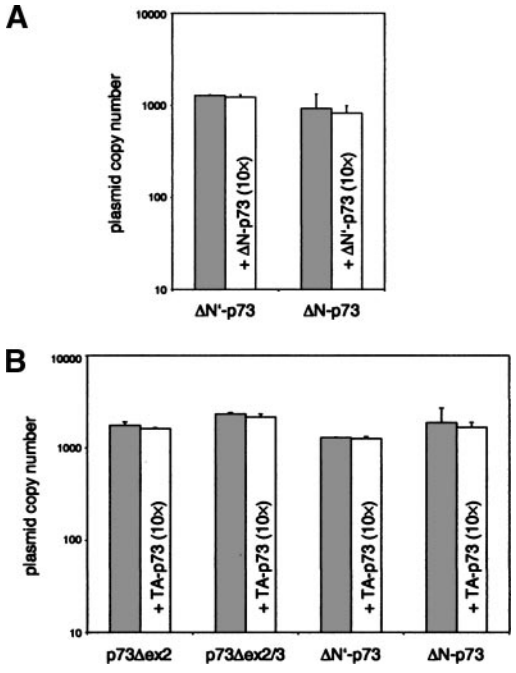


*Fig. 3* Specificity of real-time *TP73* reverse transcription (RT)-PCR assays. *A,* discrimination of related exon3B-containing $NH_2$-terminally truncated p73 protein (ΔTA-p73) isoforms ΔN′-p73 and ΔN-p73. ΔN′-p73 or ΔN-p73 were quantified by real-time PCR using either a plasmid containing the corresponding cDNA as a template or the same amount of this plasmid in the presence of a 10-fold excess of the competing cDNA. *B,* discrimination of full-length and $NH_2$-terminally truncated p73 isoforms. ΔTA-p73 isoforms were quantified using a plasmid containing the corresponding cDNA as a template in the absence (*gray bars*) or presence (*white bars*) of a 10-fold excess of transactivation-competent p73 protein (TA-p73) cDNA. Equal copy numbers were determined irrespective of TA-p73 confirming the specificity of our PCR assays. Errors bars indicate one SD. *TA,* transactivation.

and ΔTA-p73 amplicons in a 1:1 ratio, standard curves for both could be generated from the same series of dilutions, and results were directly comparable over a large dynamic range.

Initial experiments and a recent report by Grob *et al.* (16) indicated that TA-p73 transcripts are 10-to 100-fold more abundant than ΔN-p73. To verify the specificity of our ΔTA-p73 isoform-specific PCR assays, we performed quantification in the absence or presence of an excess of TA-p73 plasmid. As shown in Fig. 3*B,* all ΔTA-p73 transcripts were reproducibly quantified even in the presence of a 10-fold excess of competing full-length TA-p73 plasmid.

For quantification of *TP73* transcripts in primary tumor tissues, normal and neoplastic cells were isolated from 10 HCC patients by laser capture microdissection. The clinicopathological characteristics of the patients are shown in Table 1. Four of the 10 patients were heterozygous for the GC/AT polymorphism in exon 2 of *TP73,* but none of them showed a loss of heterozygosity (Table 1). After extraction of total RNA and random-primed reverse transcription, mRNA levels of individual transcripts (TA-p73, p73Δex2, p73Δex2/3, ΔN′-p73, and ΔN-p73) were quantified by real-time PCR (Fig. 4). In general, the TA-p73 mRNA was the most abundant *TP73* transcript in normal liver tissue with 370 ± 310 copies/250 ng total RNA. In contrast, ΔTA-p73 transcripts were only expressed in a few samples at very low levels (p73Δex2 in 3 of 10 and mean, 0.47 ± 0.93; p73Δex2/3 in 3 of 10 and mean, 5.91 ± 10.81; ΔN′-p73 in 1 of 10 and mean, 0.86 ± 2.72; ΔN-p73 in 5 of 10 and mean, 3.61 ± 5.03). In neoplastic cells, however, the TA promoter-regulated TA-p73, p73Δex2, p73Δex2/3, and ΔN′-p73 but not the ΔN promoter-driven ΔN-p73 transcript were significantly ($P < 0.01$) up-regulated. The mean copy numbers were 7.1-fold higher for TA-p73, 31.5-fold higher for p73Δex2, 7.75-fold higher for p73Δex2/3, 16.3-fold higher for ΔN′-p73, but only 2.3-fold higher for ΔN-p73, indicating that the highest levels of up-regulation are observed for the ΔTA-p73 transcripts regulated by the TA promoter. On the basis of our data, p73Δex2, p73Δex2/3, and ΔN′-p73 are the most abundant ΔTA-p73 species in neoplastic liver cells.

Looking at individual patients, up-regulation of both TA-p73 and ΔTA-p73 transcripts is a common finding in all of the samples analyzed. Although expression of single ΔTA-p73 isoforms is observed in a few normal liver samples, only neoplastic cells show a concomitant up-regulation of all ΔTA-p73 isoforms. However the extent of up-regulation for the individual ΔTA-p73 transcripts shows significant interindividual variation without demonstrating an underlying pattern (Fig. 4*A*).

These results strongly indicate that the elevated expression levels of ΔTA-p73 proteins in HCCs are derived from significantly increased mRNA levels of the TA promoter-regulated ΔTA-p73 transcripts. Together with the up-regulation of the TA-p73 transcripts, these data suggest an overall enhanced activity of the TA promoter in neoplastic liver cells.

## DISCUSSION

Increased expression levels of wild-type p73 are a frequent finding in human tumors. In some cases, overexpression of p73 could even be correlated with an advanced tumor stage or poor prognostic parameters. In hepatocellular carcinomas high-p73 expression levels were revealed as an independent marker of poor patient survival prognosis (8). Considering that p73 overexpression also correlates with poor prognostic parameters in other tumor types and is observed in approximately 20 to 90% of all cancer patients, overexpression likely contributes to the tumorigenic phenotype (1, 8, 10, 25, 26). A detailed analysis of

*Table 1* Clinicopathological data of HCC[a] patients

| Case | Age/Gender | Stage | Grade | GC/AT polymorphism | LOH |
|---|---|---|---|---|---|
| 1 | 61/M | IIIA | 2 | GC | n.i. |
| 2 | 65/M | II | 1 | GC | n.i. |
| 3 | 79/M | II | 1 | GC | n.i. |
| 4 | 63/F | IIIA | 2 | GC | n.i. |
| 5 | 64/M | IVA | 2 | GC/AT | – |
| 6 | 60/M | II | 2 | GC/AT | – |
| 7 | 65/M | II | 1 | GC/AT | – |
| 8 | 70/M | IIIA | 1 | GC/AT | – |
| 9 | 50/F | II | 3 | GC | n.i. |
| 10 | 55/M | I | 2 | GC | n.i. |

[a] HCC, hepatocellular carcinoma; LOH, loss of heterozygosity; M, male; F, female; n.i., not informative; –, no LOH.

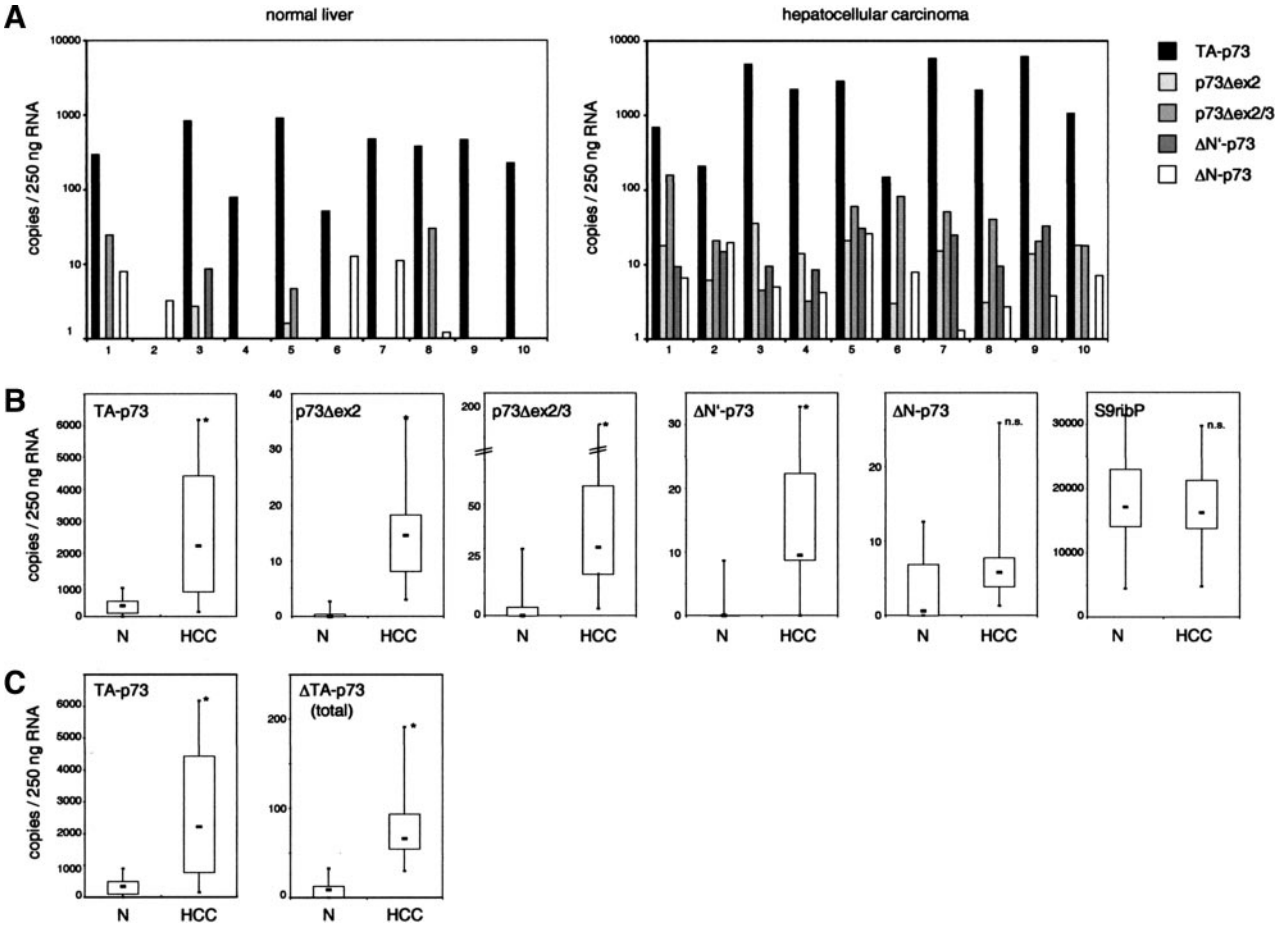


*Fig. 4* Quantification of *TP73* transcripts in HCCs. *A*, quantification results for individual patient samples (*left*, normal liver tissue; *right*, neoplastic liver tissue). Shown is the absolute copy number/250 ng of total RNA. *B*, the box plot shows the absolute copy number/250 ng of total RNA. Similar results were obtained after normalization for the S9 ribosomal protein (S9ribP) as a housekeeping gene (data not shown). The *box* shows the 25 and 75% quartile surrounding the median. The *lines* indicate the minimum and maximum copy numbers. Significant differences ($P < 0.01$; paired, two-sided *t* test) between normal and neoplastic cells are labeled with ∗; n.s., nonsignificant differences. *C*, comparison of transactivation-competent p73 protein (TA-p73) transcript number and the total copy number of all $NH_2$-terminally truncated p73 protein (ΔTA-p73) transcripts including p73Δex2, p73Δex2/3, ΔN′-p73, and ΔN-p73 (∗, $P < 0.01$ paired, two-sided *t* test). *TA*, transactivation.

p73 in tumor cells indicated the presence of ΔTA-p73 in p73-overexpressing tumor cells (11). Considering that ΔTA-p73 proteins were shown to promote malignant transformation and serve as a marker for unfavorable outcome in neuroblastoma patients (10, 11), the mechanisms that give rise to ΔTA-p73 proteins are of special interest. Thus far, several different mechanisms have been reported; some ΔTA-p73 transcripts are generated by aberrant splicing (p73Δex2, p73Δex2/3, ΔN′-p73) whereas others are derived from a second intronic promoter (ΔN-p73). In this preliminary study of 10 HCC patients, we show that ΔTA-p73 proteins are derived from significantly increased mRNA levels of the TA promoter-regulated ΔTA-p73 transcripts.

Considering that classic oncogenes like adenovirus E1A and c-myc as well as oncogenic alterations within the Rb-p16 pathway that lead to an increased activity of E2F transcription factors were shown to activate the E2F-responsive TA promoter (4, 5, 14), it is reasonable to assume a direct causal link between alterations of the RB/p16 pathway and enhanced TA-promoter activity. Importantly, the ΔN promoter-derived ΔN-p73 transcript was not significantly up-regulated in HCCs, which would be consistent with our data showing that the ΔN promoter is not directly regulated by E2F (11). It has been reported that the ΔN promoter can be activated by transactivation-competent p53 family members. However, the primary causes of HCC, exposure to aflatoxin B1 and hepatitis B virus infection, are both associated with inactivation of the p53 tumor suppressor gene by mutations and allelic deletions in about 30% of all cases (27). In addition, the HBx oncogene has been shown to interfere with p53 function (28, 29). Therefore, inactivation of p53 in HCCs might contribute to the lack of significant ΔN-p73 up-regulation in our study. We believe that the low level of ΔN-p73 induction (approximately 2.3 fold) that we observed most likely reflects a secondary effect because of autoregulatory activation of the ΔN-p73 promoter by increased levels of TA-p73 (16, 19). Considering that p53 inactivation is a common finding not only

in HCC but in most cancer types, we would expect to find similar results in other tumors as well. However, more cancer types need to be investigated to draw general conclusions about the regulation of p73 expression in tumors.

On the basis of these findings, some recent studies that correlate high levels of exon3B containing transcripts with ΔN-promoter activity need to be interpreted with appropriate caution (10, 30, 31). The high levels of ΔN-p73 expression that were reported to correlate with reduced survival of neuroblastoma patients might as well be caused by ΔN′-p73 expression, because the primers used in this study do not discriminate ΔN-p73 and ΔN′-p73 (10). In addition, Zaika *et al.* (30) reported tumor-specific up-regulation of ΔN-p73 in tumors of various origins including breast, ovary, and endometrium both on the RNA and protein levels. However, the up-regulation might just reflect changes in ΔN′-p73 expression because neither Western blot nor the combination of primers used in their RT-PCR assays are able to differentiate between both possibilities. The importance of the TA promoter for generation of ΔTA-p73 proteins is underlined by data from O'Nions *et al.* (32), which demonstrate that p73 is actually expressed mainly as the TA promoter-derived p73Δex2 isoform in vulval cancer. For future studies, it is therefore essential to use discriminating RT-PCR assays to avoid premature conclusions about the relative activity of the two promoters and the origin of $NH_2$-terminally truncated p73 species in cancer cells. Because sample number is very limited and includes hepatocellular carcinomas only, our data do not exclude up-regulation of ΔN-promoter activity in tumor tissues in general. However, they clearly show that increases in ΔTA-p73 expression can be caused by elevated TA-promoter activity so that this possibility needs to be addressed in future studies.

An important issue to consider is the ratio of the two antagonistic p73 protein classes. Unfortunately, we were not able to quantify the level of up-regulation for the ΔTA-p73 transcripts exactly and for each individual, because the ΔTA-p73 mRNAs p73Δex2, p73Δex2/3, and ΔN′-p73 are mostly undetectable in the normal liver tissue. On average, up-regulation of ΔTA-p73 (approximately 15- to 30-fold) therefore appears to exceed up-regulation of TA-p73 (approximately 7-fold), which would result in an increase in the ΔTA/TA ratio, *i.e.*, a shift to the side of the antiapoptotic ΔTA-p73. However, why increased TA-promoter activity would favor generation of aberrantly spliced transcripts as opposed to full-length transcripts remains obscure.

Still, despite higher levels of ΔTA-p73 induction, TA-p73 transcripts are approximately 20- to 100-fold more abundant than ΔTA-p73 mRNA levels. On the protein level, however, the increase in the ΔTA/TA ratio might be more prominent, because the ΔTA-p73 proteins were reported to be more stable (12, 16). Even if TA-p73 and ΔTA-p73 transcripts levels were elevated equally, the increased stability of ΔTA-p73 proteins would result in higher intracellular concentrations of ΔTA-p73 and an increased ΔTA/TA protein ratio. However, at present, it is not known how much ΔTA-p73 activity is required to outweigh the tumor suppressor function of TA-p73. Just as subtle changes in the composition of the mitochondrial membrane can decide about life or death, a slight increase in the ΔTA/TA ratio might be sufficient to convert the *TP73* gene from a tumor suppressor gene into an oncogene.

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