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Letter to the Editor

Different p73 splicing variants are expressed in distinct tumour areas of a multifocal neuroblastoma

Dear Editor,

p73 is a recently identified homologue of the p53 protein (TP53 gene) that, *in vitro*, can induce apoptosis and inhibit cell growth.[1,2] The gene coding for p73, TP73, was subregionally mapped in the 1p36.3 region,[1,3] and this localization has raised the possibility that TP73 might be relevant to the development of neuroblastoma or of other tumours where subtelomeric 1p aberrations have been described.[1,4] Mutation analysis and studies on the interaction between viral oncoproteins and p73[5–8] suggest that, if TP73 may act as an oncosuppressor gene, it does so with a mechanism different from that of TP53. Different splicing variants of p73 have been described[1,9] and since they have variable homo- and heterotypic interactions between themselves and with p53 it is likely that the several products of this gene participate, in different ways, in a complex network that regulates cell growth, death and differentiation.[9,10]

We studied the involvement of p73 in tumorigenesis by comparing the pattern of expression of this gene with the proportion of apoptotic cells in the tumour. Since the expression of TP73 shows marked interindividual and intertissue variations, our analysis was conducted in independent areas of a synchronous multifocal neuroblastoma (unpublished data). This has allowed the analysis of distinct tumours within an identical genetic background. Three tumour areas, with distinct clinico-biological characteristics, were identified and labelled as L, R1 and R2 (where L and R indicated the left or right location in the tumour). L was a stroma poor differentiated neuroblastoma, R1 was a stroma poor differentiating neuroblastoma while R2 was classified as a undifferentiated neuroblastoma.

TP73 expression was analyzed by nested RT–PCR where the amplification of the entire protein coding sequence was followed by the amplification of individual exons. As shown in Figure 1A, TP73 is expressed essentially at the same level in R1 and R2 after normalization with the housekeeping gene G3PDH (see E). No p73 was detected in the L tumour. The major variants of p73 (-α, -β, -γ and -δ) differ for the splicing of exons 11-13 at the COOH terminus of the protein outside the region of homology with p53.[9] As shown in Figure 1B, only p73α and -β are expressed in R1 while in R2 all four variants can be detected at different levels.

Another major difference in the pattern of p73 expression between R1 and R2 was identified by hybridization of a p73α cDNA probe on the PCR products derived from amplification of exons 1-4. In fact, as shown in Figure 1C, the only product detected in R1 was a fragment of 172 bp instead of the expected one of 277 bp.[1] On the contrary, in R2, both fragments were present although that corresponding to the larger one was more prominent. Direct sequencing of the PCR products demonstrated that the 172 bp fragment corresponds to the Δ2exon splicing variant previously detected in the SK-N-SH cell line.[1] This variant lacks the aminoacidic residues 1–48 that correspond to the region of homology with the p53 transactivation domain. The conclusions derived from these experiments are that R1 express only Δ2exon-p73α and -β while, in R2, multiple p73 variants (p73 and Δ2exon-p73 -α, -β, -γ and -δ) might coexist. No differences in the expression level of p53 were observed in R1, R2 and L (Figure 1D).

We utilized a TUNEL assay[11] to determine if different levels of cell death were present in the three tumour areas expressing a distinct pattern of p73 variants or not expressing this gene. As shown in Figure 1F and H, tissue sections derived from R1 and L displayed strong nuclear staining and highly condensed nuclei. Moreover, H clearly shows that the staining was typically confined to clusters of tumour cells. Cells presenting these morphological features are those having their DNA cleaved at the internucleosomal linker regions and dying of programmed cell death.[12] On the contrary, cells derived from R2 do not show apoptotic staining (G).

In vitro experiments have shown that p73 overexpression can inhibit cell proliferation and promotes apoptosis.[1,2] Our analysis revealed that *in vivo*, at least in this tumour model, the expression of this gene does not directly correlate with the presence of apoptosis. A possible explanation for this discrepancy is that the p73 functions in the apoptotic pathway can be taken up by other genes (e.g. p53). The presence of different splicing variants in the biologically distinct R1 and R2 tumour areas might also indicate that the interaction of the several p73 isoforms between themselves and with p53 might have profound effects in the control of programmed cell death.[9] Our model seems to indicate that Δ2exon-p73α and -β, when freed by the feedback negative control of the other variants, could play an important synergistic role with p53 in inducing a strong and diffuse apoptotic pathway (see Figure 1F). On the other hand they might be strong repressors of apoptosis, independently of the expression of p53, in the presence of the other variants. In this respect an important role might be played by p73γ which can strongly interact with p73α, -β and -δ but is essentially ineffective in inhibiting cell growth in SAOS cells.[9] Appropriate *in vitro* assay are being designed to clarify this point and to assign a specific function to the Δ2exon-p73 variants.

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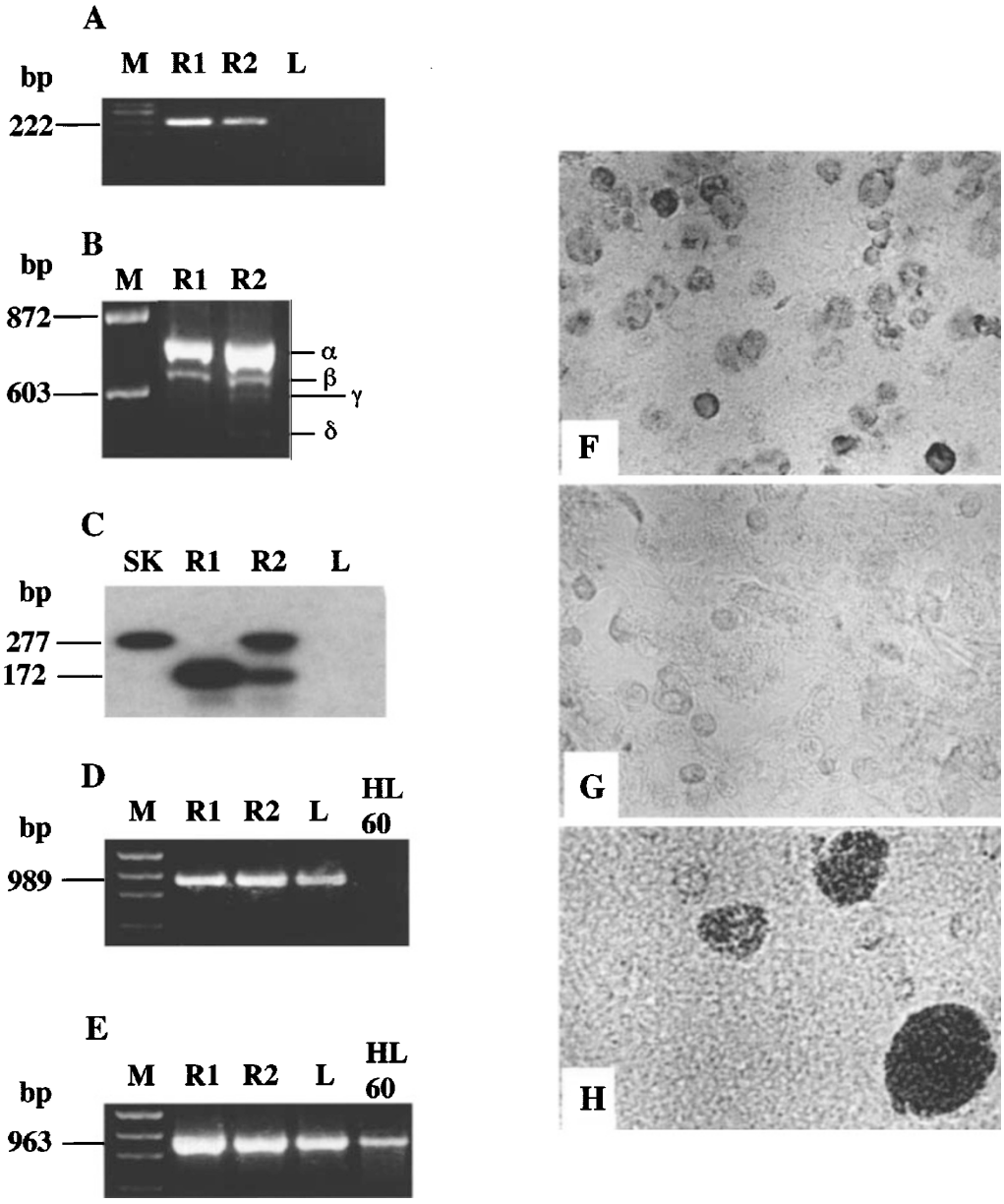

Figure 1 Analysis of TP73 expression and of apoptosis in a multifocal neuroblastoma. (**A**) Nested RT–PCR amplification with amplimers SN716-ASN938 from R1, R2 and L. tumour areas. cDNA synthesis and amplification of the TP73 entire coding sequence were performed as described by Kaghad *et al*. Two μl of the first product were reamplified with the internal set of primers (716: 5′-CTTCAACGAAGGACAGTCTGC-3′; 938: 5′-GGTGATGATGATGAGGATGG-3′) for 30 cycles (94°/58°/72° for 30'' each). (**B**) Identification of p73 splicing variants in R1 and R2 samples. Two μl of the first PCR product were reamplified with primers SN1153/ASN1899 (1153: 5′-GGCATGGAGACGAGGACACG-3′; 1899: 5′-GGATGGTGATGGTGTGGCG-3′) for 30 cycles (94°/64°/72° for 30'' each). PCR products were resolved on a 3% Metaphor gel (FMC). p73 variants were identified on the basis of their expected size[1,9] and by hybridization (not shown). (**C**) Identification of the Δ2exon p73 splicing variant in R1 and R2. The PCR products obtained by RT–PCR with amplimers SN41-ASN330 (SN41: 5′- GGACGGACGCCGATGCC-3′; ASN 330: 5′-GGTCCATGGTGCTGCTCAGC-3′) were blotted onto a nylon membrane and hybridized with a TP73 cDNA probe obtained by cloning the first PCR product (see A) into a plasmid vector. PCR conditions were identical to those of B. Sequencing of the 172 bp product revealed its identity with the Δ2exon p73 splicing variant. Note that the p73 transcript is not detectable in the L tumour not only in ethidium bromide stained gels (**A**) but also by hybridization. (**D** and **E**) TP53 and G3PDH expression in R1, R2 and L. HL60 cDNA was utilized as internal control for negative TP53 expression. (**F**, **G** and **H**) TUNEL assay on primary tumours. **F**, **G** and **H** are reported, respectively, the apoptotic stainings obtained on areas R1, R2 and on the L tumour. Note the absence of apoptotic staining in R2

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