ΔN -p73 α Accumulates in Human Neuroblastic Tumors

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Neuroblastic tumors (NTs), occurring in early childhood, display a wide spectrum of differentiation. Recurrent deletions involving the *p73* locus are frequently observed in undifferentiated NTs. To address the question of the possible implication of *p73* in neuroblastic differentiation, we investigated the status of the expression of this gene in a panel of differentiated and undifferentiated tumors. Although mutations were not found, *p73* transcript profiles differed between undifferentiated and differentiated tumors. The frequency of the transcripts lacking exon 2 (species 1–3) appeared to be higher in undifferentiated than in differentiating and differentiated NTs. In contrast, products from using an alternate promoter (ΔN -*p73*) were present in all NTs. In addition, only ΔN -*p73*, but not full-length proteins, were detected by immunoblotting, suggesting a greater stability of N-truncated isoforms. Importantly, as in the adrenal medulla, most NTs showed *p73*-positive immunohistological staining with a cellular distribution and intensity varying according to the neuronal differentiation. Surprisingly, we observed redistribution of *p73* from the nucleus to the cytoplasm during neuroblastic differentiation. Our data suggest that, in undifferentiated NTs, a link may exist between the accumulation of ΔN -*p73* α variants and the “nuclear exclusion” of *p53*. (Am J Pathol 2002, 160:631–639)

Neuroblastoma (NB), the most common extracranial solid tumor affecting children in the first years of life, is an embryonal cancer of the postganglionic sympathetic nervous system, which appears most commonly in the adrenal medulla. Neuroblastic tumors (NTs) cover a broad spectrum of lesions dependent on sympathetic ganglion

differentiation and Schwannian stroma contribution leading to the following histological classification: undifferentiated neuroblastoma (Schwannian stroma-poor), ganglioneuroblastoma (Schwannian stroma-rich), and ganglioneuroma (Schwannian stroma-dominant).¹ Malignant neuroblasts may undergo further differentiation during their natural history in the same tumor (mainly in IV-S stage in infants or in localized NB) or during the course of chemotherapy. The remarkable clinical and biological heterogeneity of these tumors resulting from neuroectodermal dysembryogenesis makes them a useful paradigm for the study of neoplastic differentiation.

Although, in contrast to many cancers,² *p53* is not mutated in human neuroblastoma,³ *p53* is sequestered in the cytoplasm of only undifferentiated tumors, strongly suggesting that functional inactivation of the *p53* protein is associated with aborted differentiation of malignant neuroblasts.⁴

Several *p53* homologous genes, including *p73*⁵ and *p63* (also called KET, *p51*, and *p40*),^{6–9} have very recently been identified. Structurally similar to *p53*, they conserve the three domains—transcriptional activation domain (TAD), DNA-binding domain (DBD), and oligomerization domain (OD)—that typify this broad spectrum sequence-specific transcription factor which plays a major role in regulating cell growth and apoptosis. DBD, the main domain usually examined for mutations of the *p53* gene in human cancers, was found to be unaltered in the *p73* gene.^{5,10,11} Unlike *p53*, which encodes a unique protein, the *p63* gene expresses an array of different N- and C-terminal isotypes (with or without TAD) in normal tissues resulting from alternate splicing. *In vitro*, these isotypes show remarkable divergent capabilities to transactivate *p53* reporter genes and to elicit growth-suppression and apoptosis-inducing activities.⁹ So far, C-terminal variants have been clearly shown to alter *p73* transcriptional activity *in vivo*.¹² In contrast, the role of N-terminal isotypes on this activity remains to be elucidated. Concerning their possible role in oncogenesis, *p53* homologues radically differ from their tumor suppressor gene

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(TSG) archetype which is mutated in most cancers,² and with germline mutations being associated with an increased risk of cancer in both man¹³ and rodents.¹⁴ Indeed, *p73* is totally devoid of mutations in many cancers,^{15–17} and homozygous *p63*–/–^{18,19} as well as *p73*–/– mouse mutants have no cancer phenotype.²⁰ It is however noteworthy that *p73*–/– mice show marked neurosensorial defects.

Among the *p53* homologues, *p73* appeared to be the most relevant candidate gene for neuroectodermal dys-embryogenesis and/or NB carcinogenesis because its locus (1p36–33) frequently exhibits loss of heterozygosity (LOH) in undifferentiated NB. No *p73* mutations have been reported, either in NB cell lines⁵ or in NB tumors.^{10,11} However, monoallelic expression has been observed in the SK-N-SH NB cell line,⁵ suggesting that the activity of the *p73* gene may be regulated by epigenetic mechanisms such as imprinting. Another possibility may be the stoichiometry of the various putative *p73* transcript variants (with and without TAD) and the functional activity of the corresponding encoded proteins.

We therefore investigated a possible association between *p73* and the differentiation process by assessing the expression of this gene in various types of NTs. We found 1) an array of transcripts including full-length and N-truncated species, 2) despite multiple transcripts, a single α -N terminally truncated protein in undifferentiated NB, and 3) immunostaining present in the cytoplasm and/or nucleus according to histological differentiation. Altogether, our data establish a relationship between *p73* gene expression and NT differentiation, and point out a role for the *p73* gene in sympathetic neuronal differentiation.

Materials and Methods

Tissue Specimens from Children

Tumor tissues were collected from primary tumors of children with clinically documented NBs,²¹ most of whom had been treated with induction chemotherapy. Tumors were snap frozen and stored in liquid N₂ until needed. Patient lymphocytes obtained at surgery were separated from peripheral blood by the Ficoll Hypaque gradient method. Before nucleic acid extraction, frozen NB tissue samples were systematically embedded in OCT (Miles Laboratories, USA) and cut into 70- μ m-thick slices with a cryostat to be used as a quantitative and qualitative histological control; 5 μ m of hematoxylin-eosin-stained tissue flanking both sides of the thick tumor slices used for nucleic acid extraction were examined histologically to determine cellularity, percentages of tumor cells, immature neuroblasts, maturing neuroblasts, and differentiated cells as well as the percentage of fibrosis and necrosis. This procedure allowed us to sort out neuroblastic tumors according to a scale of ganglionic sympathetic differentiation¹ as immature neuroblastoma (NB), maturing neuroblastoma (NB/GNB), mature ganglioblastoma (GNB), and fully mature ganglioneuroma (GN), and to select only tumor tissues presenting more

than 50% of tumor cells for molecular biology studies. gDNA heterozygosity analysis was performed from 61 patients (35, localized stage; 6, IV-S stage; and 20, IV stage) for whom matched lymphocytes/tumors were available. Due to the rarity of tumor material used for gDNA and cDNA studies, material including homozygous *p73* patients of this series was also used for *p73* protein studies (immunoblotting and immunohistochemistry).

Neuronal Differentiation of Neuroblastoma SH-SY5Y Cells

The parental human neuroblastoma SH-SY5Y cell line was provided by the European Collection of Cell Cultures (Salisbury, UK). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with L-glutamine and 10% fetal calf serum (Eurobio, Paris). Neuronal differentiation was obtained by either exogenous or endogenous growth factors. FGF1-transfected clones were obtained as previously described.²² Exogenous induction of SH-SY5Y cells differentiation started 24 hours after plating by exposing the cells to 100 ng/ml NGF (nerve growth factor, Peprotech Inc, Rocky Hill, NJ, USA) and 0.3 μ mol/L aphidicolin (Sigma, St. Louis, MO, USA) for 8 days.²³ Culture medium was changed every 2 days for the duration of the culture. Both models displayed a marked neuronal differentiation phenotype.

DNA and RNA Preparations

DNAs and total RNAs were prepared from 70- μ m thick slices of tumor tissue (about 100 mg) by the guanidinium isothiocyanate-CsCl gradient method. DNA was collected from the supernatant, dialyzed, and proteinase K-treated. After phenol-chloroform deproteinization, DNA was precipitated by ethanol and stored in 10 mmol/L Tris-HCl/1 mmol/L EDTA buffer. RNA was collected at the bottom of the centrifuge tube, washed in 70% ethanol, and precipitated with absolute ethanol. After RNase-free DNase (Gibco BRL) treatment, RNA was purified using RNeasy (Qiagen, Crawley, UK).

Determination of Allelic Polymorphism in gDNA and LOH Assessment

gDNA (500 ng) was amplified by polymerase chain reaction (PCR) using the sense 5' CAC CTG CTC CAG GGA TGC 3' and antisense 5' AAA ATA GAA GCG TCA GTC 3' primers, as described for the *p73* gene.⁵ Amplified DNA fragments were radiolabeled using α -[³²P]dATP (3000 Ci/mmol Amersham, UK); The PCR products were submitted to Styl (Boehringer, Mannheim, Germany) restriction polymorphism. D1S76 and D1S80 VNTR sequences were also analyzed for size polymorphism using primers under the conditions previously described.²⁴ Imbalance and LOH were defined as a variation in polymorphic signals between the two alleles of more than 25% and 50%, respectively.

Figure 1. Genomic organization of the *p73* gene. Diagram showing the various domains studied by RT-PCR, in **orange boxes** spanning: TAD, transactivating domain; DBD, DNA specific binding domain; OD, oligomerization domain. Exons are represented by **boxes** of various colors: N-terminal part: **green** for normal exons; **yellow** for alternate 1-bis and 3-bis; core domain, **gray**; C-terminal part, **blue**. P1, P2 and P3 indicate the three promoters of the gene. The size of amplicons is indicated in **brackets**. The **thin lines** represent introns.

Semiquantitative Determination of Global Levels of *p73* Transcript Species in Tissues

The *p73* gene can be transcribed into multiple forms using alternative promoters and/or alternate splicing. cDNA was obtained by reverse transcription (RT) of 2 μ g of total RNA using Moloney murine leukemia virus reverse transcriptase (Superscript IITM RNase H⁻, Gibco BRL kit) and random hexamers or oligodT to prime the synthesis under the conditions specified by the manufacturer. After treatment by RNase H, cDNA was submitted to PCR within the coding region (from exon 1 to exon 14) using various primer sets to obtain amplicons at different domains (Figure 1). The reaction was conducted for 40 cycles (except for core domain which was 35 cycles) of 1 minute at 95°C, 1 minute for annealing temperature (see below the annealing temperature for each amplicon) and 2 minutes 30 seconds at 72°C using a Perkin-Elmer Cetus thermocycler (PE Applied Biosystem). An aliquot (1/10) of the PCR products was analyzed on 2% agarose gel or polyacrylamide gel electrophoresis (PAGE) gel and visualized by ultraviolet illuminator.

p73 Primer Sets for Amplification of Coding Exons

E1F, E3bisF are the forward primers able to reveal transcripts initiated from P1 and P3. (Primer sequence to detect transcript initiated from P2 can be obtained on request from Dr. Caput). Quantification was performed by Phosphorimager scanning (Storm 840, Molecular Dynamics) of each *p73* transcript species using GAPDH expression as a loading control.

Immunohistochemical and Immunocytochemical Studies

Immunohistochemical (IHC) studies were performed on frozen tumor sections according to previously reported

protocols.⁴ For immunocytochemical (ICC), SH-SY5Y cells cultured on coverslips were fixed for 30 minutes in 4% paraformaldehyde. In all cases, IHC and ICC incubations were done overnight at 4°C with primary antibody using the streptavidin-biotin peroxidase technique. To detect p73 localization, the primary antibody (Ab) was a rabbit polyclonal Ab raised against p73 human (provided by Sanofi-Recherche, Labège) at a dilution of 1/300 in phosphate-buffered saline (PBS). This antibody recognizes epitopes at the C-terminal residues of the p73 protein. To detect p53 localization, two specific primary monoclonal antibodies, which recognized epitopes between amino acids 32 to 79 (Pab 1801, Oncogene Science, Boston, MA, USA), and 17 to 26 (DO-1, Oncogene Science) were used at a dilution of 1 μ g/ml. A biotinylated anti-rabbit IgG and a biotinylated rabbit anti-mouse IgG (Zymed, CA, USA) were used as secondary antibodies. A human neuroblastoma (NT no. 692) with overexpressed nuclear p53 whose gene sequence harbored a missense mutation in the fifth exon was systematically included in each set of experiments as a positive control. Rabbit IgG or normal mouse IgG was used for p73 or p53 experiments, respectively, as a negative control.

P73 α Immunoblotting

Protein lysates (50 μ g) were submitted to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto BioRad (Hercules, CA, USA) membranes. Blocking was performed in 10% milk, 0.1% Tween 20 Tris-buffered saline (TBS) for 2 hours at 25°C followed by incubation overnight at 4°C in the presence of a rabbit p73 α human polyclonal IgG (Sanofi-Recherche, Labège) at 1/1000 dilution in TBS. After three washes and a further incubation with anti-rabbit IgG-peroxidase conjugated at 25°C for 1 hour, the blot was developed by the Amersham ECL technique. Full-length p73 α and N-truncated p73 α were obtained from SK-N-AS NB cells transfected by plasmids containing *p73* full-length (1-2-3) or ΔN -p73 α (3bis-4-5) genes under CMV promoter control (Sanofi Recherche, Labège). The total protein extracts from the plasmid transfected cells yield the corresponding reference isoforms.

Results

An Array of *p73* Transcripts in NTs

A panel of 61 NTs comprising 35 localized stages, 20 IV stages, and 6 IV-S stages (classified according to a scale of ganglionic sympathetic differentiation as undifferentiated "NB," differentiating "NB/GNB," differentiated ganglioneuroblastoma "GNB," and fully differentiated ganglioneuroma "GN")¹ were screened using PCR-restriction fragment length polymorphism (PCR-RFLP) analysis for the two naturally occurring *p73* alleles denoted GC and AT.⁵ Only 20 out of the 61 NTs were determined to be GC:AT heterozygotes. Interestingly, 7 of these 20 infor-

Figure 2. RT-PCR analysis showing various transcript species spanning the three domains of the *p73* gene. GNB: ganglioneuroblastoma; NB, undifferentiated neuroblastoma. **A:** N-terminal part: P1 transcripts (lanes 2, 4, and 6, P3 transcripts (lanes 8, 9, and 10). Note that transcript 1bis-2-3 (initiated from P2) is barely detectable with no significant differences in intensity between NB and GNB according to semiquantitative RT-PCR normalized by GAPDH gene. A faint upper 1-2-3 band (lanes 2 and 6) is considered as non-specific PCR product. Occasionally, a transcript species arising from another promoter denoted as P2 (Figure 1) was expressed with faint intensity (lanes 1, 3, and 5). In lanes 2 and 6, a Δ -exon 2 band in a NB/GNB and a NB specimen is barely detectable (a ratio $r < 0.5$, is considered 0 according to the scale of Table 1). **B:** Core and C-terminal transcripts. Exons 6–8 transcript (lanes 1, 2, and 3). Exons 12–14 transcripts (lanes 5, 6, and 7). M, size marker. **C:** To illustrate the differential P1 usage in lymphocytes *versus* NTs, RT-PCR products separated in 4% polyacrylamide gel showed P1- Δ exon 2 transcript which was consistently found in various histology (NB, NB/GNB, GNB, GNB/GN) whereas it was absent or expressed at barely detectable levels in corresponding lymphocytes.

mativ tumors (35%), showed an imbalance ratio of GC:AT alleles suggesting a loss of heterozygosity at the *p73* locus, further confirmed by fluorescence in situ hybridization (FISH) analysis (data not shown). Finally, DNA sequencing performed on 15 NTs demonstrated an absence of mutations for remaining *p73* allele suggesting that Knudson's "two-hit" model for candidate tumor suppressor genes may not apply to *p73*.

To investigate whether qualitative and/or quantitative variations of *p73* transcripts correlate with differentiation defects in NTs, we analyzed transcript species using a semiquantitative PCR strategy which yields robust and short PCR products spanning the three domains of the *p73* gene, TAD, DBD, and OD (Figure 1). PCR experiments are exemplified in Figure 2 and data obtained for 20 informative NTs are compiled in Table 1.

Analysis of the core domain (exons 6–7–8) revealed detectable transcripts in all NTs, with no significant variation in expression levels independently of the gDNA *p73* status (LOH or not) as well as of the histological type (NB, NB/GNB, or GNB/GN). In the C-terminal domain, transcripts encoding α and β isoforms (Figure 2B) were found to be co-expressed in 15 of 20 NTs. No other isoforms, such as γ and δ , previously described¹² were detected. Regarding TAD, strikingly, reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed transcripts encoding *p73* proteins that lack an amino-terminal transactivation domain, in addition to the regular full-length α and β *p73* species. A study performed on transcript species in the N terminus according to the Rapid Amplification of cDNA Ends (RACE) PCR method led to identify two additional promoters, P2 and P3 (F McKeon, D Caput, data not shown). Like the transcripts encoding ΔN -*p63*,¹⁸ ΔN -*p73* transcripts (3bis-4–5) are initiated from an alternative promoter P3 located

Table 1. Fold Increases of *p73* Transcript Species

Patient no.	Histological classification	Heterozygosity status	p73 Transcript species					
			N-terminal part			Core part (DBD)	C-terminal part	
			P1-full	P1-Δex.2	3bis-4-5		α	β
		(gDNA)			Normalized by GAPDH			
913	NB	AT:GC	1	1	1	1	2	1
1103	NB	AT:GC	2	2	2	1	0	0
1106	NB	AT:GC	1	1	1	1	2	1
804	NB	AT:GC	1	0	1	1	1	0
639	NB	AT:GC	1	1	1	1	1	1
380	NB	AT:GC	1	1	1	1	2	2
202	NB	AT:GC	2	0	2	1	0	0
840	NB	AT:GC	1	0	nd	1	1	1
245	NB	AT:GC	1	2	2	nd	1	1
645	NB	AT:GC	2	0	2	2	1	1
193	NB	AT:GC	2	1	nd	nd	0	0
963	NB/GNB	AT:GC	1	0	1	1	2	1
901	NB/GNB	AT:GC	1	1	1	1	0	1
275	NB/GNB	AT:GC	1	0	1	1	1	1
1013	NB/GNB	AT:GC	1	0	1	1	2	1
860	NB/GNB	AT:GC	1	0	1	2	2	2
924	GNB	AT:GC	1	0	1	1	2	1
1008	GNB/GN	AT:GC	1	0	1	1	2	1
741	GNB/GN	AT:GC	2	1	2	1	1	1
269	GNB/GN	AT:GC	1	1	1	1	1	1

nd, not determined.

Figure 3. p73 α protein isoforms in fresh neuroblastic tumors. Immunoblot analysis of total cell lysates with p73 α polyclonal antibody. NTs are presented according to their histology: undifferentiated NB,¹⁻⁵ differentiating GNB/NB,⁶ and mature ganglionic GNB/GN.^{7,8} Protein isoforms are identified with reference to recombinant p73 isoforms (left). Arrow indicates a heavier p73 protein (130 to 150 kd).

in intron 3; consequently, exons 1, 2, and 3 in these transcripts are replaced by a unique exon named exon 3bis (Figure 1).

Interestingly, transcripts of this exon were consistently detected in all NTs (Figure 2A, right panel; Table 1). Finally, a substantial amount of p73-encoding transcripts were splice variants lacking exon 2 (Figure 2A, left panel) as already reported in the human NB cell line SK-N-SH.⁵ It is noteworthy that exons 1 and 3bis, corresponding to two distinct origins of transcription, were equally transcribed in NT, suggesting a similar and/or coordinated transcriptional control at the promoter level (Figure 2A). The frequency of the transcripts lacking exon 2 (species 1-3) appeared to be higher in undifferentiated (7 of 11) than in differentiating and differentiated tumors (3 of 9). RT-PCR experiments performed on matched lymphocyte/tumor pairs and representing the histological spectrum NB, NB/GNB, GNB, GNB/GN showed that the P1- Δ exon2 (1-3) transcript species of lymphocytes was mostly absent or only faintly expressed when compared to the corresponding tumors (Figure 2C).

Overall, the RT-PCR data indicate that the two promoters P1 and P3 are consistently used in NTs, regardless of the histological status. By contrast, the p73 transcripts lacking exon 2 typify undifferentiated NBs. To ascertain the identity of the transcript variant species, we performed Northern blot analyses from total RNA using α -[³²P] probes corresponding to amplified exons. Despite a 2 week exposure, the radiolabeled bands obtained were barely detectable even in the core domain (data not shown). This implies that p73 transcript levels are very low and are only detectable by RT-PCR.

p73 Protein Expression in Neuroblastic Tumors

To characterize p73 isoforms in NTs, lysates obtained from large amounts of frozen tumor specimens from 15

patients were successively analyzed by immunoblotting and immunohistochemistry. Surprisingly, immunoblotting using the p73 α polyclonal antibody revealed a single band of similar migration to ΔN -p73 reference isoform (Figure 3). In undifferentiated NB, only the ΔN -truncated isotype band was detected, often accompanied by a p73 cross-reacting lighter band (at about 50 kd), very likely to represent a product of specific proteolysis. A heavier protein species (130 to 150 kd) was also observed. Importantly, this protein species was present at high levels in differentiating-differentiated NTs (NB/GNB and GNB/GN, respectively), but rarely in undifferentiated NTs (Figure 3, lane 5). This heavy protein was the only species found in the fully differentiated GNB/GN of this series (Figure 3, lane 8). The nature of this SDS-PAGE resistant protein remains to be determined.

To localize the p73 protein at the subcellular level in NTs, we performed an immunohistochemical study using a polyclonal antibody that recognizes C-terminal residues of the α isoform.⁵ Formalin-fixed tissues were analyzed including five specimens of normal human adrenal medulla from which most NTs originate, and 23 NTs including 6 undifferentiated, 16 poorly differentiated NTs, and one GN. Histological criteria of sympathetic differentiation in NTs are based on cell morphology:¹ undifferentiated neuroblasts appear as small round cells with a prominent nucleus, whereas very large mature ganglionic cells have an abundant and distinct cytoplasm, marked nucleoli and a prominent nuclear membrane. In normal human adrenal medulla tissue, conspicuous immunoperoxidase staining appeared to be restricted to the nuclei of the chromaffin cells, but it was also intense in the nuclear membrane and associated with faint but significant staining in the cytoplasm (Figure 4a). Nineteen (83%) of the 23 NTs showed positive p73 α staining. p73 immunoperoxidase staining was found in neuroblasts and ganglionic cells, but was absent in S100-positive Schwann cells (Figure 4, b and d). Nuclear staining was intense in small cells (undifferentiated neuroblasts lacking visible cytoplasm), and still present, but less intense in fully differentiated ganglionic cells (very large cells with a prominent cytoplasm, Figure 4d). Faint but significant cytoplasmic staining was also noted in differentiated and fully differentiated ganglionic cells (Figure 4, c and d). Ganglionic maturation is often observed after induction chemotherapy in NT patients. In a particular patient, comparison of the GNB tumor specimen taken before (Figure 4c) and after (Figure 4d) chemotherapy indicated more intense cytoplasmic staining in fully differentiated ganglionic cells. This subcellular localization may suggest that a nuclear-cytoplasm shuttling of active p73 forms could mark the neuronal differentiation process. Strikingly, p73 subcellular localization in NTs differed from that of p53. Indeed undifferentiated NTs readily showed detectable p53 cytoplasmic retention (Figure 5, A and B), as previously reported,⁴ while differentiating and differentiated NTs show only minimal and no staining, respectively (Figure 5, C and D).

To confirm the p73 cellular localization observed in

Figure 4. p73 immunostaining in normal human adrenal medulla tissue and neuroblastic tumors. **a:** Normal adrenal medulla. Note p73-stained chromaffin cells (**large arrow**) with conspicuous staining of nuclei (scored ++) and more intense staining at the inner membrane level associated with faint but significant cytoplasmic staining (scored +/-) (**small arrow**). Cells from the reticular zone of the adrenal cortex denoted RZC are totally devoid of staining for p73. Similar data are observed in the glomerular zone. **b:** Poorly differentiated, Schwannian stroma-poor neuroblastoma, (denoted NB): all neuroblasts (**large arrow**) show diffuse and homogeneous nuclear staining for p73 (scored +++), while rare slender Schwann cells are p73-negative (**small arrow**). **c:** Ganglioneuroblastoma nodular type (denoted GNB), at diagnosis: neuroblasts at different stages of ganglionic maturation, the most mature (**large arrow**) showing p73 positivity in the nucleus (scored +) as well as the abundant cytoplasm (scored +/-). **d:** Ganglioneuroma, having undergone sympathetic ganglionic maturation after chemotherapy (adriamycin/vincristine), from the GNB presented in **c**: typical fully differentiated large cells with positive nuclei (scored + , **large arrow**) and abundant cytoplasm (scored +/-) and neuropils (**very small arrows**) are surrounded by slender Schwann cells which are consistently p73-negative (**small arrow**). (Magnification, $\times 400$).

NTs, FGF1-stable transfected SH-SY5Y cells and NGF exogenous induction were used as models of neuronal differentiation.^{22,23} Neuritic outgrowth at the morphological level (see Figure 6D for stable FGF-transfected cells and Figure 6G for NGF-exogenous induction) and N-myc down-regulation associated with *gap43* up-regulation at the molecular level were used as standard differentiation markers.²² In differentiated SH-SY5Y cells, ICC studies revealed a nuclear staining as well as a p73 punctuated cytoplasmic staining (Figure 6, F and I, black arrows); by contrast, in control cells, the p73 α staining was exclusively nuclear (Figure 6C).

Discussion

The C-terminal domain of the *p73* gene has been shown to be subject to splicing in human tumors leading to major α and β variants⁵ and other minor species.^{12,17} In this study, we showed that the N-terminal domain of the gene is also subject to regulation. Based on NT transcript

Figure 5. p53 immunostaining in various types of neuroblastic tumors. Undifferentiated NB tumor stained with PAb 1801 (**A**) or DO-1 (**B**). **C:** Differentiating tumor NB/GNB stained with PAb 1801. **D:** Differentiated tumor GNB/GN stained with PAb 1801. Note cytoplasmic overexpression of p53 in an undifferentiated NB tumor (**A** and **B**). A very slight staining is observed in differentiating tumor (**C**) whereas no staining is observed in differentiated tumor (**D**). (Magnification, $\times 400$).

and protein analyses, we propose a role in sympathetic differentiation for the *p73* gene with regards to N. terminal domain.

Transcript Profiles and Stoichiometry Discriminate NTs

RT-PCR screening of the first three exons yielded the expected products from the two main promoters (P1 and P3), but also revealed a new variant in which exon 2 had been spliced out (Table 1). Transcripts initiated from P1 and P3 promoters were equally frequent, irrespective of the tumor differentiation status. By contrast, the spliced-out exon 2 variant initiated from P1 (ie, the 1-3 species) was far more expressed in undifferentiated than in differentiating and differentiated NTs. These data strongly suggest that the amount of the compiled transcript isotypes 1-3 and 3bis-4-5 with putative dominant-negative activity, is probably higher in undifferentiated NTs than in NTs in the process of differentiation. As DBD was not subjected to splicing, it appeared to be consistently transcribed in all NTs. The α and β COOH-terminal splice variants were found to be frequently co-expressed in NTs; however 4 of 11 undifferentiated NBs lack β isotype while 9 of 9 differentiating and differentiated NTs express this variant (Table 1). This evidence provides an additional link between the C-terminus integrity of the *p73* gene and sympathetic differentiation.

Figure 6. p73 α immunocytochemistry in SH-SY5Y cells. **A–C:** Control SH-SY5Y cells transfected by empty plasmid. Note a nuclear p73 α immunostaining in (C) compared to the negative control without p73 antibody in (B). **D–F:** FGF1 overexpression mediates neuritic outgrowth in a FGF1-transfected clone (D); negative control (E). **Arrows** indicate punctuate cytoplasmic p73 α dotted staining in differentiated cells (F). **G–I:** SH-SY5Y cells treated by exogenous NGF + aphidicolin for 8 days. Note the strong p73 α cytoplasmic staining which contrasts with the light nuclear staining; **H:** negative control. **B, E, H:** magnification, $\times 200$; **A, C, D, F, G, I:** magnification, $\times 400$.

An Array of Multiple Transcripts, but a Single Protein Profile

In undifferentiated NBs, a single α -N truncated p73 iso-type was identified which contrasts sharply with the multiple transcripts putatively encoding an array of isoforms. These data strongly imply a marked difference in the p73 protein turnover or highly stable N-truncated iso-types compared to full-length iso-types. With regards to ΔN -p63, known to yield a dominant negative activity on p63 itself and/or p53 transactivation,¹⁸ a stable truncated protein would exert a similar activity while a labile full-length protein would elicit transient transcriptional activity. In full agreement with this hypothesis, it has been recently shown that in the development of sympathetic neurons, N-truncated p73 is an essential anti-apoptotic protein which, by directly binding to p53, counteracts the pro-apoptotic function of p53.²⁵ We are unable to specify the identity of the single ΔN -p73 protein band since mRNA

species (either 3bis-4-5 initiated from P3 or Δ exon2¹⁻³ initiated from P1) encode proteins of similar theoretical M_r (64 kd). However, the products of 3bis-4-5 transcript should be favored given its consistent occurrence in all NTs except one. Whether the ΔN -p73 isoform and the one encoded by the spliced-out exon 2 variant mostly found in undifferentiated NBs exert similar biological role remains to be elucidated, as it has been recently described in other models.^{26,27}

The fact that ΔN -p73 α protein was detected but not p73 α full-length can be explained by its higher stability. Another possibility is the association of this isoform with another protein to be identified. Therefore, this sheds light on an interesting parallel with the wild type p53 stability, in view of data demonstrating that i) full-length p73 interacts via its N-terminus, thereby involving the TAD with p53, MDM2, and p300/CBP, and that, ii) in contrast to p53, MDM2-p73 complex inactivates p73 without degrading it.²⁸⁻³¹ Moreover, recent studies have identified

a larger molecular weight product proposed to be the sumulated version of ΔN -p73 α .³²

p73 Is an Attractive Candidate for a Differentiation Function

Immunohistochemistry revealed specific p73 expression localized within the nucleus, in particular at the nuclear membrane as well as in the cytoplasm of chromaffin cells of normal adrenal medulla. Moreover, most NTs, including malignant neuroblasts and ganglionic cells, but not Schwann cells, also exhibited significant p73 expression. p73 is mainly localized in the nucleus of undifferentiating NBs, with a low intensity in the perinuclear and cytoplasmic area of differentiated GNB/GN. Similar data were observed between parental SH-SY5Y cells and differentiated cells resulting from either FGF1 endogenous activity or exogenous NGF/aphidicolin induction. By combining these findings with the expression of an N-truncated α -isotype in undifferentiated NBs and a large protein complex in fully differentiated GN, a plausible postulate would be that the full-length cytoplasmic form of p73 is involved in sympathetic differentiation, while the nuclear N-truncated α form of p73 expresses oncogenic properties in the nucleus with a dominant-negative impact on p53 transactivation of target genes. In full agreement with *in vivo* results strongly favoring p73 involvement in neuronal differentiation is the recent *in vitro* finding reported by De Laurenzi et al³³ who showed that transfected full-length p73 cDNA induced neuronal differentiation in the murine N1E-115 neuroblastoma model, while these cells do not undergo differentiation when co-transfected with full-length and ΔN -p73 cDNAs. The mechanisms conferring cytoplasmic localization to p73 protein in differentiated sympathetic cells are currently unknown. We propose that full-length p73 is instrumental in the differentiation process. This hypothesis could be tested using *in vitro* studies. Importantly, the p73 cellular localization appears to be opposite to that of p53 which is overexpressed and localized in the cytoplasm in undifferentiated NBs,⁴ and down-regulated (not visible by ICC) in differentiated GNB or GN. Differentiation of malignant neuroblasts is accompanied by a decrease in the p53 level *in vitro*.³⁴ The nucleus-cytoplasm transfer of p53 and p73 occurs in opposite directions and it could be hypothesized that a single protein or proteic complex is in charge of the transfer for both proteins. Transgenic p73^{-/-} mice show lack of tumors, in contrast to p53^{-/-} mice, but exhibit neurosensorial defects and hydrocephaly²⁰ and a decrease of urinary catecholamines (D Caput, personal communication). In nullizygous p73^{-/-} mice, ΔN -p73, the physiological p53 antagonist²⁵ being absent would explain the lack of NTs because of early p53-dependent death of sympathetic neurons.

p73 does not possess the properties of a typical tumor suppressor gene, but its versatile regulation of transcription and translation shows some correlation with the pathogenesis of NTs. The equilibrium between the various isotypes with divergent biological activities could

determine whether or not the sympathetic differentiation process occurs.

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