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N-*myc* Modulates Expression of *p73* in Neuroblastoma

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Abstract

The human *p73* gene is a homolog of *p53*, which has been localized to chromosome 1p36 in a region that is frequently deleted in neuroblastoma. Transfection of the *p73* gene into neuroblastoma cells that lack detectable *p73* protein has been shown to result in growth suppression and to induce neuronal differentiation. In this study, we have identified by means of restriction landmark genome scanning (RLGS) a genomic fragment that was frequently reduced in intensity in neuroblastomas. The cloned fragment contained exon 1 of *p73* as well as intronic and promoter sequences. We investigated the genomic and expression status of *p73* and N-*myc* in 34 neuroblastoma tumors and 12 neuroblastoma cell lines. Approximately a third of neuroblastomas in our series exhibited deletion of *p73*. Most tumors analyzed exhibited reduced expression of *p73*, as determined by quantitative RT-PCR, in the absence of detectable *p73* gene deletion. The reduced expression of *p73* correlated with overexpression of N-*myc* in a statistically significant manner. The N-*myc* gene was transfected into two neuroblastoma cell lines that lacked N-*myc* amplification to determine its effect on *p73* RNA levels. *p73* was detectable at low level by RT-PCR in untransfected SK-N-AS cells and became undetectable following N-*myc* transfection, whereas in SH-EP1 cells, *p73* levels were substantially reduced following transfection but remained detectable. Our data suggest that the N-*myc* gene modulates expression of *p73*, allowing neuroblastoma cells to escape the growth suppressing properties of *p73*.

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Keywords: neuroblastoma, N-*myc*, *p73*, amplification, chromosome 1p36.

Introduction

The *p73* gene encodes a protein with significant amino acid sequence homology and functional similarity with *p53* [1]. Overexpression of *p73* activates the transcription of *p53* target genes and inhibits cell growth by inducing apoptosis [2]. *p73* maps to chromosome 1p36, in a region frequently deleted in neuroblastomas and other human cancers [3–6] raising the possibility that in neuroblastoma, inactivation of *p73* may contribute to tumorigenesis.

Neuroblastoma is a tumor of neural-crest–derived cells of variable severity. Amplification of the N-*myc* oncogene occurs in approximately one-third of neuroblastomas and correlates with aggressive disease [7]. There is conclusive evidence that targeted expression of N-*myc* in transgenic mice results in neuroblastomas that exhibit features of human neuroblastomas [8]. The identity of genes that play a role in neuroblastoma development in the absence of N-*myc* amplification is largely unknown. There is evidence that the 1p36 region may contain at least two tumor-suppressor genes [9,10]. Neuroblastoma tumors lacking N-*myc* amplification frequently contain relatively small 1p36 deletions, suggesting that this region, which encompasses *p73*, contains a tumor suppressor. Tumors with N-*myc* amplification contain larger deletions that extend proximally beyond the location of *p73*, suggesting that the extended region contains another tumor-suppressor gene. Whereas expression analysis supports a potential role for *p73* in neuroblastoma [11], conclusive functional evidence that links *p73* inactivation to the development of neuroblastoma is currently lacking. *p73*-deficient mice have neurological defects but lack spontaneous tumors [12]. There is also lack of genetic evidence that points to inactivation of *p73* by point mutations [5,6,13]. Thus, the identity of tumor-suppressor gene(s) that play a role in neuroblastoma tumor initiation or progression and that may reside in the 1p36 region remains to be determined. Nevertheless, given the evidence that *p73* activates the transcription of *p53* target genes and inhibits neuroblastoma cell growth and induces neuronal differentiation [1,14], analysis of the expression patterns of *p73* in neuroblastoma may be relevant to our understanding of abnormal proliferation in this malignancy.

We have utilized restriction landmark genome scanning (RLGS) to detect novel genomic alterations in neuroblastoma cell lines and primary tumors [15,16]. The RLGS approach to scan the whole genome allows the identification and isolation of as yet unknown amplified, deleted or methylated sequences. The use of the enzyme *Not*I to digest

Abbreviations: RLGS, restriction landmark genome scanning; RT-PCR, reverse transcription polymerase chain reaction; GAPDH, glyceride-3-phosphate dehydrogenase

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DNA and the tagging of the fragments at the *Not*I ends allows preferential visualization and quantitative analysis of fragments containing CpG islands. Using this approach, we have cloned a novel gene referred to as *NAG*, that is frequently co-amplified with N-*myc* in neuroblastoma [17]. We have developed informatics tools for comparisons of sample derived RLGS patterns with patterns predicted from the human genome sequence and displayed as virtual genome scans (VGS) [18]. The tools developed allow sequence prediction of fragments in RLGS patterns obtained with different restriction enzyme combinations. The utility of VGS has been demonstrated by the identification of restriction fragment length polymorphisms, and of amplifications, deletions, and methylation changes in tumor-derived CpG islands [19]. We have also developed a strategy for the analysis of RLGS patterns of neuroblastomas and control tissues for the detection of chromosome 1 fragments that are either absent or that exhibit diminished intensity in tumor patterns [20]. In this study, one such genomic fragment was identified as encompassing exon 1 of the *p73* gene, leading us to determine the relationship between N-*myc* amplification and overexpression on the one hand and *p73* deletion and reduced expression on the other. We provide evidence that N-*myc* plays a role in regulating expression of *p73*. N-*myc* amplified tumors exhibited reduced expression of *p73*, independent of *p73* gene deletion. In addition, transfection of neuroblastoma cell lines with N-*myc* resulted in reduced expression of *p73*.

Materials and Methods

Two-Dimensional Gel Electrophoresis

High-molecular-weight DNA was extracted from 34 neuroblastoma tumor specimens obtained at the time of diagnosis and 12 neuroblastoma cell lines. DNA was subjected to RLGS analysis, as previously described [16]. Briefly, 3 μg of DNA was used for digestion with *Not*I and *Eco*RV. The *Not*I-derived 5' protruding ends were labeled with ^{32}P-marked nucleotides (*Eco*RV yields blunt ends). The labeled fragments were directly applied onto first-dimension agarose gels before electrophoresis. Separated fragments were treated *in situ* with *Hin*fI for further cleavage, before second-dimension separation in acrylamide slab gels. The gels were subsequently dried and exposed to PhosorImager plates (Molecular Dynamics, Piscataway, NJ). Images were obtained after scanning of the PhosphorImager plates. Analysis of *p73* spot intensity was undertaken by an investigator that was blinded to the N-*myc* and *p73* expression status of the samples from which the RLGS were derived. The intensity of the *p73* fragment in RLGS was estimated relative to the intensity of invariant neighboring fragments.

Southern Blot Analysis

Genomic DNA was digested with *Not*I and *Eco*RV before electrophoresis and blotting. Filters were hybridized with a 1.4-kb random-labeled *Not*I–*Hind*III *p73* genomic fragment derived from a 1.7-kb *Not*I–*Eco*RV fragment cloned from preparative RLGS patterns. The same filters were also hybridized with a 1.4-kb *Not*I–*Dpn*II unrelated chromosome 1–derived genomic fragment that contains part of the GAC1 gene [21]. N-*myc* amplification was also determined by Southern blotting using an N-*myc* cDNA as probe.

Construction of a Chromosome 1 NotI– EcoRV Fragment Library

Metaphase chromosomes were isolated from the human lymphoblastoid cell line GM-1130B obtained from the American type Culture Collection (Rockville, MD), using the polyamine isolation buffer described previously [22]. Isolated chromosomes were stained with two fluorochromes, Hoechst 33258 and chromomycin A3 (Calbiochem, La Jolla, CA), and allowed to stand for 48 hours using a combination of slow centrifugation and sedimentation. Two million chromosomes 1 were sorted into siliconized glass tubes. Chromosome 1 genomic DNA was digested first with *Not*I and ligated to the *Not*I and *Eco*RV digested plasmid vector Bluescript SK+ (Strategene, La Jolla, CA) with T4 ligase (Roche Molecular Biochemicals, Indianapolis, IN). After the first ligation and phenol/chloroform extraction, the DNA was digested with *Eco*RV and purified with phenol/chloroform extraction–ethanol precipitation. The *Eco*RV ends were ligated. The circular DNA was introduced into Epicurian coli, XL1-blue MRF′ cells by electroporation according to the conditions recommended by the manufacturer. Genomic library DNA was subjected to RLGS analysis as described above.

Cloning of a Not 1 Containing p73 Genomic DNA Fragment

For cloning of fragment Ch1-113, 1.1 μg of the library DNA was digested with *Not*I and *Eco*RV. An aliquot of digested DNA was labeled at the *Not*I ends with [^{32}P]dCTP and dGTP. The labeled DNA was mixed with unlabeled library DNA and subjected to electrophoresis. DNA labeling allowed detection of the fragment in RLGS pattern. Unlabeled DNA served to increase the yield for cloning of fragments extracted from gels. The separated fragments were treated with *Hin*fI for further cleavage, *in situ*. The *Hin*fI-digested DNA fragments were separated in the second dimension as for tumor DNA, and the resulting gel was exposed against X-ray film for 3 days at -80°C. A fragment in the position of Ch1-113 was extracted from the gel and ligated into modified Bluescript SK-vector sets, followed by electroporation into Epicurian coli, XL1-blue MRF′ cells and transformed bacteria colony selection. The corresponding *Not*I–*Eco*RV fragment was obtained by using the ^{32}P-labeled *Not*I–*Hin*fI fragment as probe for screening the chromosome 1 library.

RNA Samples and RT-PCR

Total RNA was extracted from neuroblastoma tumors and cell lines using Trizol reagent (Life Technologies, Rockville, MD). Two micrograms of each total RNA was reverse transcripted into single-stranded DNA with Superscript II reverse transcriptase (Life Technologies) and random

primers according to the conditions recommended by the manufacturer. One microliter of the products was amplified with Expand High Fidelity PCR System (Roche Molecular Biochemicals) in 25 μl of reaction that contained 1.75 U of polymerase, 20 pM of each primer, 1$\times$ buffer (with 1.5 mM $MgSO_4$), 12.5 nM of each dNTP, and 5% DMSO. The amplification process was as previously described [23] and consisted of 30 cycles of 60°C/0.5 minute, 71°C 2 minutes, and 93°C/0.5 minute after starting with a denaturation step at 93°C for 3 minutes and ended at 72°C for 10 minutes. PCR was undertaken using double primers as follows. Primers were designed to achieve the same parameters for primer Tm, GC content and length. The size of the targeted regions was chosen to achieve a comparable size while allowing resolution of the PCR products by agarose gel electrophoresis.

p73 primers: forward primer 5′GAACCAGACAGCACCTAGCACCTACTTCG; reverse primer: 5′ GACAGGGTCATCCACATACTGC.

N-*myc* primers: forward primer 5′ TCCACCAGCAGCACAACTATG; reverse primer: 5′ GTCTAGCAAGTCCGAGCGTGT.

Alternatively one set of double primers consisted of ubiquitin primers relied upon as a control: forward primer: 5′ TGACACAATCGAGAACGTCAAG; reverse primer: 5′ ATGCCTTCCTTGTCTTGGATCT.

All PCR reactions were undertaken at least twice to ensure consistency.

N-myc Transfection

Neuroblastoma cell lines were maintained in MEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. SH-EP1 and SK-N-AS cells were stably transfected with the pCMV*myc* expression plasmid or native pSMneo vector (gifts of William Fahl, McArdle Laboratory for Cancer Research, Madison, WI) using Lipofectamine (Life Technologies) as described by the manufacturer. Cells (5×10^4) were plated on 35-mm dishes in serum-free media and transfected with 10 μg of plasmid DNA (pCMV*myc* or pSM*neo*). Twenty-four hours following transfection, 20% MEM was added to the plates. At 48 hours, cells were trypsinized and transferred to 100-mm dishes and the media was supplemented with G418. Individual G418 colonies were transferred using filter paper soaked in trypsin and subcultured in six-well dishes. N-*myc* expression was determined by Western blotting. Following stable transfection, cells were cultured in 10% MEM containing G418 (Geneticin; Gibco, Carlsbad, CA) at 500 μg/ml.

Results

A NotI Fragment with Diminished Intensity in Neuroblastoma RLGS Patterns Contains a p73 CpG Island

We focused our analysis of neuroblastoma RLGS patterns on chromosome 1 fragments that exhibited absence or reduced intensity in tumor patterns compared to control peripheral blood DNA from the same patients. We had previously ascertained that 280 DNA fragments in our standard genomic RLGS patterns were derived from chromosome 1 [20]. The standard patterns are obtained using *Not*I and *Eco*RV as the initial cutters before the first-dimension separation and *Hin*fI as the enzyme cutter for *in situ* digestion before the second-dimension separation. A fragment designated Ch1-113 frequently occurred at decreased intensity in tumors relative to controls (Figure 1). This fragment was predicted from VGS analysis to be derived from the *p73* gene and its identity was further verified by cloning.

To obtain sufficient amounts of DNA of chromosome 1 fragments of interest for their cloning, as in the case of fragment Ch1-113, we constructed a chromosome 1 *Not*I–*Eco*RV fragment library. For the preparation of the library, chromosome 1 DNA was obtained as described in Materials and Methods. DNA from the library was used for preparative RLGS gels of chromosome 1 fragments. Fragment Ch1-113 was localized in chromosome 1 library RLGS patterns, leading to its extraction and cloning. We first cloned the Ch1-113 406-bp *Not*I–*Hin*fI fragment directly from RLGS gels, as described in Materials and Methods. Subsequently, we used the cloned 406-bp fragment as a probe to screen the chromosome 1 *Not*I–*Eco*RV library. Six *Not*I–*Eco*RV-positive clones were obtained. All six clones had the same 1.7-kb size insert and hybridized with the Ch1-113 406-bp probe in a Southern blot of cloned DNA. Two of the six clones were sequenced at the *Not*I end, and one was sequenced in its entirety, yielding a 1682-bp insert. The 406-bp sequence occurred in its entirety in the 1682-bp fragment, beginning at the *Not*I end. DNA from the fully sequenced clone was used to spike a whole genomic DNA preparation before RLGS analysis. The "spiked" RLGS pattern contained an increased intensity of spot Ch1-113 (Figure 2).

The full 1682-bp sequence was found to correspond to bases 3921–5604 of a subsequently derived sequence for part of the *p73* gene (GenBank AF235000). The 1682-bp DNA fragment contained 119 bp of the *p73* 5′ nontranslated region, a 77-bp first exon, and 1486 bp of the first intron.

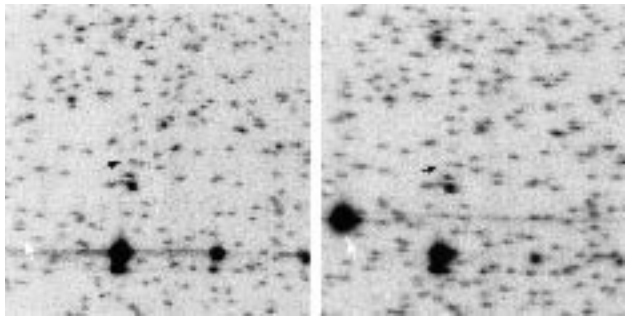

Figure 1. *Close-up sections of RLGS patterns from a neuroblastoma tumor (right) and normal DNA from the same subject (left). The black arrow points to reduced intensity of the p73 genomic fragment in the tumor relative to normal DNA. The white arrow points to an amplified fragment derived from the N-myc amplicon in the tumor [17].*

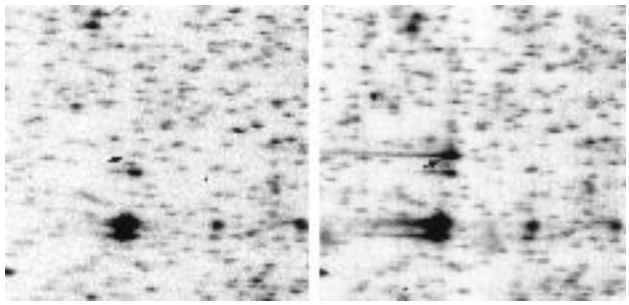

Figure 2. *Close-up sections of RLGS patterns from normal whole genomic DNA (left) and from the same DNA after spiking with cloned p73 genomic DNA (right), demonstrating increased intensity of the p73 genomic fragment.*

Approximately 1 kb of the 1682-bp fragment, beginning at the *Not*I site, was GC rich and contained 90 CpG pairs, consistent with a CpG island. A TATA-box–like element occurred 70 bp upstream of the transcribed sequence in *p73* mRNA. A MetInd and MatInspector search [24] uncovered numerous putative binding sites for known transcription factors in the 1682-bp sequence. Interestingly, two N-*myc* binding consensus sequence sites occurred inside the first intron, at 723 and 1158 bp from the transcription initiation site (at bases 4095 and 4438 of AF235000).

p73 Deletion and N-myc Amplification in Neuroblastoma Tumors and Cell Lines

A total of 34 neuroblastoma tumors and control DNAs were analyzed by RLGS for *p73* genomic loss. N-*myc* amplification in these tumors was previously analyzed by Southern blotting. As a control for variation in *p73* spot intensity in RLGS patterns that may be due to polymorphism at the cut sites, particularly for cell lines that did not have normal DNA for comparison, we analyzed the *p73* spot intensity in 126 normal RLGS patterns (67 of these DNA samples were peripheral blood cells from normal individuals; 47 were from various normal adult tissues including brain, breast, muscle, lung, stomach, pancreas, and colon; and 12 samples were from various fetal tissues including adrenal). All RLGS patterns from normal tissues exhibited a *p73* spot intensity that corresponded to two copies in the genome based on our prior quantitative analysis of spot intensities in RLGS patterns [25]. These results indicate lack of polymorphism at the cut sites for the *p73* fragment visualized by RLGS and further point to lack of significant methylation at the *Not*I cut site in normal tissues. Sufficient DNA was available for 17 tumors to also allow Southern blot analysis of *p73* genomic status. In the case of tumors for which both RLGS analysis and Southern blot analysis were available, there was a good agreement between *p73* fragment intensity in RLGS patterns and *p73* band intensity in Southern blots. Twelve neuroblastoma cell lines were also analyzed by RLGS and Southern blotting. Eleven tumors (32.4%) and seven cell lines (58.3%) showed evidence of deletion in our combined RLGS and Southern blot analysis (Table 1; Figure 3). No tumor or cell line demonstrated complete loss of both genomic copies of *p73*. Seven tumors (20.6%) and seven cell lines (58.3%) showed N-*myc* amplification. *p73* deletion and N-*myc* amplification frequently occurred in the same tumors as 5 of 7 tumors and 4 of 7 cell lines with N-*myc* amplification had *p73* deletion, while 5 of 11 tumors and 4 of 6 cell lines with *p73* deletion had N-*myc* amplification. The correlation between N-*myc* amplification and *p73* deletion in tumors was statistically significant (P=.0004, 1-tailed Fisher's exact test). The correlation remained statistically significant when cell lines were also included in the statistical analysis. Both N-*myc* amplification and *p73* deletion in neuroblastoma tumors occurred preferentially in advanced stage tumors (Table 1).

Table 1. *p73* and N-*myc* Genomic Status and Expression in Neuroblastoma Tumors and Cell Lines.

	Tumor Stage	*p73* Deletion	N-*myc* Amplification	*p73* Expression	N-*myc* Expression
Tumors					
NBT 1	II	No	No	↑	↓
NBT 2	I	No	No	↓	↑
NBT 3	I	No	No	↓	↑
NBT 4	III	No	No	↑	No change
NBT 5	IV	Yes	Yes	↓	↑
NBT 6	III	No	No	↓	↑
NBT 7	II	No	No	No change	No change
NBT 8	I	No	No	↓	↑
NBT 9	II	No	No	↓	↑
NBT 10	II	No	No	↓	No change
NBT 11	I	No	No	No change	↑
NBT 12	IV	Yes	Yes	↓	↑
NBT 13	I	No	No	↓	No change
NBT 14	IV	No	No	No change	No change
NBT 15	IV	No	No	↓	↓
NBT 16	III	Yes	Yes	↓	↑
NBT 17	II	No	No	↓	↑
NBT 18	II	No	Yes	↓	↑
NBT 19	IV	Yes	Yes	↓	↑
NBT 20	IV	No	No		
NBT 21	III	Yes	No		
NBT 22	II	No	No		
NBT 23	II	No	No		
NBT 24	IIb	Yes	No		
NBT 25	IV	No	No		
NBT 26	III	Yes	No		
NBT 27	IVs	No	No		
NBT 28	III	No	No		
NBT 29	IV	Yes	Yes		
NBT 30	III	No	no		
NBT 31	IV	Yes	yes		
NBT 32	IIb	No	No		
NBT 33	IIa	Yes	No		
NBT 34	III	Yes	yes		
Cell lines					
STA-NB 3		Yes	Yes	↑	↑
STA-NB 8		No	Yes	↓	↑
STA-NB 9		Yes	Yes	↓	↑
STA-NB 11		Yes	Yes		
IMR 32		Yes	Yes	↓	↑
LAN 1		Yes	No		
KCNR		No	Yes	↓	↑
NGP127		No	Yes	↓	↑
SHEP		No	No	No change	No change
SKNAS		Yes	No	↓	No change
SKNSH		No	No	No change	No change
SY5Y		No	No	No change	No change

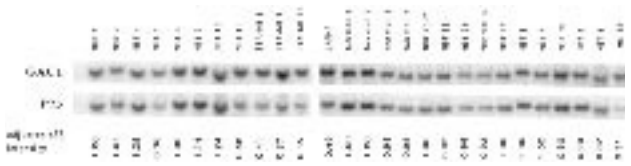

Figure 3. *Southern blot analysis of p73 in tumors and cell lines. The filters were also hybridized with a probe for a gene (GAC1) also located on chromosome 1. The adjusted ratio of intensity of the p73 band relative to the GAC1 is indicated. The designation NBT refers to tumors. Other designations refer to cell lines and four normal DNA samples.*

Relationship Between Expression of p73 and of N-myc

We relied on semiquantitative RT-PCR to study the expression of *p73* and N-*myc* in 19 tumors, 4 of which contained *p73* deletion. *p73* and N-*myc* expression was also investigated in 10 cell lines. The results are summarized in Table 1. RNA from fetal adrenal and brain was used as a reference for judging the intensity of RT-PCR–derived N-*myc* and *p73* products. Double primers for N-*myc* and *p73* were used in the same PCR reactions for each sample to better allow comparison of the relative abundance of N-*myc* and *p73* (Figure 4). Additionally, PCR analysis of *p73* and of N-*myc* was undertaken using internal primers for ubiquitin as control. Analysis of normal fetal adrenal and brain tissue, used as controls, yielded a *p73* ratio of approximately 3 to 1. A majority of neuroblastomas analyzed (15 of 19 tumors), including the 4 tumors with *p73* deletion, showed decreased expression of *p73*. Likewise, 7 of 10 cell lines including four cell lines analyzed that had *p73* deletion, showed decreased expression of *p73*. There was a statistically significant inverse correlation between *p73* and N-*myc* levels as detected by RT-PCR (P=.03, 1-tailed Fisher's exact test). Increased expression of N-*myc* was observed in 11 of 19 tumors, only 4 of which had N-*myc* gene amplification. Likewise, increased expression of N-*myc* was observed in 6 of 10 cell lines, all 6 of which had N-*myc* gene amplification. In most tumors and cell lines, there was an obvious inverse correlation between *p73* and N-*myc* RNA levels (Figure 4). One noticeable exception was cell line STA-NB3, in which both N-*myc* and *p73* were highly expressed. In one tumor (NBT 24) and one cell line (SKNAS) reduced *p73* expression was associated with *p73* deletion in the absence of N-*myc* overexpression. The decreased *p73* expression in the other tumors and cell lines was associated with N-*myc* overexpression with or without *p73* deletion.

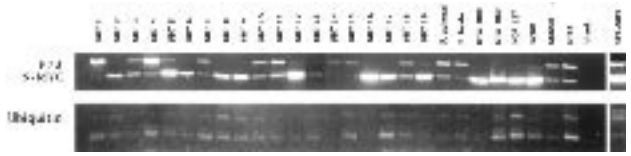

Figure 4. *RT-PCR analysis of p73 and N-myc expression in neuroblastoma tumors and cell lines. Ubiquitin RT-PCR products were used as control. A fetal adrenal and a fetal brain are included for reference, and the band intensity of their RT-PCR products for p73, N-myc, and ubiquitin were used as controls.*

Reduced p73 Expression in Neuroblastoma Cell Lines Following N-myc Transfection

Two neuroblastoma cell lines, SH-EP1 [26] and SK-N-AS [27], were stably transfected with the N-*myc* gene. These cell lines were chosen as they are not N-*myc* amplified. One cell line (SK-N-AS) contained a *p73* deletion, while SH-EP1 contained two *p73* gene copies. *p73* and N-*myc* expression in the N-*myc* transfected cell lines was examined by semiquantitative RT-PCR. After N-*myc* transfection, both cell lines contained increased amount of N-*myc* mRNA and protein (Figure 5*A*). *p73*, which was detectable at low level in untransfected SK-N-AS, becomes undetectable following N-*myc* transfection, whereas in SH-EP1 cells, *p73* levels were substantially

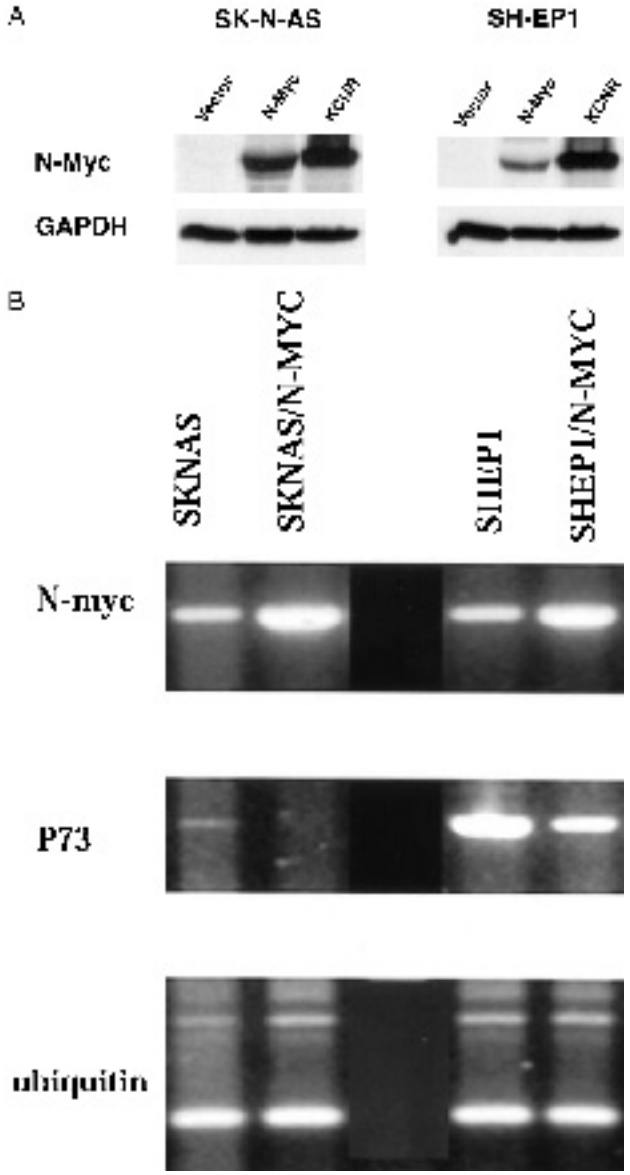

Figure 5. *(A) N-Myc expression by Western analysis in SH-EP1 and SK-N-AS transfectants. The KCNR neuroblastoma cell line with amplified N-myc was used as a positive control. Equivalent protein loading is confirmed by GAPDH detection. (B) Reduced intensity of p73 RT-PCR products in SK-N-AS and SH-EP1 transfectants expressing N-Myc.*

reduced following transfection but remained detectable (Figure 5*B*).

Discussion

The identification of *p73* as a novel gene with significant homology to *p53* has evoked the possibility that loss of *p73* function may play a role in neuroblastoma and other cancers that exhibit 1p36 deletion. Evidence that *p73* is a candidate tumor-suppressor gene in neuroblastoma included demonstration that transfection of the *p73* gene into a neuroblastoma cell line that lacks detectable *p73* protein resulted in growth suppression and induced neuronal differentiation. [1,14] However, there has been a paucity of evidence for mutation based inactivation of *p73* in neuroblastoma [5,6,28] and a lack of homozygously inactivated *p73* in neuroblastoma tumors with a single copy N-*myc* gene [13,29,30]. Nevertheless, a number of studies have shown reduced expression of *p73* in neuroblastoma [5,6,11,29]. In one study of 28 neuroblastoma tumors, *p73* expression levels were found to vary widely among tumors [6]. Given the potential tumor suppressor role of *p73* in neuroblastoma and the demonstrated role of the N-*myc* oncogene in neuroblastoma carcinogenesis, it was of interest to determine the genomic and expression status of both genes in neuroblastoma tumors and cell lines. Our prior quantitation studies of fragment intensities in RLGS patterns have demonstrated a high measure of sensitivity of fragment intensity to gene dosage changes [25]. Approximately a third of neuroblastomas in our series, exhibited deletion of *p73* as could be ascertained by analysis of the intensity of the *p73* genomic fragment in RLGS patterns and by Southern blot analysis. This frequency is comparable to the frequency of 1p deletions as determined by Southern analysis reported by others [31]. None of the tumors exhibited a complete absence of the *p73* genomic fragment in RLGS patterns. Likewise, *p73* remained detectable in Southern blots of tumors. Whether this is related to lack of homozygous deletions or to contaminating normal tissue could not be determined. It should be pointed out, however, that neuroblastoma cell lines also did not exhibit a complete absence of *p73* genomic DNA by RLGS and Southern analysis. In our study, some tumors exhibited reduced expression of *p73* in the absence of an obvious deletion, leading us to determine the relationship between *p73* expression and N-*myc* status.

The reduced expression of *p73* correlated with overexpression of N-*myc*. This inverse correlation was observed in both tumors and cell lines, a majority of which exhibited high expression of N-*myc* and decreased expression of *p73*. High expression of N-*myc* was not limited to tumors and cell lines with N-*myc* gene amplification. Other groups have previously reported high-level expression of N-*myc* in some neuroblastomas without N-*myc* gene amplification [32,33]. Our data suggested that *p73* levels may correlate with N-*myc* expression rather than N-*myc* amplification status. It was, therefore, of interest to demonstrate the effect of manipulating N-*myc* levels on *p73* expression. Transfection of N-*myc* into two neuroblastoma cell lines resulted in a readily detectable decrease in *p73* RNA levels, providing *in vitro* evidence that N-*myc* downregulates *p73* expression. Our data suggest that as part of its normal function, the N-*myc* gene may inhibit *p73* expression, thus preventing *p73*-induced growth suppression. This inverse relationship between N-*myc* and *p73* appears to be maintained in some neuroblastoma tumors in the absence of either N-*myc* amplification or *p73* deletion. Expression of either gene may remain subject to regulatory control in tumors that do not contain *p73* or N-*myc* genomic alterations. However, N-*myc* genomic amplification, with or without *p73* deletion results in N-*myc* overexpression and downregulated levels of *p73* that are characteristic of aggressive tumors.

The initial studies of *p73* suggested that it may be a candidate for the imprinted tumor-suppressor gene at 1p36, based on its frequent deletion and monoallelic expression [1]. Allele-specific methylation is a common feature of imprinted genes [34]. In addition, tissue-specific imprinting correlates with differential allele-specific DNA methylation [35]. In our study, the use of the methylation-sensitive enzyme *Not*I for both RLGS analysis and Southern analysis allows assessment of methylation at the *Not*I site. There was no evidence in either tumors or normal DNA of any methylation at the *Not*I cut site, which is located in a part of the *p73* promoter, close to exon 1. Thus, it is likely that any methylation that affects allele specific expression of the *p73* gene is located elsewhere in the gene.

It remains to be determined whether *p73*, through its inactivation, plays a role in neuroblastoma development, independent of N-*myc* overexpression. Several studies to date have failed to detect *p73* point mutations more than occasionally [5,36,37]. One cell line (STA-NB3) in our study contained an amplified and overexpressed N-*myc* gene, yet expressed *p73* at a high level. We amplified a 1.1-kb *p73* genomic DNA fragment from this cell line and sequenced three clones containing *p73* from this tumor. No sequence changes were detectable. The high-level expression of *p73* in a small number of neuroblastoma tumors and cell lines may be related to activation of the *p73* silent allele, as has been recently reported to occur in lung cancer [38].

It should be noted that in a recent study [39], the c-*myc* gene was found to upregulate *p73* expression, while in our study a downregulation by N-*myc* was observed. Therefore, there is a need to reconcile these observations given the overlap among c-*myc* and N-*myc* target genes. These opposite findings may relate to cell type and differentiation state. Pertinent to our observations in neuroblastoma are prior reports that are concordant with our findings. It is well known that retinoic acid induces neuronal differentiation of neuroblastoma cells. In one study, treatment of neuroblastoma cells with retinoic acid was found to induce *p73* [14] and in prior studies, treatment with retinoic acid was found to decrease N-*myc* levels [40]. These findings do indicate a negative correlation between N-*myc* levels and *p73* in neuroblastoma, which is consistent with our findings.

The N-*myc* gene is known to affect a variety of signaling pathways [41,42]. An inhibitory effect of N-*myc* on the expression of certain genes has previously been reported. Overexpression of the N-*myc* gene in a rat neuroblastoma cell line following gene transfer resulted in reduced expression of class I histocompatibility antigens [43]. Overexpression of transfected N-*myc* in the human SKNSH neuroblastoma cell line resulted in reduced expression of beta 1 integrin [44]. Thus, it appears that the ability of the N-*myc* gene to induce neuroblastoma formation, through its overexpression, is related to its ability to positively, as well as negatively, control the expression of a large number of target genes that play a role in neuroblast proliferation and differentiation.

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