p16/p14\textsuperscript{ARF} Cell Cycle Regulatory Pathways in Primary Neuroblastoma: p16 Expression Is Associated with Advanced Stage Disease\textsuperscript{1}

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ABSTRACT

p16 regulates the G\textsubscript{1}-S cell cycle transition by inhibiting the cyclin D-cyclin-dependent kinase (CDK)\textsubscript{4}/CDK\textsubscript{6}-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 overexpression of MDM2, and loss of p14\textsuperscript{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 GN\textsuperscript{3} (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 (CDK\textsubscript{4} and CDK\textsubscript{6}) induce the phosphorylation of pRb to promote the G\textsubscript{1}-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 G\textsubscript{1}-S cell cycle transition by inhibiting the cyclin D-CDK\textsubscript{4}/CDK\textsubscript{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 G\textsubscript{1} into S-phase. Similarly, alterations of pRb itself may also lead to G\textsubscript{1}-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, p14ARF, with a distinct reading frame (36–38). Recent studies have demonstrated that p14ARF 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 p14ARF-MDM2-p53 pathway appears to also be inactivated in human cancers through alteration of various components such as deletion and silencing of p14ARF, 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 p14ARF-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 premi- amplification 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: p14ARF cDNA, 5′-CTGAGGCGGCGCGAGAACA-3′ (sense) and 5′-CTACGAAACGGGGTGTTGCTG-3′ (antisense); and MDM2 cDNA, 5′-CTGGGGAGTCTGAGGGACC-3′ (sense) and 5′-CAGTTGTCTAAATTCCTTAG-3′ (antisense). The p14ARF 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× High Fidelity PCR buffer (60 mM Tris-SO4 pH 8.9), 18 mM (NH4)2SO4 (Life Technologies), 2 mM MgSO4, 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 p14ARF, 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 1% agarose gel in 1× 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 μg/ml mouse monoclonal anti-human p16 monoclonal antibody Z31 (NeoMarker, Fremont, CA) and 0.5 μg/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 µg/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 µg/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 SI-S/A 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 samples 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 PABs, 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
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.

### Table 1 p16-CDK/cyclin D-pRb

| ID | Stage | N-myC | Age at diagnosis (yr) | p16 mRNA | Protein | IH | cycl2 mRNA | CDK4 mRNA | pRb status | p14ARF mRNA | MDM2 mRNA | p53 DNA |
|----|-------|-------|------------------------|-----------|---------|----|------------|------------|-------------|-------------|------------|-----------|---------|
| 1632 | D     | NA | 1.2                  | −         | −       | −  | −          | −          | +           | +           | −          | +        | +       |
| 1723 | D     | AMP | 1.4                  | +         | +       | ++ | +          | +          | +           | −          | −          | −        | −       |
| 1724 | D     | NA | 1.03                 | +/−       | −/−     | +  | +          | +          | −           | +          | +          | +        | +       |
| 1785 | D     | NA | 5.1                  | +         | ++      | −  | +          | +          | −           | −          | +          | +        | −       |
| 3163 | D     | NA | 12.0                 | +         | +       | +++ | +          | +          | +           | −          | +          | +        | +       |
| 3169 | D     | NA | 4.2                  | +         | ++      | −  | +          | +          | +           | +          | +          | +        | +       |
| 4013 | D     | AMP | 0.6                  | +/−       | −       | +  | +          | +          | +           | +          | +          | +        | +       |
| 4152 | D     | NA | 5.2                  | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 4177 | D     | AMP | 0.0                  | +/−       | −       | +  | +          | +          | +           | +          | +          | +        | +       |
| 4316 | D     | AMP | 4.2                  | +         | +       | d  | +          | +          | +           | +          | +          | +        | +       |
| 4348 | D     | AMP | 1.0                  | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 839  | C     | AMP | 2.8                  | +/−       | −/−     | d  | +          | +          | +           | +          | +          | +        | +       |
| 1176 | C     | AMP | 3.8                  | +         | +++     | d  | +          | +          | +           | +          | +          | +        | +       |
| 1307 | C     | NA | 2.8                  | +/−       | −/−     | −  | +          | +          | +           | +          | +          | +        | +       |
| 1367 | C     | NA | 1.1                  | +         | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 2076 | C     | NA | 1.5                  | +         | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 2095 | C     | NA | 0.2                  | −         | +       | +  | +          | +          | +           | +          | +          | +        | +       |
| 4098 | C     | AMP | 2.8                  | +         | −       | +  | +          | +          | +           | +          | +          | +        | +       |
| 1898 | B     | NA | 5.8                  | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 4133 | B     | NA | 0.3                  | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 4145 | B     | NA | 1.9                  | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 4175 | B     | NA | 5.8                  | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 4349 | B     | NA | 0.1                  | +         | −       | +  | +          | +          | +           | +          | +          | +        | +       |
| nANW | A     | NA | 2.5                  | −         | −       | +  | +          | +          | +           | +          | +          | +        | +       |
| 253  | A     | NA | 0.98                 | +         | +       | +  | +          | +          | +           | +          | +          | +        | +       |
| 2090 | A     | NA | 0.0                  | −         | −       | +  | +          | +          | +           | +          | +          | +        | +       |
| 3031 | A     | NA | 10.4                 | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 3066 | A     | NA | 0.1                  | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 3072 | A     | NA | 12.2                 | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 3059 | A     | NA | 1.4                  | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 3124 | A     | NA | 0.1                  | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 4052 | A     | NA | 1.4                  | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 4111 | A     | NA | 0.7                  | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 4142 | A     | NA | 3.6                  | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 1767 | Ds    | NA | 0.1                  | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 2025 | Ds    | NA | 0.6                  | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 3087 | Ds    | NA | 0.0                  | −         | −       | +  | +          | +          | +           | +          | +          | +        | +       |
| 4082 | Ds    | NA | 0.3                  | +         | −       | +  | +          | +          | +           | +          | +          | +        | +       |
| 4423 | Ds    | NA | 0.1                  | +/−       | −/−     | +  | +          | +          | +           | +          | +          | +        | +       |
| 595  | GN    | +       | +/−                   | +         | +       | +  | +          | +          | +           | +          | +          | +        | +       |
| 516  | GN    | +       | +/−                   | +         | +       | +  | +          | +          | +           | +          | +          | +        | +       |

* Expression: −, undetectable; +/−, barely detectable; +, low; ++, moderate; ++++, high.

* IH, immunohistochemistry; AMP, amplification; NA, no amplification; na, not available; cyclD2, cyclin D2; wt, wild type; mut, mutant.

* Phosphorylation status of Rb: hypo, hypophosphorylated Rb; hyper, only hypophosphorylated Rb.

d Discrepancy between the results of Western blot analysis and immunohistochemistry.

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
Mature RNA levels were determined as a ratio to GAPDH, Lane 1, normal adrenal gland (Adrenal), Lane 2, GN, Lanes 3–16, neuroblastoma tumors obtained at different stages. The expression of GAPDH versus p16 in neuroblastoma was determined by using RT-PCR. All of the levels of gene expression are in comparison with that of GAPDH. Neuroblastoma tumors that retain p16 protein expression, pRb (80%) and favorable stages was not statistically significant [12 of 15 (80%) 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 G1-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 G1-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 sample 3072, stage A and CGC283TGC (Arg-Cys) in samples 4145 and 4082, stage B and Ds, respectively (data not shown). There were no CDK4 or CDK6 mutations detected in any of these tumor samples. There was no apparent correlation between the expression levels of either p14ARF or MDM2 and disease stage.

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

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<thead>
<tr>
<th></th>
<th>p16 mRNA (+)</th>
<th>p16 protein (+)</th>
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<tbody>
<tr>
<td>Unfavorable stage</td>
<td>9/18 (50%)</td>
<td>5/16 (31%)</td>
</tr>
<tr>
<td>Favorable stage</td>
<td>2/22 (9%)</td>
<td>0/18 (0 %)</td>
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\(^{a}\) Statistical analysis, Fisher’s Exact Test.

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., p595; Fig. 6, Lane 11), suggesting that cell cycle progression was properly regulated, which is consistent with the benign and self-limited proliferation of GN.

**p14ARF-MDM2-p53 Pathway in Primary Neuroblastoma.** We also examined each component of the p14ARF-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 p14ARF and MDM2 were investigated by using RT-PCR (Fig. 5B). In contrast to p16, p14ARF 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 p14ARF 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 p14ARF-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-

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**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. LN, normal adrenal gland (Adrenal); LN 1, normal adrenal gland; LN 2, GN; LN 3–16, neuroblastoma tumors obtained at different stages. The p16 mRNA levels were determined as a ratio to GAPDH mRNA.
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...
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. 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.
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 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 contribute 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

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**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.
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, p14ARF-CDK4-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 CDK2; loss of p14ARF 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 upregulated 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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p16/p14ARF Cell Cycle Regulatory Pathways in Primary Neuroblastoma: p16 Expression Is Associated with Advanced Stage Disease


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