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Letter to the Editor

Role of methylation in the control of Δ*Np73* expression in neuroblastoma

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Dear Editor,

The *p53* gene plays a crucial role in the control of cell growth and is frequently inactivated by mutations in cancer cells. Differently from most tumor types, *p53* mutations in neuroblastoma (NB) are rare suggesting that p53 status is irrelevant in the pathogenesis of this tumor. The biological functions of p53 in NB, however, are inhibited by the accumulation of the protein in the cytoplasm. Moreover, after DNA damage, p53 translocates to the nucleus but its capacity to induce G1 cell-cycle arrest is attenuated.[1–3]

A considerable interest in NB research was recently raised by the discovery of *p73*, a *p53* homologue located at 1p36.3, which transactivates several *p53* target genes, induces apoptosis and inhibits cell proliferation. The contribution of p73 to tumor suppression however, is still unclear.[4] p73 is a direct downstream effector of E2F-1 and is an essential component of the p53-independent apoptotic pathway. In developing sympathetic neurons of mice, p73 is predominantly expressed as a truncated anti-apoptotic isoform (ΔNp73), lacking the transactivation domain of the full length protein (TAp73). ΔNp73 antagonizes p53 and suppresses the transactivating activity of TAp73 by oligomerization and competition for DNA binding.[5,6]

We have demonstrated that the expression of the anti-apoptotic Δ*Np73* variant in primary neuroblastoma is a strong, independent, predictor of unfavorable outcome.[7] To gain further insights into the role of *p73* in this tumor we investigated the control of Δ*Np73* transcription in human neuroblastoma. Our results indicate that the expression of this p73 isoform is, at least in part, controlled by epigenetic changes in NB cell lines and tumor tissues.

Mouse Δ*Np73* is transcribed from an internal promoter in the third intron of the *p73* gene, upstream of an alternative exon 3 (exon 3′). We have cloned the human Δ*Np73* cDNA and promoter by similarity with the mouse transcript. In Figure 1A, we report the genomic organization of the *p73* locus. Nucleotide alignment between human and mouse Δ*Np73* exon 3′ indicated that the two sequences share 78% homology.

Promoter prediction by neural network analysis detected a TATA box and a CAP signal immediately upstream from the putative human *p73* exon 3′. The preliminary characterization of this region demonstrated that a 1400 bp fragment, that includes the entire exon 3′ cloned into a CAT reporter vector, indeed has promoter activities in the neuroblastoma cells lines SK-N-BE2(c) and LAN5 but not in U937, a myeloid cell line that constitutively does not express TA or ΔNp73 (Figure 1A).

RT–PCR analysis of a panel of cell lines showed that Δ*Np73* is transcribed in 8 out of 11 NB cell lines tested but not in the myeloid HL60 or U937 cells. We were also unable to observe Δ*Np73* mRNA expression in PBL from six healthy donors (data not shown). Sequencing of the RT–PCR products confirmed that the transcript was authentic Δ*Np73* with a 1760 bp open reading frame coding for a protein with 92% homology to the mouse protein. Western blot analysis conducted with a pan-p73 monoclonal antibody on NB cell lines and on U937 cells (Figure 1B), detected a major band at 62 kDa only in the NB cells that express Δ*Np73*. This result indicates that ΔNp73α is the prevalent isoform present in these cell lines (Figure 1B and data not shown).

The 5′ end of ΔNp73, differently from that of the TA variant, does not contain a cluster of the consensus E2F binding sequence TT(C/G)(C/G)CG(C/G). The two regions, however share a similar structure. In both cases the first transcribed exon (exon 1 for TA and exon 3′ for Δ*Np73*) is part of a CpG island and a second CpG-rich region is located approximately 6 kb upstream from the putative transcription startpoint. Although the CpG-rich region at the Δ*Np73* 5′ end is considerably smaller than that of *TAp73*, it qualifies as a true island (length 336 bp; C+G content 64.2%; CpG/GpC=0.91, chi-square obs/exp=0.27) (Figure 1C).

In the mammalian genome, methylation of the cytosine residues within CpG dinucleotides plays an important role in the regulation of transcription and the importance of epigenetic modifications in the control of tumor cell growth is now well recognized. In leukemia and lymphomas, *p73* silencing is associated with the aberrant methylation of the *TAp73* promoter.[8,9] In neuroblastoma, however, *TAp73* transcription is subject to a different mode of regulation. In fact we did not observe a different level of methylation in primary NB expressing or not expressing *TAp73*.[10] In an attempt to understand the mechanisms regulating Δ*Np73* expression we have analyzed the methylation status of the Δ*Np73* 5′ region in neuroblastoma and control cell lines by Methylation Specific PCR (MSP). This technique relies upon the selective PCR amplification of methylated or unmethylated sequences after chemical conversion of the unmethylated C into T. The presence of an amplification band only with the primer set designed to bind to the methylated sequence indicated that the Δ*Np73* 5′ end is fully methylated in the Δ*Np73*-negative cell lines, U937, HL60 and ACN. Conversely neuroblastoma cell lines GI-ME-N, HTLA230 and SK-N-BE2(c), that express Δ*Np73*, displayed only partial methylation as witnessed by the presence of amplification bands with both sets of primers (Figure 1D). To determine if this epigenetic modification was present also in fresh normal and tumor cells we have analyzed the methylation status of Δ*Np73* in T and B acute

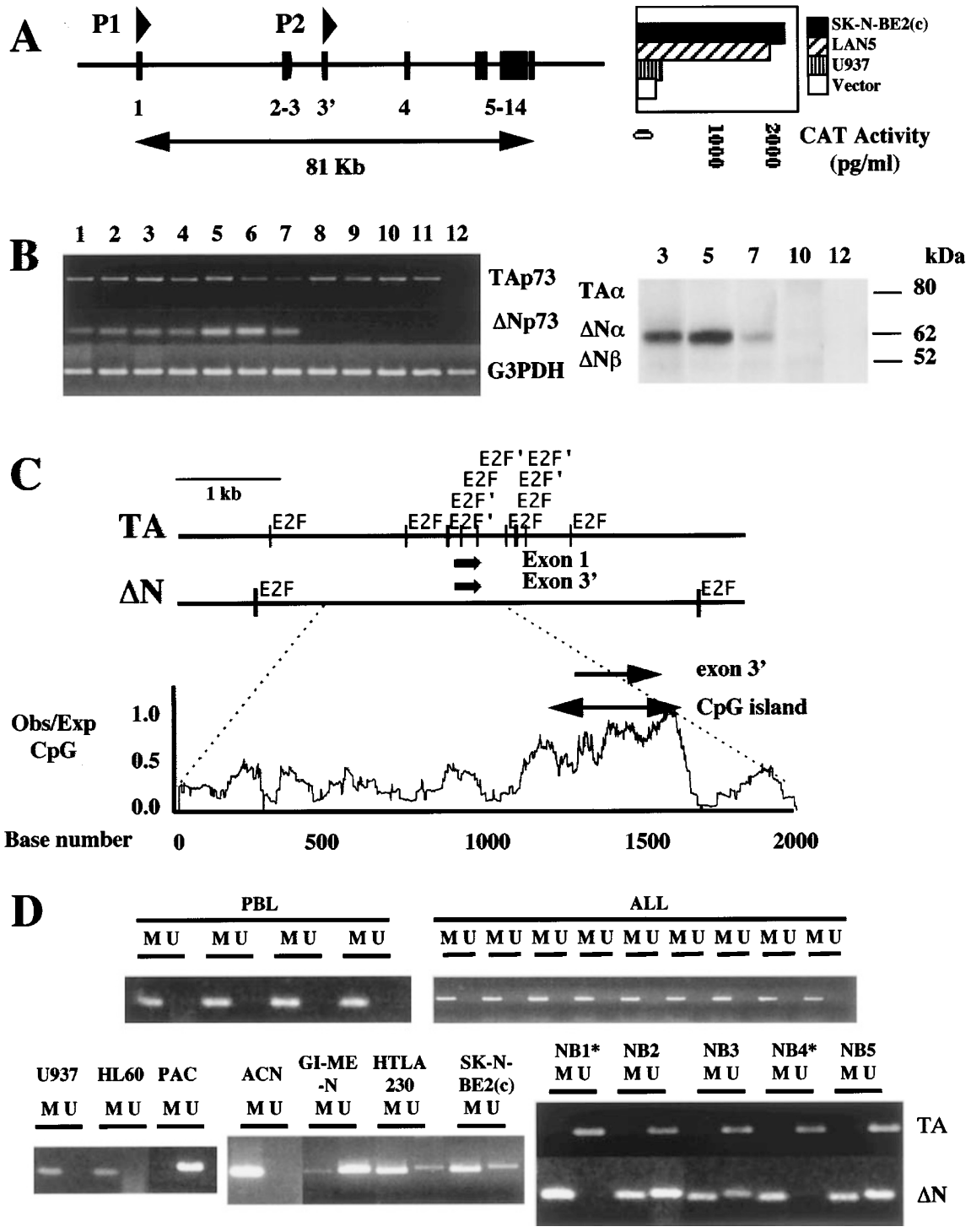

Figure 1 (**A**) Structure and organization of the *p73* locus. A human DNA sequence with 78% homology to nucleotides 1–274 of the mouse ΔNp73 was identified by blast query against the human genome sequence. The human exon 3' and flanking sequences were subcloned from mini-libraries constructed from PAC clones 863N7 and 967O8 as described[10] utilizing a probe derived from the mouse cDNA sequence. The left part of the panel reports the schematic representation of the structure of the *p73* locus; P1 indicates the *TA* promoter and P2 is the putative Δ*Np73* promoter. A 1422 bp fragment containing the entire exon 3' (corresponding to nucleotides 471–1893 of sequence AF443116) in a CAT reporter vector was transfected into SK-N-BE2(c), LAN5 and U937 cell lines. In the right part of the panel is reported the CAT expression induced by transfection of this construct. Background expression (designed as Vector) represents the average value obtained by transfection of the promoterless vector into the three cell lines. (**B**) RT–PCR and Western blot analysis for *p73* expression. *p73* expression was semiquantitatively determined with primers designed to discriminate between *TA* and Δ*Np73* as described.[7] Authenticity of the RT–PCR products was determined by sequencing. For Western blot analysis, cell lines were lysed in RIPA buffer in the presence of protease inhibitors and 300 μg of cleared lysate were applied to a 7–14% acrylamide gel. p73 was revealed with the monoclonal antibody ER15 (Neomarkers Inc. Union City, CA, USA) diluted at 1:200, utilizing the reaction conditions indicated by Pozniak *et al.*.[6] The cell lines are: (1) SK-N-BE2(c); (2) GI-LI-N; (3) GI-ME-N; (4) LAN1; (5) LAN5; (6) HTLA230; (7) IMR32, (8) GI-CA-N; (9) SK-N-SH; (10) ACN; (11) HL60; (12) U937. The SK-N-SH derivative SH-SY5Y, that express ΔNp73, was not included in this picture. (**C**) CpG island at p73 exon 3. The upper part of the

lymphocytic leukemia cells and in neuroblastoma tumor tissues and PBL from normal donors. As shown in Figure 1D, normal and leukemic lymphocytes display complete methylation of the ΔN 5′ region, a result consistent with the lack of Δ*Np73* expression observed in these cells ([7] and this report). On the contrary, in most primary NB expressing Δ*Np73* this sequence is only partially methylated whereas it is fully methylated in two samples not expressing this isoform. These findings support the hypothesis that Δ*Np73* expression is epigenetically regulated in NB.

Neuroblastoma is a childhood tumor that presents a high frequency of spontaneous regression likely due to the activation of apoptotic pathways. The *p73* gene is a *p53* homologue that can exert opposite functions: the full-length protein TAp73, can in fact induce apoptosis and neuronal differentiation[4], while the truncated ΔN isoform is a dominant-negative inhibitor of programmed cell death. It thus appears that the *p73* gene can functionally act as a tumor suppressor or as an anti-tumor suppressor.

Over the last few years it has become increasingly clear that epigenetic changes are essential for the normal development and that alterations of the pattern of DNA methylation are common in cancer cells. Hypermethylation of CpG residues within gene promoter regions can inhibit transcription and can participate in the inactivation of tumor-suppressor genes by acting, alternatively or synergistically to mutations or chromosomal deletions, in the multistep process of carcinogenesis. Another frequent epigenetic alteration of cancer cells is the decreased overall level of 5-methylcytosine. The implications of this modification in tumor progression are not yet completely understood although hypomethylation likely modifies the structural integrity of the chromosomes.[11] The demethylation of specific loci can also reactivate gene expression and significantly alter the functionality of the cell as it was shown for the Bcl-2 gene in chronic lymphocytic leukemia.[12] DNA methylation is a dynamic, reversible signal whose pattern depends largely on sequential methylation and demethylation events. Our results indicate that the expression of Δ*Np73*, another regulator of apoptosis, is associated with epigenetic changes. We propose that this mechanism finely modulates apoptotic signals in neuroblastoma tumors.

Note added in proof

After acceptance of this paper Grob *et al.*[13] reported the functional characterization of the ΔNp73 promoter.

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panel reports the putative E2F binding sites in a 6 kb genomic fragment surrounding p73 exons 1 and 3. E2F and E2F′ represent the sites of the top or on the bottom DNA strand. The position of exon 1 and 3′ is indicated. The lower part of the panel reports the observed/expected CpG ratio in a 2 kb fragment, delineated by dotted lines, that corresponds to the 5′ end of Δ*Np73*. The double headed arrow indicates the CpG island and the single headed arrow indicates exon 3′ and the direction of transcription. (**D**) Methylation analysis in cell lines and tumor samples. The methylation status of Δ*Np73* was determined by MSP as described.[10] Primers sets produced a 157 or a 168 bp fragment respectively from the methylated and the unmethylated DNA after bisulfite treatment. Forward and Reverse primers were: (Met-FW) GTTGTCGGGCGGTTACGATC; (Met-Rev) TCACACCGTAACGAAATACCG; (UnMet -FW) GGTTTATGTTGTTGGGTGGTTATGATTG; (UnMet-Rev) CACATCACACCTACCATAACAAAATACCATAC. Annealing temperature was 63°C for both sets, extension was 72°C for the methylated primers and 68°C for the unmethylated reaction. Amplification of DNA from HL60, U937, and from PAC clone 863n7 was utilized in each experiment as internal control for methylation. The presence of a band of amplification with both primers denotes partial methylation at that site. The completely methylated NB samples 1 and 4 (indicated by an asterisk) do not express Δ*Np73* as the ACN cell line (sample 10, B). Note that the TA promoter is completely unmethylated