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Letter to the Editor

Increased ΔN-p73 expression in tumors by upregulation of the E2F1-regulated, TA-promoter-derived ΔN′-p73 transcript

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Dear Editor,

p53 is certainly one of the most efficient tumor-suppressor proteins, yet its two siblings, p63 and p73, appear to have fundamentally different functions. Multiple reports in a wide variety of tumor types failed to show that inactivating mutations were as common as in p53.[1] Instead, overexpression of wild-type p73 is a common finding in tumor tissues. In addition, mouse knockout studies indicated that loss of p73 or p63 does not predispose to cancer.[2]

One possible explanation for the differences in function despite a significant degree of homology lies in differences in gene architecture. Whereas the *TP53* gene encodes one major protein, both *TP63* and *TP73* give rise to a constantly increasing number of different isoforms.[1,3,4] Starting with the description of an N-terminally truncated, transactivation-deficient p73-isoform (termed ΔN-p73) in the developing mouse by Yang *et al.* the N-terminus of *TP73* has become a focus of scientific investigation.[2] This ΔN-p73 protein is generated from an alternative promoter in intron 3 and plays an essential antiapoptotic role during neuronal death in the developing brain.[2,5] Subsequently, several groups reported on the cloning of the human homolog of ΔN-p73, which can also be generated from an alternative promoter in intron 3.[6–12] However, because of the excitement about a second intronic promoter other possible sources of N-terminally truncated p73 species have been largely ignored. Already in the cloning paper, Kaghad *et al.* described an aberrantly spliced variant of p73 that lacks exon 2 (p73Δex2).[13] Subsequently, two other N-terminal splice variants (p73Δex2/3 and ΔN′-p73) have been identified.[7,9,10] Importantly, the ΔN′-p73 transcript is generated from the first promoter (TA-promoter) but aberrantly includes 198 bp from exon 3' (Figure 1a). This inclusion generates a premature 'stop' in the regular reading frame. Translation of the ΔN′-p73 therefore starts in exon 3' resulting in the production of a protein indistinguishable from the ΔN-p73 protein generated from the alternative promoter transcript. Therefore the same N-terminally truncated protein species (ΔN-p73) is encoded by two differently regulated transcripts, which can only be differentiated on the basis of mRNA sequence but not on the protein level (Figure 1a).

Especially, the antiapoptotic function of ΔN-p73 has raised the possibility that ΔN-p73 might act as an oncogene in human cancers and prompted a number of studies addressing this point. In this respect, we have recently shown that N-terminally truncated p73 isoforms are expressed in both tumor cell lines and primary tumor tissues and act as oncogenes by transforming NIH3T3 cells and turning them

tumorigenic in nude mice.[10] Zaika *et al.*[12] reported tumor-specific up-regulation of ΔN-p73 in various tumors including breast and ovarian cancer. In addition, Casciano *et al.*[14] have reported that overexpression of ΔN-p73 in neuroblastoma patients is significantly associated with reduced survival and serves as an independent prognostic marker for poor outcome. However, the RT-PCR primers used in the latter studies amplify sequences common to both the ΔN-p73 and ΔN′-p73 transcripts and therefore measure the total amount of exon 3' containing transcripts but do not allow to differentiate the origin of the detected transcripts. So the question remains, whether the oncogenic and prognostically relevant p73 species are the product of the alternative ΔN-promoter or whether they are rather the result of aberrant splice processes involving transcripts regulated by the TA-promoter.

To address this question we have designed primers for real-time quantification of ΔN-p73 and ΔN′-p73 transcripts (Figure 1a). The upstream primer for ΔN-p73 was placed within the first 78 bases of exon 3', which are unique to ΔN-p73 and are not included in ΔN′-p73. For quantification of ΔN′-p73 we designed a primer pair which specifically amplifies the characteristic exon3–exon3' splice junction, which is only found in ΔN′-p73. In combination with hot start technology and cycling programs optimized for the LightCycler we obtained specific products that appeared on agarose gels as single bands of the correct size and sequence (Figure 1b). This allowed us to use SYBR Green I reaction chemistry, thus eliminating the need for relatively expensive hybridization probes. Quantification of ΔN-p73 and ΔN′-p73 transcripts in Saos-2 cells expressing E2F1 and p53, respectively, demonstrated six-fold induction of ΔN-p73 by p53, but only low-level induction by E2F1 (Figure 1c). In contrast, the ΔN′-p73 transcript was significantly upregulated by E2F1 but not by p53. These data demonstrate differential regulation of the two ΔN-p73 encoding transcripts consistent with the regulation by two independent promoters. Quantification of microdissected samples from 10 hepatocellular carcinoma patients clearly shows that the difference between tumor and normal cells is most prominent for the ΔN′-p73 transcript (Figure 1d). 7/10 tumors have a more than five-fold increased expression of ΔN′-p73, whereas changes in ΔN-p73 expression are only modest. Just one tumor showed a more than five-fold increased expression of ΔN-p73. In these tumor samples, upregulation of ΔN′-p73 is therefore the basic mechanism underlying increased expression of exon 3' containing transcripts.

Our data suggest that in human hepatocellular tumors, aberrantly spliced TA-promoter derived transcripts are the predominant source of potentially oncogenic p73 proteins. This hypothesis is in line with the well-established positive regulation of the TA-promoter by proliferative signals (E2F1, c-myc, E1A).[15–17] Although it casts some doubt on the relevance of the ΔN-promoter, it has to be considered that the sample number in our study is limited and comprises only one tumor entity. However, the data clearly show that it is a premature conclusion to attribute elevated tumor levels of exon 3' containing transcripts to an increase in ΔN-promoter activity. At least in our hepatocellular carcinoma samples, this increase is due to upregulation of ΔN′-p73 transcripts and not due to increased ΔN-p73 promoter activity. A careful primer design is therefore essential to avoid misleading interpretations especially with genes like *TP73*, which encode a multitude of structurally and functionally diverse isoforms.

Considering the high frequency of p73 overexpression in most cancer types, the possible role of oncogenic p73 isoforms for various aspects of tumorigenesis, and the resulting prognostic and therapeutic implications, it has become more than an academic question how the different p73 isoforms are regulated. We suggest that future studies on p73 expression in cancer address this point and carefully differentiate N-terminally truncated p73 isoforms according to their origin.

Figure 1 (**a**) Complexity of *TP73* splice variants. Shown is the genomic structure of the *TP73* gene and the composition of the full-length TA-p73 transcripts and the two ΔN-p73 encoding transcripts ΔN-p73 and ΔN′-p73. The two different promoters, TA- and ΔN-promoter, are indicated by black triangles, exons are shown as boxes. Coding regions are highlighted in color. *Red*, transactivation domain; *blue*, DNA-binding domain; *orange*, 13 amino-terminal amino acids of the ΔN-p73 protein. Relevant start (ATG) and stop codons are labelled. Primers for isoform-specific transcript quantification are depicted as arrows. (**b**) Specificity of PCR primers for real-time quantification of ΔN-p73 and ΔN′-p73 transcripts. Specific products appear on agarose gels as single bands of correct size (bp). Smart Ladder SF (Eurogentec) is indicated. TA-p73 and S9 ribosomal protein (S9ribP) amplicons are shown as controls. (**c**) Differential regulation of ΔN-p73 and ΔN′-p73 transcripts. For analysis of regulation by p53, Saos-2 cells were infected with 50 moi (multiplicity of infection) of a p53-encoding recombinant adenovirus for 12 h (left panel). For analysis of regulation by E2F1, Saos-2 cells that stably express the 4-hydroxytamoxifen inducible ER-E2F1 fusion protein (previously described) were induced with 4-OHT (*right panel*).[13] Total RNA 1 μg was reverse transcribed with Omniscript RT (Qiagen) using random examers (Applied Biosystems). Transcript levels of ΔN-p73 and ΔN′-p73 were measured by real-time RT-PCR using isoform-specific primers as depicted in (**a**). PCR reactions contained 1 × Lithos qPCR™ SYBR Green I Master Mix (Eurogentec), 150 nM of each primer, 3.5 mM $MgCl_2$ and 0.5 mg/ml bovine serum albumin (Roche Diagnostics) and were carried out in triplicates using a LightCycler (Roche Diagnostics). Amplification products were verified by melting curves, agarose gel electrophoresis and direct sequencing. Primer sequences: ΔN-p73 sense: 5'-CAAACGGCCCGCATGTTCCC-3', antisense: 5'-TGGTCCATGGTGCTGCTCAGC-3'; ΔN′-p73 sense: 5'-TCGACCTTCCCCAGT-CAAGC-3', antisense: 5'-TGGGACGAGGCATGGATCTG-3'. (**d**) Quantification of ΔN-p73 and ΔN′-p73 transcripts in microdissected samples from 10 hepatocellular carcinoma patients. For absolute quantification of transcript numbers standard curves were obtained with plasmids containing the various amplicons. Exon 3' containing transcripts represent the sum of ΔN-p73 and ΔN′-p73 transcripts. The difference in transcript numbers between neoplastic and normal liver cells was calculated for each patient. The fold difference tumor *vs.* normal represents the ratio of this difference and the average copy number in normal liver tissues

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