

p16/p14^{ARF} Cell Cycle Regulatory Pathways in Primary Neuroblastoma: p16 Expression Is Associated with Advanced Stage Disease¹

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ABSTRACT

p16 regulates the G₁-S cell cycle transition by inhibiting the cyclin D-cyclin-dependent kinase (CDK)4/CDK6-mediated phosphorylation of retinoblastoma protein (pRb). We examined the possible derangement of the p16-CDK/cyclin D-pRb pathway in 40 primary neuroblastomas including 18 samples in the unfavorable stages (C and D) and 22 in the favorable stages (A, B, and Ds) by PCR, reverse transcription-PCR, Western blot, and immunohistochemistry and correlated the results with clinical outcome. No samples harbored alterations of the *p16* gene. Interestingly, the samples in the unfavorable stages exhibited expression of p16 mRNA and protein more frequently than those in the favorable stages [mRNA, 9 of 18 (50%) versus 2 of 22 (9%), *P* = 0.006; protein, 5 of 16 (31%) versus 0 of 18 (0%), *P* = 0.013]. Alterations of the downstream components of the pathway were infrequent. pRb was deregulated in the majority of samples investigated [27 of 33 (82%), 24 with hyperphosphorylated pRb and 3 with no pRb protein]. The phosphorylation status of pRb did not correlate with p16 protein expression, suggesting that the elevated p16 protein may not be functioning properly to regulate the pathway. Among patients of all stages, p16 expression was significantly associated with a lower overall survival. There was no overex-

pression of MDM2, and loss of p14^{ARF} expression and *p53* mutation were infrequent events. Taken together, these findings suggest that up-regulated p16 expression may represent a unique feature of aggressive neuroblastoma.

INTRODUCTION

Neuroblastoma is one of most common solid tumors in children and has a wide spectrum of clinical and biological features. Prognosis of patients with advanced disease (stages C and D) remains poor despite intensive multimodal treatment including bone marrow transplantation, whereas most patients with localized disease (stages A and B) and stage Ds disease can be cured. A young age at diagnosis is also known to be associated with a favorable outcome (1, 2). Furthermore, tumors that develop in infants often undergo spontaneous regression or maturation to benign GN³ (3). Amplification of the *N-myc* proto-oncogene is well known as one of the unique genetic changes in neuroblastoma and a negative predictor for prognosis (4, 5). Deletion of the short arm of chromosome 1 is also frequently found in unfavorable-stage neuroblastoma (6, 7). The exact nature of gene alterations in this region, however, remains elusive despite intensive investigation. Few neuroblastoma cell lines and primary samples harbor alterations of the major tumor suppressor genes, *p53* and *p16*, which are affected in a variety of human tumors (8–11). Overall, molecular mechanisms of the pathogenesis or progression of neuroblastoma and the relationship between molecular and clinical features other than the *N-myc* oncogene remain to be delineated.

The p16-CDK/cyclin D-pRb pathway plays a critical role in cell cycle progression (12, 13). Protein complexes of D-type cyclins and CDKs (CDK4 and CDK6) induce the phosphorylation of pRb to promote the G₁-S-phase transition (14, 15). The phosphorylated pRb releases transcriptional factors such as E2F, which activate the expression of genes essential for S-phase entry (16). CDK inhibitor proteins, including p16, play critical roles in the G₁-S cell cycle transition by inhibiting the cyclin D-CDK4/6-mediated pRb phosphorylation (17, 18). Alterations of any component of the pathway, such as deletion/mutation of the *p16* gene (19–21), amplification/overexpression of CDKs or D cyclins (22–24), and mutations to CDKs that affect p16 binding (25, 26), result in pRb phosphorylation and subsequent progression of G₁ into S-phase. Similarly, alterations of *pRb* itself may also lead to G₁-S-phase transition (27, 28). These alterations have been found frequently in various human tumors,

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³ The abbreviations used are: GN, ganglioneuroma; CDK, cyclin-dependent kinase; pRb, retinoblastoma protein; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAb, polyclonal antibody; POG, Pediatric Oncology Group.

suggesting that inactivation of the pathway p16-CDK/cyclin D-pRb may play an important role in their pathogenesis.

We and others (8, 11, 29–32) have reported deregulation of the p16-CDK/cyclin D-pRb pathway in neuroblastoma. In these studies, almost all of the neuroblastoma cell lines and primary tumors were shown to retain the wild-type *p16* gene. Interestingly, we have demonstrated that 7 of 19 neuroblastoma cell lines displayed very high *p16* expression at both the mRNA and protein levels (33). In addition, a preliminary study of 6 primary neuroblastoma samples revealed elevated *p16* expression in 3 samples, suggesting that our finding from cell lines may represent a general feature of neuroblastoma *in vivo*. We have also demonstrated that in neuroblastoma cell lines, alterations in components downstream of *p16*, which may negate the regulatory effect of p16, were limited to infrequent *CDK4* gene amplification and *cyclin D2* expression. All 19 cell lines exhibited various degrees of phosphorylated pRb protein; *p16* expression was independent of the pRb phosphorylation status (33). These results suggest that the elevated p16 protein may not be functioning properly to regulate the pathway and that *p16* transcription is induced by a pRb-independent mechanism, in contrast to a pRb/p16 feedback regulatory loop that has been reported in other cancers (21, 34, 35).

The *p16INK4a* locus has been found to also encode a second protein, p14^{ARF}, with a distinct reading frame (36–38). Recent studies have demonstrated that p14^{ARF} regulates the cell cycle progression through an interaction with p53 and MDM2 (39–41); it physically interacts with MDM2 and blocks both MDM2-induced p53 degradation and transcriptional silencing of *p53* (42). Similar to the p16-CDK/cyclin D-pRb pathway, the p14^{ARF}-MDM2-p53 pathway appears to also be inactivated in human cancers through alteration of various components such as deletion and silencing of *p14^{ARF}*, amplification of *MDM2*, or *p53* mutation (42, 43). The deregulation of this novel cell cycle pathway in neuroblastoma has not been explored yet.

In this study, we hypothesized that elevated *p16* expression or other alterations of the p16-CDK/cyclin D-pRb pathway may have correlations with clinical features and patient outcome. To address this hypothesis, we investigated the alterations of the pathway in 40 primary neuroblastomas at different clinical stages. We also performed a preliminary analysis of the p14^{ARF}-MDM2-p53 pathway. Such information may offer a better understanding of the molecular biology of neuroblastoma and help to identify patients at high risk of treatment failure.

MATERIALS AND METHODS

Primary Samples. Forty primary neuroblastoma samples collected at the time of diagnosis were obtained from the POG Neuroblastoma Tumor Bank. The distribution by stage of the samples (28% A, 12% B, 17% C, 28% D, and 15% Ds) is fairly representative of the distribution of stages across the general population of patients with neuroblastoma. The clinical stages were classified as favorable stage (A, B, and Ds) or unfavorable stage (C and D) based on POG staging criteria. Altogether, there were 18 samples in the unfavorable stage and 22 in the favorable stage. The majority of samples contained 80–90% or more tumor cells. Two GN samples were also obtained from the POG Neuroblastoma Tumor Bank. Tumor

samples were collected as part of the Neuroblastoma Biology Study POG9047 after informed consent was obtained.

DNA, RNA, and Protein Extraction. Frozen samples were crushed over dry ice and homogenized in Trizol (Life Technologies, Inc., Gaithersburg, MD). RNA, DNA, and protein were extracted according to the manufacturer's protocol and quantified by using a spectrophotometer. Two μg of RNA were reverse transcribed into cDNA by using the Superscript preamplification system (Life Technologies).

PCR. PCR amplifications were performed by using 50 ng of genomic DNA or 1–2 μl of cDNA in a 50- μl reaction volume. Primers and conditions for semiquantitative PCR and RT-PCR for *p16*, *GAPDH*, *CDK4*, *CDK6*, and *cyclin D2* and mutation analysis by PCR-single-strand conformational polymorphism and sequencing for *p16*, the *CDKs*, and *p53* were as we described previously (33, 44). For PCR analysis of *p16* in this study, 28 cycles of amplification were used to avoid possible overestimation because of contamination with surrounding nonneoplastic tissue. Other primers used were as follows: *p14^{ARF}* cDNA, 5'-CTGGAGGGCGGCGAGAACA-3' (sense) and 5'-CTACGAAAGCGGGGTGGGTTGT-3' (antisense); and *MDM2* cDNA, 5'-CTGGGGAGTCTTGAGGGACC-3' (sense) and 5'-CAGGTTGTCTAAATTCCTAG-3' (antisense). The *p14^{ARF}* amplifications were performed in 10 mM Tris-HCl (pH 9.1), 25 mM KCl, 1.5 mM MgCl₂, 50 μM deoxynucleotide triphosphate mix and 1% DMSO, with 10 pmol of each primer and 1.25 units of Taq DNA polymerase. The *MDM2* amplifications were performed in 1 \times High Fidelity PCR buffer [60 mM Tris-SO₄ (pH 8.9), 18 mM (NH₄)₂SO₄ (Life Technologies), 2 mM MgSO₂, 80 μM deoxynucleotide triphosphate mix and 2% DMSO], with 10 pmol of each primer and 0.5 unit of High Fidelity Taq DNA polymerase (Life Technologies). For p14^{ARF}, reactions were cycled with a 3-min initial denaturation at 95°C, followed by 31 30-s cycles of denaturation at 95°C, annealing at 60°C, and extension at 72°C. For *MDM2*, reactions were cycled with a 2-min initial denaturation at 95°C, followed by 36 30-s cycles of denaturation at 95°C, annealing at 58°C, and extension at 68°C. PCR products were resolved on a 2% agarose gel in 1 \times TAE (40 mM Tris-acetate and 1 mM EDTA). Gene expression levels were estimated as – to +++ by two of the authors independently. Alternatively, signal intensities of the bands of *p16* and *GAPDH* were measured by using densitometry, and the *p16* expression was estimated by using the ratio of these intensities. Each RT-PCR was performed at least in triplicate.

Western Blot Analysis for pRb, p16, and β -Actin. For protein analysis, we used 20–50 μg of protein in lysate prepared from samples. Western blot analysis for p16, pRb, CDK4, and β -actin was as described previously (33). Two different antibodies were used for p16: 0.25 $\mu\text{g}/\text{ml}$ mouse monoclonal anti-human p16 monoclonal antibody ZJ11 (NeoMarker, Fremont, CA) and 0.5 $\mu\text{g}/\text{ml}$ rabbit polyclonal antihuman p16 PAb C-20 (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunohistochemical Staining for p16 Protein. Immunohistochemical staining was performed on 2- μm sections cut from frozen tumors embedded in OCT as described previously for neuroblastoma cell lines (33) with slight modification. Briefly, after the tumor section was fixed in 1:1 ethanol:acetone, endogenous peroxidase activity was quenched in 0.03% H₂O₂ in PBS for 30 min at room temperature. After treatment with

blocking solution, the slide was incubated with rabbit antihuman p16 PAb (C-20; Santa Cruz Biotechnology) at 1.0 $\mu\text{g/ml}$ for 1 h at room temperature. Whereas immunodetection was performed by using the Supersensitive Multilink system (BioGenex, San Ramon, CA) as described previously, 0.5 $\mu\text{g/ml}$ normal human serum was added to the secondary antibody solution to reduce nonspecific antibody binding. As a negative control for immunohistochemistry, the primary antibody was replaced with rabbit immunoglobulin or PBS. The osteosarcoma cell line SJ-SA 1 and neuroblastoma cell line Be2c/ADR5 were used as positive and negative controls, respectively, for immunohistochemical analysis (33). All of the samples were evaluated by a pathologist who was blind to the result of *p16* RT-PCR and Western blot analysis as well as the clinical information of the patients.

Statistical Analysis. Gene analysis and clinical features of the patients were examined by using Fisher's Exact Test. The survival curves for each group of the patients were estimated by using the Kaplan-Meier method, and the resulting curves were compared by using the generalized Wilcoxon test.

RESULTS

Analyses of the p16-CDK/cyclin D-pRb pathway were performed in 40 primary neuroblastoma samples, which included 6 samples that had been reported previously as to their gene status and mRNA expression of *p16*, *CDK4*, and *cyclin D2* (33). Two primary GNs, which are known as a benign counterpart of neuroblastoma, were also examined. All of the results are summarized in Table 1.

p16 Gene Status and Expression at mRNA and Protein Levels. Alteration of the *p16* gene is rare in neuroblastoma (8, 11, 29, 31, 32). Consistent with previous reports, neither deletion nor mutation of the *p16* gene was found in any of the 40 primary tumors (data not shown).

Next, *p16* mRNA expression was examined by using RT-PCR. The results are summarized in Table 1, and representative samples are shown in Fig. 1. Eleven of the 40 primary samples expressed significant levels of *p16* (scored + to +++ in Table 1). Expressions of the remaining 29 samples were barely detectable or undetectable (scored +/- or -). Interestingly, 9 of the 18 (50%) unfavorable-stage samples expressed *p16* (e.g., Fig. 1, Lanes 11 and 13-15), whereas only 2 of the 22 (9%) in the favorable stage did so (e.g., Fig. 1, Lanes 3 and 8). The difference in the rate of *p16* mRNA expression between unfavorable and favorable stages was statistically significant (Fisher's Exact Test, $P = 0.006$; Table 2). Both of the two primary GNs expressed *p16* mRNA (e.g., Fig. 1, Lane 2).

p16 protein expression was examined by using Western blot analysis. Sufficient tissue was available from 34 of the 40 tumors (16 unfavorable-stage and 18 favorable-stage tumors). The results are summarized in Table 1, and representative samples are shown in Fig. 2. Only 5 of the 34 samples (1723, 3163, 4316, 3169, and 1176; Fig. 2, Lanes 3-5, 8, and 9) exhibited both mRNA and protein expression of p16. All of these samples were obtained from unfavorable-stage patients [5 of 16 (31%)], whereas none of the favorable-stage samples [0 of 18 (0%)] expressed p16 protein (e.g., Fig. 2, Lanes 10, 11, 14, and 15). The difference in the rates of p16 protein expression between unfavorable and favorable stages was also statistically significant

(Fisher's Exact Test, $P = 0.013$; Table 2). Consistent with our finding in neuroblastoma cell lines (33), expression of p16 mRNA and protein correlated in most primary samples (Table 1). However, 4 samples (1367, 2076, 4098, and 253) that expressed low levels of p16 mRNA (scored +) did not have detectable p16 protein expression (e.g., 4098; Fig. 2, Lane 6), a discrepancy that may be attributable to the known higher sensitivity of RT-PCR than that of Western blot analysis. Expression of p16 mRNA or protein did not correlate with either *N-myc* amplification or an age of >1 year at diagnosis. Two GNs that expressed p16 mRNA showed barely detectable levels of the protein (data not shown).

RT-PCR and Western blot analysis of p16 expression in primary tumor samples may be attributable to contamination from surrounding nonneoplastic stromal cells. Therefore, we examined the localization of p16 protein by using immunohistochemical analysis in 7 samples in which sufficient quantities of specimen were available. The representative pictures of staining are shown in Fig. 3. Three samples that expressed p16 protein as determined by using Western blot (1723, 3163, and 1176) also demonstrated strong p16 staining in the majority (60-90%) of tumor cell nuclei by using immunohistochemistry (e.g., 1723 and 3163; Fig. 3). Of the remaining 4 samples, 3 (839, 3031, and 3072) exhibited very little or no p16 staining, consistent with the results of RT-PCR and Western blot analysis (e.g., 3072 and 839; Fig. 3). In the remaining 1 sample (1767), however, there was a discrepancy between the protein expressions detected by two methods; this sample exhibited moderate p16 staining in the nuclei by immunohistochemistry (data not shown) despite undetectable levels of p16 expression with RT-PCR and Western blot analysis. Analysis of this sample by using Western blot for β -actin, as well as for pRb, assured us that the discrepancy was not attributable to degradation of the protein extract from this tumor. Because it may be attributable to cross-reactivity of the PAb, the p16 status of this sample was regarded as negative in the analysis of clinical correlation.

Clinical Outcome and p16 Expression. To determine the prognostic significance of p16 expression, we examined the relationship between p16 expression and patient survival. The information on patients' outcome was available for 37 of the 40 samples. In contrast to the understood role of p16 as a tumor suppressor gene, expression at both the mRNA and protein levels was paradoxically associated with a significantly lower rate of survival when patients of all stages are included in the analysis (Fig. 4, A and C; $P = 0.018$ for mRNA and 0.002 for protein, respectively). Because we observed a significantly higher rate of p16 expression in unfavorable-stage than favorable-stage neuroblastoma samples, we sought to determine whether p16 expression status correlates with survival among unfavorable-stage neuroblastoma. In this analysis, there was a trend for the patients expressing p16 to have a poorer prognosis than those patients not expressing p16. However, the difference did not reach statistical significance (Fig. 4, B and D; $P > 0.3$ for mRNA, $P = 0.1$ for protein), perhaps because of small sample sizes.

Downstream Component of p16. Alterations of downstream components of the pathway, such as overexpression of cyclin D1-3, CDK4/6, and E2Fs, may negate the regulatory effect of p16 and could drive pRb phosphorylation leading to

Table 1 p16-CDK/cyclin D-pRb

ID	Stage	N-myc	Age at diagnosis (yr)	p16 ^a			cycD2 mRNA ^a	CDK4 mRNA ^a	pRb status ^c	p14 ^{ARF} mRNA ^a	MDM2 mRNA ^a	p53 DNA
				mRNA	protein	IH ^b						
1632	D	NA	1.2	-	-		-	+	no Rb	+	+	wt
1723	D	AMP	1.4	+	++ ^d	++++/90%	+/-	+	hyper	++	+	wt
1725	D	NA	1.03	+/-	+/-		+	+	hypo	+	+	wt
1785	D	NA	5.1	++	na		+	+	na	+	-	wt
3163	D	NA	12.0	+	++ ^d	++++/80%	-	+	hyper	+	+	wt
3169	D	NA	4.2	++	+++		+	+	na	+	+	wt
4013	D	AMP	0.6	+/-	-		+	+	hyper	++	+	wt
4152	D	NA	5.2	+/-	+/-		+/-	+	hypo	+	+/-	mut
4177	D	AMP	0.0	-	-		+/-	+	hyper	+	+	wt
4316	D	AMP	4.2	++	+		+/-	+	hyper	++	+	wt
4348	D	AMP	1.0	+/-	+/-		+	+	hypo	++	+	wt
839	C	AMP	2.8	+/-	- ^d	-	+/-	+	hyper	+	+	wt
1176	C	AMP	3.8	+++	+++ ^d	+++++/60%	+/-	+++	hyper	++	+	wt
1307	C	NA	2.8	+/-	-		+/-	+	hyper	-	+	wt
1367	C	NA	1.1	+	+/-		+	+	no Rb	+	+	wt
2076	C	NA	1.5	+	-		+	+	hyper	++	+	wt
2095	C	NA	0.2	-	na		+	+	na	++	+	wt
4098	C	AMP	2.8	+	-		+	+	hyper	+	+	wt
1898	B	NA	5.8	+/-	+/-		+	+	hyper	+	+	wt
4133	B	NA	0.3	+/-	-		+	+	hyper	+	+	wt
4145	B	NA	1.9	-	+/-		+	-	hyper	+	-	wt
4175	B	NA	5.8	+/-	+/-		+	+	hyper	++	+/-	wt
4349	B	NA	0.1	-	-		+	+	hyper	+	+	wt
nANW	A	NA	2.5	-	na		+	+	na	+/-	+	wt
253	A	NA	0.98	+	-		+	+	hypo	+	+	wt
2090	A	NA	0.0	-	na		+	+	na	+	+	wt
3031	A	NA	10.4	+/-	+/- ^d	+/20%	+	+	hyper	++	+	wt
3066	A	NA	0.1	+/-	-		+	+	hypo	+	+	wt
3072	A	NA	12.2	+/-	- ^d	+/5%	+	+	no Rb	+	+	mut
3059	A	NA	1.4	+/-	+/-		-	+	hyper	+	+	wt
3124	A	NA	0.1	+/-	+/-		+	+	hyper	++	+	wt
4052	A	NA	1.4	+/-	+/-		+/-	+	hypo	+	+/-	wt
4111	A	NA	0.7	-	+/-		+	+	hyper	++	+	wt
4142	A	NA	3.6	+/-	+/-		+/-	+	hyper	+	+	wt
1767	Ds	NA	0.1	-	- ^e	+++/80%	-	+	hyper	+	+	wt
2025	Ds	NA	0.6	+/-	-		+	+	hyper	+	+	wt
3087	Ds	NA	0.0	-	-		+	+	hyper	+	+	wt
4082	Ds	NA	0.3	+	na		+	+	na	++	+	mut
4301	Ds	NA	0.0	-	-		-	+	hyper	++	+	wt
4423	Ds	NA	0.1	+/-	na		+	+	na	+	+	wt
595	GN			++	+/-		+	+	hypo	+	+/-	wt
516	GN			+	+/-		+	+	hypo	+	+/-	wt

^a Expression: -, undetectable; +/-, barely detectable; +, low; ++, moderate; +++, high.

^b IH, immunohistochemistry; AMP, amplification; NA, no amplification; na, not available; cycD2, cyclin D2; wt, wild type; mut, mutant.

^c Phosphorylation status of Rb; hyper, hyperphosphorylated Rb; hypo, only hypophosphorylated Rb.

^d The results were consistent with those of immunohistochemistry.

^e Discrepancy between the results of Western blot analysis and immunohistochemistry.

G₁-S deregulation. In 19 neuroblastoma cell lines, these alterations were limited to rare *CDK4* gene amplification (2 of 19) and infrequent cyclin D2 expression (2 of 19; Ref. 33). Thus, expressions of these two genes were investigated in the 40 primary neuroblastomas. The results are summarized in Table 1, and representative samples are shown in Fig. 5A.

Gene expression of *CDK4* was examined by using RT-PCR (Fig. 5A). As reported in our previous study, 1 sample (1176; Fig. 5A, Lane 11) displayed very high levels of *CDK4* expression that could be detected even at low cycles of amplification. The level of *CDK4* expression in this sample was similar to those in neuroblastoma cell lines with amplification of this gene (33). One sample (4145, stage B) lacked detectable

CDK4 mRNA expression (data not shown). Expression levels of the remaining 38 samples were somewhat variable, with none exhibiting the gross elevation of expression as was observed in *CDK4* gene-amplified cell lines. Western blot analysis for *CDK4*, performed in 17 of the 38 samples of which sufficient protein was available, demonstrated comparable protein expression among samples (data not shown).

Using RT-PCR, we examined the 40 samples for mRNA expression of cyclin D2 (Fig. 5A). In contrast to the infrequent cyclin D2 expression observed in neuroblastoma cell lines, 26 of the 40 primary samples (60%) displayed significant levels of cyclin D2. The difference in the rates of cyclin D2 expression between unfavorable and favorable stages was not statistically

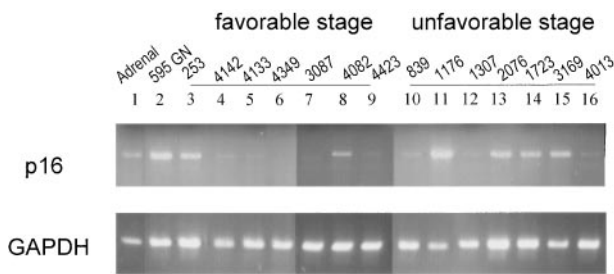


Fig. 1 Expression of *p16* mRNA in primary neuroblastoma. The mRNA expression of *p16* in neuroblastoma was determined by using RT-PCR. All of the levels of gene expression are in comparison with that of *GAPDH*. Lane 1, normal adrenal gland (Adrenal); Lane 2, GN; Lanes 3–16, neuroblastoma tumors obtained at different stages. The *p16* mRNA levels were determined as a ratio to *GAPDH* mRNA.

significant [9 of 18 (50%) versus 17 of 22 (77%)]; Fisher's exact test, $P = 0.1$).

Mutation to the p16-binding site of CDK4 or CDK6 is another mechanism to deregulate the p16-CDK/cyclin D-pRb pathway (25, 26). Easton *et al.* (30) have demonstrated a CDK6 mutation in a neuroblastoma cell line that disrupts p16 binding to CDK6 and prevents p16 from inhibiting CDK6 protein kinase activity. However, in our study, no mutation to the p16-binding site of CDK4 or CDK6 was detected in any of the 40 primary samples. Furthermore, no CDK4 or CDK6 mutations were detected in any of the 19 neuroblastoma cell lines used in our previous study (33; data not shown), which included the cell line reported by Easton *et al.* (30) to harbor a CDK6 mutation, suggesting that this may be a cell culture-acquired mutation.

pRb Phosphorylation of Primary Neuroblastoma.

Lack of p16 is expected to result in the hyperphosphorylation of pRb, the end point of the G_1 -S cell cycle progression. In neuroblastoma samples, which retain p16 protein expression, pRb deregulation either by hyperphosphorylation or by loss of pRb protein may also lead to a deregulation of G_1 -S transition. To investigate the relationship between p16 and pRb status, we examined pRb protein expression in 33 primary neuroblastomas, which were also examined for p16 protein with the exception of 3169. Among these 33 samples, 29 did not express p16 protein, whereas 4 retained expression as determined by using Western blot analysis. The results are summarized in Table 1, and representative samples are shown in Fig. 6. Of the 33 samples, 24 displayed significant levels of pRb protein in the hyperphosphorylated state (*e.g.*, Fig. 6, Lanes 4–6 and 8–10). All 4 samples expressing p16 protein displayed significant levels of the hyperphosphorylated form of pRb (*e.g.*, Fig. 6, Lanes 4–6). Of the remaining 9 samples, 6 expressed predominantly hypophosphorylated pRb despite the absence of p16 protein (*e.g.*, Fig. 6, Lanes 2 and 7) and 3 had no detectable pRb protein (1632, 1367, and 3072 at stages D, C, and A, respectively; *e.g.*, 1632; Fig. 6, Lane 1). When pRb hyperphosphorylation ($n = 24$) and loss of pRb protein ($n = 3$) were combined, the majority of samples [27 of 33 (82%)] investigated displayed pRb deregulation. The difference in the rates of pRb deregulation between unfavorable and favorable stages was not statistically significant [12 of 15 (80%) versus 15 of 18 (83%); Fisher's Exact Test, $P = 1.0$].

Table 2 Relationship between *p16* gene expression and clinical stages

	<i>p16</i> mRNA (+) ^a	<i>p16</i> protein (+) ^a
Unfavorable stage ^b	9/18 (50%)	5/16 (31%)
Favorable stage	2/22 (9%)	0/18 (0%)
	$P = 0.006$	$P = 0.013$

^a Statistical analysis, Fisher's Exact Test.

^b Unfavorable stage, stages C and D; favorable stage, stages A, B, and Ds.

Although a correlation between pRb deregulation and *p16* transcription has been observed in other types of cancer (21, 34, 35), there was no such relationship in the 33 primary neuroblastomas (Fisher's Exact Test, $P = 1.0$). The two GN samples displayed only very low levels of the hypophosphorylated form of pRb (*e.g.*, 595; Fig. 6, Lane 11), suggesting that cell cycle progression was properly regulated, which is consistent with the benign and self-limited proliferation of GN.

p14^{ARF}-MDM2-p53 Pathway in Primary Neuroblastoma. We also examined each component of the p14^{ARF}-MDM2-p53 pathway in the 40 neuroblastoma samples. Consistent with previous reports (9, 10), gene alteration of *p53* was infrequent in the primary neuroblastoma; only 3 of the 40 samples harbored *p53* mutations [CGT273CTT (Arg-Leu) in sample 3072, stage A and CGC283TGC (Arg-Cys) in samples 4145 and 4082, stage B and Ds, respectively (data not shown)]. Expression of p14^{ARF} and *MDM2* were investigated by using RT-PCR (Fig. 5B). In contrast to *p16*, p14^{ARF} was expressed in all except 1 sample (1307, stage C; Fig. 5B, Lane 12), although expression was somewhat variable among samples. *MDM2* expression was also variable among samples (Fig. 5B), with 1 low-stage and 1 high-stage sample lacking detectable expression of the *MDM2* gene (Table 1). The absence of any sample expressing very high levels of *MDM2* suggests that *MDM2* gene amplification, observed in some neuroblastoma cell lines, was not present in any of these tumor samples. There was no apparent correlation between the expression levels of either p14^{ARF} or *MDM2* and disease stage.

DISCUSSION

This is the first report of a systematic analysis of the entire p16-CDK/cyclin D-pRb cell cycle regulatory pathway as well as the p14^{ARF}-MDM2-p53 regulatory pathway in primary neuroblastoma. We demonstrated a significant correlation between *p16* expression and clinical stage in primary neuroblastoma; samples in the unfavorable stage expressed *p16* more frequently than those in the favorable stage. In general, neuroblastoma cell lines are established from tumors of the unfavorable stages, which tend to be those with an aggressive phenotype (45). Consistent with this, the rates of p16 expression in neuroblastoma samples in unfavorable stages [5 of 16 (31% at protein levels)] agree well with those of published studies of neuroblastoma cell lines [7 of 18 (39%), Ref. 33 and 6 of 19 (30%), Ref. 32]. The finding that p16 expression occurs mainly in unfavorable-stage neuroblastoma and cell lines suggests that the expression of p16 may represent a unique feature of aggressive neuroblastoma cells. Although alterations of downstream com-

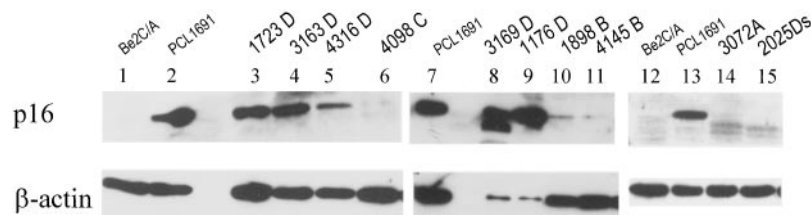


Fig. 2 Expression of p16 protein in primary neuroblastoma. Protein expression of p16 in neuroblastoma was determined with Western blot analysis. All of the levels of protein expression are in comparison with that of β -actin. *Lanes 1 and 12*, p16-deleted Be2c/ADR5 neuroblastoma cell line shown as a negative control (8, 33); *Lanes 2, 7, and 13*, pronounced p16 expression in the PCL1691 neuroblastoma cell line shown as a positive control (33); *Lanes 3–6, 8, and 9*, primary neuroblastoma samples that expressed p16 mRNA; and *Lanes 10, 11, 14, and 15*, samples that expressed no or barely detectable level of p16 mRNA.

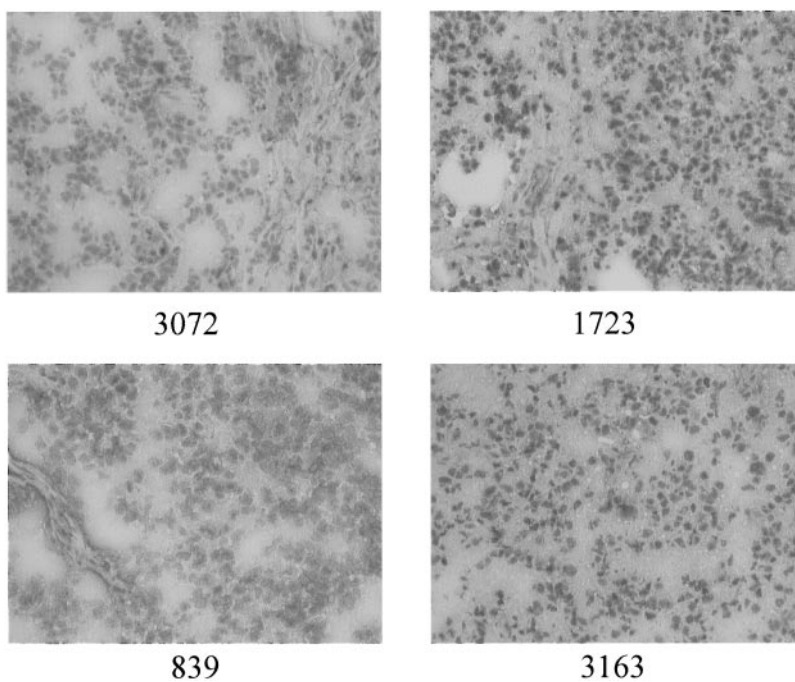


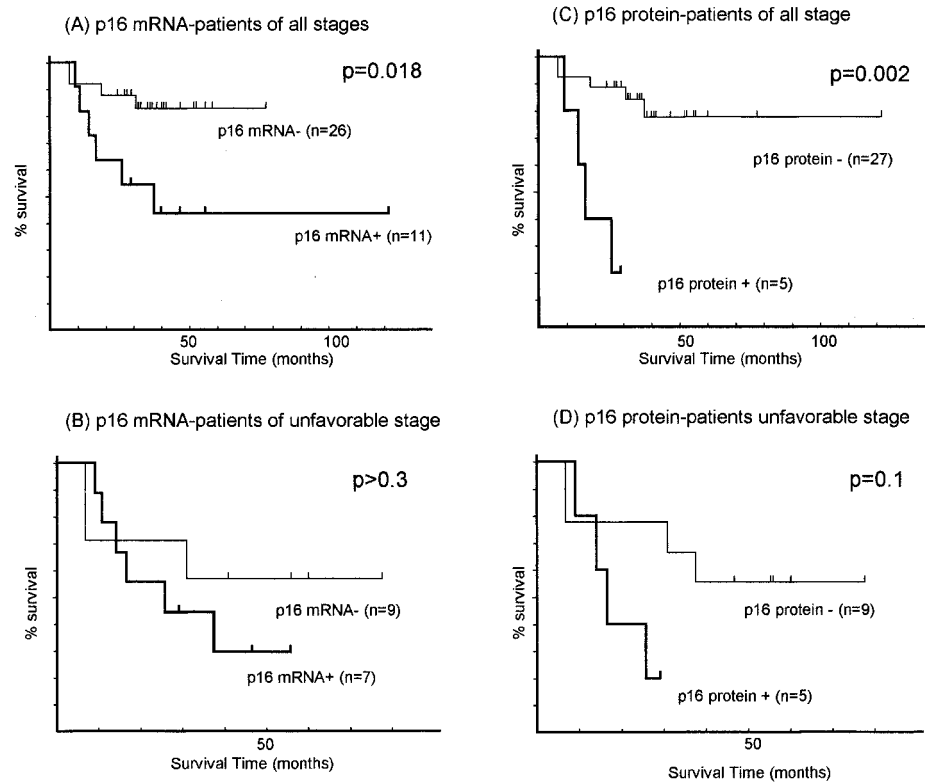
Fig. 3 Immunohistochemical analysis of p16 in primary neuroblastoma. Samples 1723 and 3163 showed strong staining in the majority of tumor nuclei. p16 protein was also detected in these samples by Western blot. Samples 3072 and 839 showed predominantly negative staining. p16 protein was not detected in either of these samples by Western blot.

ponents of CDK and cyclins were rare events, the majority of samples investigated showed a phosphorylated pRb regardless of p16 protein/mRNA expression status. These findings suggest that p16 protein may not be operative to regulate the pRb phosphorylation and that p16 transcription may be induced by a pRb-independent mechanism, in contrast to a pRb/p16 feedback regulatory loop that has been reported in other cancers (21, 34, 35). These findings are in line with those of our study of neuroblastoma cell lines (33), confirming that elevated p16 expression reflects characteristic features of neuroblastoma *in vivo*. Furthermore, p16 expression may delineate a subset of unfavorable-stage neuroblastoma patients who fare worse prognostically. Among patients of all stages, p16 expression was significantly associated with a lower overall survival and may be attributable to the association between p16 expression and unfavorable stages (Fig. 4, A and C). Among the unfavorable-stage patients, we found a trend toward lower survival for those tumor samples expressing p16, although the differences did not reach statistical significance, perhaps because of small sample

sizes (Fig. 4, B and D). As shown in our study, transcription of p16 is not always followed by translation. In either an analysis of all stages or an analysis restricted to unfavorable stages only, the expression of p16 protein had a more significant impact on survival than that of mRNA. Perhaps p16 protein expression may be a better prognostic factor for future analyses. On the other hand, we found that the expression of p16 mRNA or protein did not associate with either N-myc amplification or an age of >1 year at diagnosis, which have been known to be associated with an unfavorable outcome prognosis (4, 5, 46, 47). A lack of such correlation has also been observed in our recent study of neuroblastoma (33), as determined by using p16 protein and mRNA, similar to this present study, or by using protein (30) or immunohistochemistry alone (48). Therefore, p16 expression may be a potentially important prognostic indicator independent of these known predictors.

p16 is known to be one of the major tumor suppressor genes and is inactivated in a variety of human tumors. In neuroblastoma, however, we found frequent p16 expression in

Fig. 4 Prognostic significance of *p16* mRNA (A and B) and protein (C and D) expression in survival of neuroblastoma patients. Survival curve analysis for neuroblastoma patients of all stages (A and C) and unfavorable stage (B and D) from the time of diagnosis in relation to *p16* expression status is shown. Statistical analysis was performed by using the generalized Wilcoxon test of equality. Patients were subdivided into groups based on *p16* expression status.



primary tumors at the unfavorable stages. Similar findings of paradoxically elevated *p16* expression in advanced-stage tumors have been reported in other types of cancer. For example, the immunohistochemical study of a large number of ovarian cancer samples revealed that the high level of *p16* protein expression was associated with advanced-stage, high-grade tumors and a poor prognosis of the patients (49–51). Consistent with our findings, a more recent study of ovarian cancer with Western blot analysis demonstrated that both *p16* and *pRb* were expressed in most of the samples investigated. Furthermore, 3 ovarian cell lines expressing both *pRb* and *p16* proteins were refractory to the inhibitory effects of adenovirus-transfected *p16*, suggesting alterations downstream of *p16* or independent of the *p16* cell cycle regulatory pathway (52). Immunohistochemical analysis of *p16* in prostate cancer shows that high levels of expression of this tumor suppressor gene is also associated with poor clinical outcome (53–54). In a separate study, high levels of *p16* mRNA in prostate cancer were unrelated to *pRb* expression status as measured with immunohistochemistry (55), which is in agreement with our results. Finally, poor prognosis has also been associated with *p16* mRNA and protein expression in childhood acute lymphoblastic leukemia (56), in which *pRb* alterations are rare (57). Alterations of *CDK4/6* and cyclin D have not been reported for these tumors.

Although these findings suggest that elevated *p16* expression without downstream alterations may be characteristic of an aggressive phenotype of human tumors, they fail to answer the paradox of how this cell cycle regulatory gene can be expressed so highly whereas tumor cells continue to rapidly proliferate.

One possibility may be the presence of a *p16* inhibitor protein. In addition to the *CDKs*, several proteins have been identified that bind to *p16*, including RNA polymerase II (58) and *v-Tax*, a regulatory protein of human T-cell virus type 1 (59). *v-Tax* is particularly interesting in that binding to *p16* resulted in a reduction of the *p16*-*CDK4* complexes, with a subsequent activation of *CDK4* kinase (59, 60). Easton *et al.* (30), who have reported high levels of *p16* expression in neuroblastoma cell lines as we have (33), hypothesize that a cellular *Tax* may account for the inability of *p16* to effectively inhibit neuroblastoma, although no reports or precedent for a cellular *Tax* has been documented.

An alternative explanation for the inability of highly expressed *p16* to inhibit neuroblastoma growth may be the expression of certain proteins that interact with downstream components of the cell cycle, such as the *CDKs*, cyclins, or *pRb*. *pRb* is a particularly strong candidate because it lies at the apex of a regulatory pathway containing multiple proteins with analogous functions, such as *CDK* inhibitors (*p15*, *p16*, and *p18*), the *CDKs* (*CDK4* and *CDK6*), and the D family of cyclins. Although many proteins have been demonstrated to bind to *pRb*, the helix-loop-helix protein *Id2* may be a particularly strong candidate for a deregulatory role in neuroblastoma. *Id2* can antagonize the growth-suppressive activities of *p16* and abolish *p16*-imposed cell cycle arrest by binding the hypophosphorylated form of *pRb*, releasing transcriptional factors and driving the cell through the cell cycle (61). *Id2* levels have been shown recently to be very high in neuroblastoma cell lines harboring *N-myc* amplification (62). Because unfavorable-stage neuro-

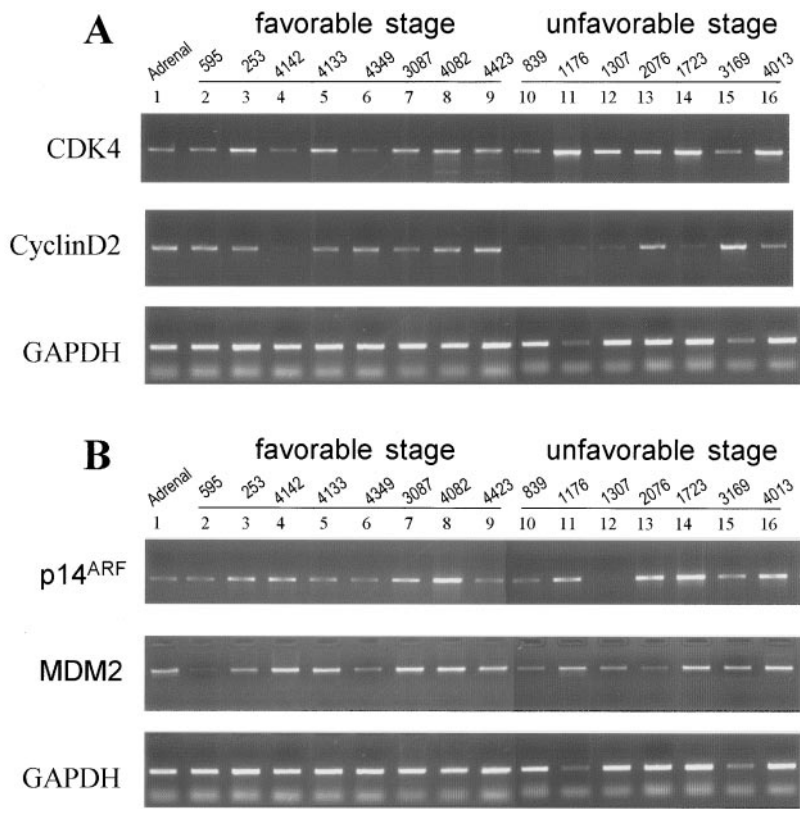


Fig. 5 Expression of *CDK4* and *cyclin D2* (A) and *p14^{ARF}* and *MDM2* (B) in primary neuroblastoma. mRNA expression of these genes in neuroblastoma was analyzed by using RT-PCR. *GAPDH* is shown as a control. Lane 1, normal adrenal gland; Lane 2, GN; and Lanes 3–16, neuroblastoma samples obtained at different stages.

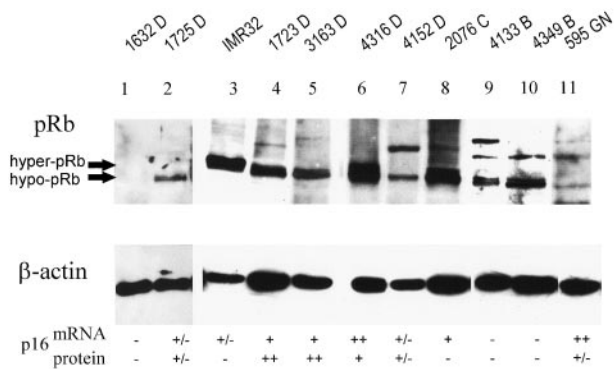


Fig. 6 Expression of pRb protein in primary neuroblastoma. Protein expression of p16 in neuroblastoma was determined by using Western blot analysis. All of the levels of protein expression are in comparison with that of β -actin. Lanes 1, 2, and 4–10, neuroblastoma samples obtained at different stages; Lane 3, IMR32 (neuroblastoma cell line) showed predominantly hyperphosphorylated pRb (33); and Lane 11, GN.

dispute a putative role for Id2 involvement. None of the neuroblastoma patient samples in our study that expressed a predominantly hypophosphorylated pRb, the form that binds Id2, also expressed *p16*. Whereas these data argue against a relationship between p16, pRb, and Id2 in neuroblastoma, there exists the possibility that a fraction of the pRb is present in the hypophosphorylated form, although the samples expressed predominantly hyperphosphorylated protein. It may be this fraction that is bound and inactivated by Id2, resulting in the release of pRb-bound transcription factors that contributes to cell cycle deregulation as well as *p16* expression in a feedback loop mechanism. This possible role of Id2 in the deregulation of the G₁ pathway in neuroblastoma is currently under investigation.

In this study, we have shown that *p16* expression is significantly associated with unfavorable-stage neuroblastoma. Takita *et al.* (48), on the other hand, have analyzed p16 expression by using immunohistochemistry in a large number of primary neuroblastomas and demonstrated that the lack of p16 expression significantly correlated with the unfavorable stage of the disease. Although the basis for this discrepancy with our result is unclear, it may reflect the difference in methodology between the studies. Immunohistochemistry has the advantage of delineating the cellular distribution of signals but may be inadequate for quantitative analysis. In addition, the proportion of patients >1 year of age and of *N-myc* amplification, both of which are well-known adverse prognostic factors, were lower in their study than in ours (age >1 year, 38% in Takita's study versus 60% in ours; *N-myc* amplification, 9% versus 20%, respectively). Overall, the patient population in their

blastoma contains frequent *N-myc* amplification, it is conceivable that high levels of *p16* expression in some neuroblastoma patients may reflect pRb inactivation by Id2. The data in our study, however, suggest no such relationship between *N-myc* amplification and *p16* expression. The 6 unfavorable-stage neuroblastomas highly expressing *p16* were split evenly between those *N-myc* amplified and nonamplified. Our pRb data also

study appears to represent lower risk neuroblastomas compared with that in our study, where the stage (risk) distribution is fairly representative of the distribution of stages across the general population of patients with neuroblastoma. Confounding the interpretation further, Iolascon *et al.* (31) have shown recently a higher rate of *p16* expression [60% (12 of 18)] with RT-PCR that did not correlate with stage. Overall, none of the studies published previously included a systematic analysis of *p16* at the transcriptional and translational levels as performed in this study. Such systematic analysis in a larger number of patients may be necessary to define the prognostic significance of *p16* expression.

Recent studies (42, 43) have demonstrated that there is another distinct pathway, *p14^{ARF}*-MDM2-*p53*, for cell cycle regulation and that alteration of this pathway also contributes significantly to human cancer. However, in primary neuroblastoma, there was no evidence of overexpression of MDM2; loss of *p14^{ARF}* expression and *p53* gene mutation were infrequent events (1 of 40 and 3 of 40, respectively). These findings suggest that alterations of this pathway may not be involved in the pathogenesis of neuroblastoma and appear to have no clinical correlation, in contrast to *p16*.

Because the outcome of patients with unfavorable disease remains poor despite aggressive and multimodal treatment, better prognostic indicators for these patients are needed. In this study, we have demonstrated that *p16* expression is associated with unfavorable-stage neuroblastoma, suggesting that up-regulated *p16* expression may represent a unique feature of aggressive neuroblastoma cells. We are now undertaking a study of a larger sample size to address the possibility of *p16* expression as a prognostic factor in unfavorable-stage neuroblastoma.

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