Title

Expression profiles and clinical relationships of ID2, CDKNIB, and CDKN2A in primary neuroblastoma

Journal

Genes Chromosomes & Cancer, 41(4)

ISSN

1045-2257

Authors

Gebauer, S
Lu, A L
Omura-Minamisawa, M
et al.

Publication Date

2004-12-01

Peer reviewed

tapraid5/z3v-genes/z3v-genes/z3v01104/z3v0954d04g | sangreyj | S=3 | 8/13/04 | 18:32 | Art: 03120NJZ

GENES, CHROMOSOMES & CANCER :000–000 (2004)

RESEARCH ARTICLE

Expression Profiles and Clinical Relationships of *ID2*, *CDKN1B*, and *CDKN2A* in Primary Neuroblastoma

Sigrun Gebauer,[1] Alice L. Yu,[1] Motoko Omura-Minamisawa,[2] Ayse Batova,[1] and Mitchell B. Diccianni[1]*

[1]Division of Pediatric Hematology/Oncology, UCSD Medical Center, San Diego, California
[2]Yokohama City University School of Medicine, Department of Radiology, Yokohama, Japan

AQ: 1

Despite considerable research into the etiology of neuroblastoma, the molecular basis of this disease has remained elusive. In contrast to the absence of expression of the known tumor suppressor *CDKN2A* (also known as *p16* and *INK4A*) in a wide variety of tumor types because of its frequent deletion, paradoxically, expression of CDKN2A protein in many advanced stage neuroblastomas unrelated to RB1 status was found in our previous studies. In the present study, we sought to identify the mechanistic relationships that might influence *CDKN2A* expression and negate its influence on tumor cell proliferation. In this regard, we examined the role of the tumor-suppressor gene *CDKN1B* (also known as *p27* and *Kip1*) and the oncogene *ID2* in relationship to *CDKN2A* expression, *MYCN* amplification, and neuroblastoma pathogenesis in 17 neuroblastoma cell lines and 129 samples of primary tumors at all stages. All neuroblastoma cell lines expressed the *ID2* transcript and protein. However, although the majority of primary neuroblastomas also expressed the *ID2* transcript, expression of the ID2 protein was undetectable or only barely detectable, regardless of transcript expression. In both cell lines and primary tumors, *ID2* expression was independent of both *CDKN2A* and *MYCN* expression. In primary neuroblastomas, CDKN1B protein was expressed in significantly fewer advanced-stage neuroblastomas than early-stage neuroblastomas, but its expression had no relationship with *CDKN2A* expression or *MYCN* amplification. We concluded that the paradoxical expression of *CDKN2A* in neuroblastoma cannot be explained by inactivation of the tumor-suppressor gene *CDKN1B* or overexpression of the oncogene *ID2*. We further concluded that *ID2* is not a target of *MYCN* regulation nor is it a prognostic factor for neuroblastoma. Finally, the loss of *CDKN1B* in advanced-stage neuroblastoma suggests this protein may play a role in the neuroblastoma disease process.

INTRODUCTION

The CDKN2A–CDK/CCND1 (cyclin D1)–RB1 pathway plays a critical role in cell-cycle progression (Sherr, 2004). Protein complexes of D-type cyclins and cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) induce phosphorylation of the retinoblastoma protein (RB1) in order to promote the G1/S-phase transition. Phosphorylated (inactivated) RB1 releases transcriptional factors such as E2F1, which activate the expression of genes essential for S-phase entry. Regulators of this pathway include the CDK inhibitor proteins CDKN1B (also known as p27 and Kip1) and CDKN2A (also known as p16 and INK4A). CDKN1B has a dual role as a G1-phase stabilizer and a G2-phase inhibitor (Sherr and Roberts, 1999). In the G1 phase, CDKN1B binds and stabilizes the CDK4/CCND1 complex, facilitating RB1 phosphorylation and entry into the early G1 phase of the cell cycle. Upon binding and inhibition of CDK4/CCND1 by CDKN2A, CDKN1B is released and becomes available for binding and inhibition of CDK2/CCNE (cyclin E) or CDK2/CCNA1 (cyclin A1), complementing CDKN2A-induced G1 inhibition with inhibition at the G2 phase. Inactivation of *CDKN2A* and *CDKN1B* has been found frequently in various human tumors (for recent reviews, see Rocco and Sidransky, 2001; Nho and Sheaff, 2003), suggesting inactivation of this pathway plays a role in many neoplasms. *CDKN2A* is inactivated at the molecular level in a wide variety of neoplasms but in general is not prognostic, whereas *CDKN1B* is generally inactivated at the posttranslational level and frequently is prognostically significant.

Neuroblastoma is one of most common solid tumors in children and has a wide spectrum of clinical and biological features. The prognosis for patients with advanced disease (stages III and IV) remains poor despite intensive multimodality treat-

Supported by: FDA; Grant numbers: FD-R-00952 and 95-5470 (to A.L.Y.); Cindy Matters Funds; General Clinical Research Center program of the National Center for Research Resources, NIH; Grant number: MO1-RR00827.

*Correspondence to: Mitchell B. Diccianni, Division of Pediatric Hematology/Oncology, UCSD Medical Center, 200 West Arbor Drive, San Diego, CA 92103-8447.
E-mail: mdiccianni@ucsd.edu

Received 19 December 2003; Accepted 9 July 2004
DOI 10.1002/gcc.20096
Published online 00 Month 2004 in Wiley InterScience (www.interscience.wiley.com).

ment including bone marrow transplantation, whereas most patients with localized disease (stages I and II) and stage IV disease can be cured. Amplification of the *MYCN* protooncogene is a well-known unique genetic change in neuroblastoma and an important predictor of negative outcome (Brodeur et al., 1984; Seeger et al., 1985). Nevertheless, the mechanism by which *MYCN* contributes to neuroblastoma pathogenesis remains largely unknown.

A number of genetic alterations have been associated with adverse outcomes of neuroblastoma, including deletions of 1p or 11q, unbalanced gain of 17q, and amplification of *MYCN* (Brodeur, 2002), whereas high expression of *HRAS* has been associated with the disease being at an early stage of disease and having a better outcome (Tanaka et al., 1988). Unlike with most cancers, finding molecular alterations of *TP53* (Tweddle et al., 2003) and *CDKN2A* (Beltinger et al., 1995; Diccianni et al., 1996; Kawamata et al., 1996) is rare in neuroblastoma. In contrast, we demonstrated that *CDKN2A* paradoxically is expressed in neuroblastoma and is associated with an advanced stage of the disease (Omura-Minamisawa et al., 2001). Mutation, deletion, and rearrangement of the *CDKN1B* gene also rarely occur in neuroblastoma (Kawamata et al., 1996) and other cancers (Sgambato et al., 2000; Viglietto and Fusco, 2002). However, many neoplasms exhibit posttranslational inactivation of *CDKN1B*, which, in breast, colorectal, and gastric cancers, among others, results in a loss of protein that is associated with a poor prognosis (Philipp-Staheli et al., 2001). Loss of CDKN1B protein also was reported to be prognostically significant in neuroblastoma (Bergmann et al., 2001).

The basic helix–loop–helix (b-HLH) proteins are a family of eukaryotic transcription factors that have been shown to play key roles in the differentiation of a number of cell lineages, including muscle cells, B and T lymphocytes, pancreatic β cells, osteoblasts, and neurons (Jan and Jan, 1993; Olson and Klein, 1994; Weintraub, 1993). These proteins generally contain an HLH dimerization domain and an adjacent basic amino acid DNA-binding domain (Ellenberger et al., 1994; Murre et al., 1989). The transcriptional activity of b-HLH proteins is dependent on their ability to form heterodimers and to bind DNA (Lassar et al., 1991). Proteins of the ID class, another class of HLH proteins, lack a basic DNA-binding domain. These proteins act as negative regulators of b-HLH transcription factors by forming heterodimers that make the transcription factors unable to bind DNA (Benezra et al., 1990; Sun et al., 1991), thereby regulating cell growth and differentiation. *ID2* has been of particular interest in tumor cell regulation. The *ID2* level is up-regulated as cells progress out of G0 and remain elevated through the S phase. In general, expression of *ID2* is high in proliferating cells and low or absent in nonproliferating cells. ID2 has been shown to influence cell-cycle progression, not by its inhibition of DNA binding but rather by its ability to bind and inactivate RB1 (Iavarone et al., 1994; Lasorella et al., 1996). Recently, it was suggested that the overexpression of *ID2* may be involved in neuroblastoma pathophysiology. Lasorella et al. (2000) demonstrated that *ID2* can be transactivated by *MYCN* and that its expression correlates with *MYCN* amplification, neuroblastoma stage, and poor prognosis (Lasorella et al., 2002). However, whereas one research group corroborated this finding in a microarray analysis of *ID2* and *MYCN* in neuroblastoma (Raetz et al., 2003), at least three other groups found no correlation between the 2 genes at the transcriptional level (Sato et al., 2003; Vandesompele et al., 2003; Wang et al., 2003), and although ID2 protein has been investigated by immunohistochemistry (Lasorella et al., 2002), no studies that quantitatively or semiquantitatively measured ID2 protein levels by Western blot analysis in primary neuroblastomas have been reported.

In the current study, we investigated the mechanistic relationships that may be responsible for the paradoxical expression of *CDKN2A* in advanced-stage neuroblastoma, which we (Diccianni et al., 1999; Omura-Minamisawa et al., 2001) and others (Easton et al., 1998) have reported, especially in light of our observations that *CDKN2A* expression was not related to the phosphorylation state or to the protein status of *RB1* and that no alterations of other components of the G1 regulatory pathway, including CCND1, CDK4, and CDK6, were detectable (Diccianni et al., 1999; Omura-Minamisawa et al., 2001). We hypothesized that changes in the expression of proteins that directly or indirectly inactivate RB1 could lead to compensatory nonfunctional overexpression of *CDKN2A*. In this regard, we considered the overexpression of *ID2* or the loss of *CDKN1B* as candidate deregulatory events. ID2 has been shown to inactivate RB1 and also has been purported to be transactivated by MYCN, whose amplification is a well-known negative prognostic factor in neuroblastoma. The loss of the CDK inhibitor CDKN1B also could lead to an increase in RB1 phosphorylation and a compen-

satory increase in *CDKN2A* transcription through the *CDKN2A*/RB1 feedback regulatory loop.

Our results demonstrated that *ID2* mRNA is expressed in all neuroblastoma cell lines and in the majority of primary neuroblastomas. ID2 protein also was expressed in all neuroblastoma cell lines but was undetectable or just barely detectable in most primary neuroblastoma samples. Neither expression of *ID2* mRNA nor of protein correlated with *MYCN* amplification, disease stage, or *CDKN2A* expression. *CDKN1B*, on the other hand, was expressed significantly less frequently in advanced-stage neuroblastoma than in early-stage neuroblastoma, but was unrelated to *CDKN2A* expression. We concluded that (1) the paradoxical expression of *CDKN2A* in neuroblastoma cannot be explained by elevated levels of *ID2* expression or by loss of CDKN1B protein, (2) decreased expression of *CDKN1B* is associated with a poor prognosis in neuroblastoma, and (3) *ID2* is not involved in neuroblastoma pathobiology.

MATERIALS AND METHODS

Cell Lines and Primary Sample Accrual

The HL60 leukemia cell line, the SK-N-MC neuroepithelial cell line, and the IMR32 and SK-N-SH neuroblastoma cell lines were obtained through the American Type Culture Collection (Manassas, VA). The NMB7 neuroblastoma cell line was graciously provided by S.K. Liao (McMaster University, Hamilton, Ontario, Canada). The sms-KAN, sms-SAN, and sms-KCNR cell lines were a gift from C. Patrick Reynolds (Naval Medical Research Institute, Bethesda, MD). The NB and PCL neuroblastoma cell lines were obtained from the Children's (formerly Pediatric) Oncology Group (COG) cell bank (Neuroblastoma Biology Protocol, POG # 9074). The cell lines Be2C and Be2C/ADR5 (Be2C with a developed resistance to adriamycin at 5 ng/ml) were generously provided by J. Biedler (Memorial Sloan-Kettering Cancer Center, New York, NY). The osteosarcoma cell line SJ SA-1 was kindly provided by D. Shapiro (St. Jude Children's Research Hospital, Memphis, TN). Primary neuroblastoma samples were obtained from the COG tumor bank or the Cooperative Human Tissue Network (CHTN). The samples were fully encoded to protect patient confidentiality and to conform to HIPAA standards and were utilized under a University of California, San Diego–approved IRB protocol (# 021410). Each sample was determined to have a tumor cell content of 80% or greater, according to the report of the tissue section analysis by the institution submitting the sample or by a CHTN or COG tumor bank pathologist.

RNA and Protein Preparation

RNA and protein were extracted from the primary tumors, which were crushed over dry ice, lysed in Trizol® (Invitrogen, Carlsbad, CA), and extracted according to the manufacturer's instructions. Coextraction of RNA and protein from the same tumor fragment ensured that mRNA and protein expression profiles represented identical cross sections of the tumor. In some samples, protein was extracted directly from a tumor sample by pulverization on dry ice followed by lysis in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM sodium fluoride, 1 mM phenylmethanesulfonyl fluoride, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin). Cell lines, harvested at approximately 60%–80% confluence, were lysed directly in Trizol® for RNA and RIPA lysis buffer for protein preparation.

Western Blot Analysis

For Western blotting, 20 μg of protein from whole-cell lysates was denatured in sample buffer, separated on a 10% NuPage gel (Invitrogen, Carlsbad, CA), transferred to an Immobilin-P membrane, blocked in 5% nonfat milk in TBST (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.05% Tween 20), and incubated for 1 h at room temperature (RT) or overnight at 4°C with primary antibody. In some experiments, nitrocellulose membrane was used, with analogous results. After TBST washing, blots were incubated for 1 h at RT with 1:1000 alkaline phosphatase-conjugated antimouse or antirabbit secondary antibody (KPL, Gaithersburg, MD), followed again by TBST washing, dH_2O rinsing, and incubation with ECF substrate (Amersham Biosciences Corp., Piscataway, NJ) for 5 min at RT. Proteins were visualized by a STORM imager (Amersham Biosciences Corp., Piscataway, NJ). The following primary antibodies were used: for CDKN2A, ZJ11 (0.5 μg/ml); for CDKN1B, MS-256 (0.5 μg/ml), both from Neomarkers Inc. (Fremont, CA); for ID2, C20 (1 μg/ml), from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and for ACTB (β-actin), AC15 (1:100,000), from Sigma-Aldridge (St. Louis, MO).

ID2 RT-PCR

Two micrograms of total RNA were reverse-transcribed by use of the SUPERSCRIPT™ First

tapraid5/z3v-genes/z3v-genes/z3v01104/z3v0954d04g | sangreyj | S=3 | 8/13/04 | 18:32 | Art: 03120NJZ

TABLE 1. Expression of *ID2, CDKN2A,* and *CDKN1B* in Primary Neuroblastoma

Transcript	Stage I	Stage II	Stage IVs	Stage III	Stage IV	Total	I, II, IVs vs. III, IV	*P* value
ID2	13/17 (76%)	18/20 (90%)	8/9 (89%)	12/15 (80%)	26/31 (84%)	77/92 (84%)	39/46 (85%) vs. 38/46 (83%)	1.00
CDKN2A	4/21 (19%)	7/29 (24%)	5/12 (42%)	9/26 (35%)	26/41 (63%)	51/129 (40%)	16/62 (26%) vs. 35/67 (52%)	0.0024
CDKN2A[a]	2/17 (12%)	6/20 (30%)	4/9 (44%)	7/15 (46%)	22/31 (71%)	41/92 (45%)	12/46 (26%) vs. 29/46 (63%)	0.003
Protein	Stage I	Stage II	Stage IVS	Stage III	Stage IV	Total	I, II, IVs vs. III, IV	*P* Value
ID2	3/8 (38%)	7/12 (58%)	1/4 (25%)	2/8 (25%)	4/20 (20%)	17/52 (33%)	11/24 (46%) vs. 6/28 (21%)	0.08
CDKN2A	0/15 (0%)	1/19 (5%)	0/5 (0%)	1/15 (7%)	9/27 (33%)	11/81 (14%)	1/39 (3%) vs. 10/42 (24%)	0.007
CDKN1B	11/14 (79%)	10/17 (59%)	1/4 (25%)	7/13 (54%)	5/24 (21%)	34/72 (47%)	22/35 (63%) vs. 12/37 (32%)	0.018

Expression of *ID2, CDKN2A,* and *CDKN1B* transcript and protein were assessed by semiquantitative RT-PCR or Western blot assays, respectively, and compared across neuroblastoma stages. For statistical purposes, stages I, II, and IV are compared to stages III and IV by use of Fisher's exact test. *ID2* transcript, but not protein, was expressed in most primary neuroblastomas, whereas *CDKN2A* was preferentially expressed in advanced-stage neuroblastomas and *CDKN1B* in early-stage neuroblastoma. The statistical significance of the difference between stages is noted for *CDKN2A* and *CDKN1B* only. There were no statistically significant differences in the rate of CDKN2A protein expression in stage IV neuroblastoma (9 of 27, 33%) or in neuroblastoma cell lines harboring an intact *CDKN2A* gene 7 of 16 (Table 2), 44%; $P = 0.53$, Fisher's exact test.
[a]*CDKN2A* expression in only those 92 samples coanalyzed for *ID2* expression.

Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). *ID2* PCR was performed in a 50-μl reaction volume containing 1 μl of cDNA, 1× reaction buffer (Qiagen, Valencia, CA), 0.75 mM $MgCl_2$, 0.04 mM dNTP mix, 0.2 pmol/μl of each primer (sense: ACTCGCATCCCACTATTG-TCAG; antisense: AAAGGTCCATTCAACTTG-TCCTC), and 1 U Taq polymerase (Qiagen) for 26 cycles at 95°C, 62°C, and 72°C for 30 sec in each cycle. Primers were designed against the *ID2* mRNA sequence (NM_002166); they were intron spanning and thus did not amplify the intronless *ID2* pseudogene (Kurabayashi et al., 1993). *CDKN2A* reverse-transcription PCR (RT-PCR) was performed as previously described (Omura-Minamisawa et al., 2001).

Statistical Analysis

Statistical analysis was done with Fisher's exact test.

RESULTS

ID2 and *CDKN2A* in Neuroblastoma Cell Lines

In our previous study of 40 primary neuroblastomas, we reported that advanced-stage neuroblastomas had more frequent expression of the *CDKN2A* gene than did early-stage neuroblastoma (Omura-Minamisawa et al., 2001). In this study, this paradoxical finding was substantiated further with the analysis of 89 additional primary neuroblastomas. This combined analysis of *CDKN2A* transcripts in 129 primary neuroblastoma samples (Table 1) again confirmed that the frequency of expression *CDKN2A* was significantly higher in advanced-stage neuroblastomas (35 of 67, 52%) than in early-stage neuroblastomas (16 of 62, 26%; $P = 0.0024$, Fisher's exact test). Western blot analysis of 81 of the primary neuroblastoma samples (Table 1) confirmed this significant association between *CDKN2A* and advanced stage at the protein level; only 1 of 39 (3%) early-stage neuroblastomas had detectable CDKN2A protein, versus 10 of 42 (23%) advanced-stage neuroblastomas ($P = 0.007$, Fisher's exact test). T1

Having confirmed our previous finding that *CDKN2A* is expressed in advanced-stage neuroblastoma in a large number of patient samples, we sought to understand the mechanistic relationship that could be responsible for the apparent lack of tumor suppression by this protein. Because ID2 had been reported to bind and inactivate the hypophosphorylated form of RB1 (Lasorella et al., 1996), we speculated that the release of transcription factors by RB1 inactivation might explain this paradoxical expression of *CDKN2A* and that CDKN2A would have no effect on cell growth because RB1 is already inactivated. We first investigated transcript and protein levels of *ID2* in 17 neuroblastoma cell lines and 1 neuroepithelial cell line. As shown in Figure 1A and summarized in

TABLE 2. *ID2* and *CDKN1B* Are Expressed in All Neuroblastoma Cell Lines

Cell line	Type	*MYCN* amplification	*ID2* mRNA	ID2 protein	CDKN2A protein[a]	CDKN1B protein
NMB 7	NB	n/d	+	+++	−	+++
NB 5	NB	A	+	++	+++	+++
NB 14	NB	A	+++	+++	+++	++
NB 17	NB	A	++	++	+++	++
NB 20	NB	A	+++	+++	+++	+++
PCL 1691	NB	A	+++	+++	+++	+++
PCL 2021	NB	A	+++	+++	−	+++
PCL 1643	NB	A	+++	+++	−	+++
PCL 3014	NB	A	+++	++	−	+++
PCL 4199	NB	A	+++	+++	+++	+++
SK-N-SH	NB	N	+++	+++	−	++
IMR 32	NB	A	+++	++	−	+
Be2c	NB	A	+++	+++	++	+++
Be2c/ADR5	NB	A	+++	+++	del	+++
sms-KAN	NB	A	+++	+++	−	+++
sms-SAN	NB	n/d	++	++	−	+++
sms-KCNR	NB	A	+++	+++	−	n/d
SK-N-MC	NE	N	+++	+++	+++	++

ID2, CDKN2A, and *CDKN1B* transcript/protein expression was semiquantitatively assessed as very high (+++), high (++), weak (+), or undetectable (−) by RT-PCR and Western blot assays. *CDKN2A* was expressed in 7 of 16 (44%) neuroblastoma cell lines with an intact *CDKN2A* gene (Be2CADR5 is *CDKN2A* deleted and SK N-MC is a neuroepithelial cell line). *ID2* and *CDKN1B* were expressed in all cell lines investigated.
NB, neuroblastoma; NE, neuroepithelial; del, deletion; A, *MYCN* amplified; N, *MYCN* not amplified; n/d, not determined.
[a]Data from Diccianni et al. (1999).

Table 2, all cell lines expressed the *ID2* transcript and protein. *ID2* can be induced in some cell lines by induction of differentiation with agents such as dimethyl sulfoxide (DMSO). To understand what levels the expression of *ID2* in neuroblastoma represents, we compared the levels of *ID2* expression in neuroblastoma to the levels found in the HL60 cell line before and after induction of differentiation by 1.25% DMSO. Consistent with published reports (Ishiguro et al., 1996), treatment of HL60 cells with DMSO resulted in increases in *ID2* transcript and protein expression (Fig. 1A). When we compared the level of *ID2* in neuroblastoma cell lines with that in the differentiated HL60 cells, we observed that transcript levels of *ID2* in the neuroblastoma cell lines were at least comparable with, if not higher than, the induced level observed in the HL60 cells. A similar result was observed at the protein level, where many of the neuroblastoma cell lines expressed ID2 protein at levels comparable to those in the induced HL60 cell line. Correlation of *ID2* expression with *MYCN* amplification also has been reported (Lasorella et al., 2000; 2002). However, the 2 cell lines included in this study that did not harbor an amplified *MYCN* gene, the neuroblastoma cell line SK-N-SH and the neuroepithelial cell line SK-N-MC, expressed similar levels of *ID2*, as did the 14 neuroblastoma cell lines with *MYCN* amplification (Fig 1A and Table 2). Although all cell lines expressed *ID2*, we sought to determine whether a relationship between the level of *ID2* expression and *CDKN2A* expression could be established. However, as shown in Table 2, no correlation between *ID2* and *CDKN2A* expression was observed. For example, cell lines IMR32 and NB17 both expressed *ID2* (Fig. 1A). However, the IMR32 cell line showed no expression of *CDKN2A*, whereas NB17 expressed very high levels of the protein. Also, whereas NMB7 and NB5 both had low levels of *ID2* transcript expression, only NB5 expressed *CDKN2A* (Table 2). T2, F1

ID2 and *CDKN2A* in Primary Neuroblastoma

We next assessed *ID2* expression at the transcript and protein levels in primary neuroblastoma samples obtained from all stages (representative samples are shown in Fig. 2A and B). *ID2* transcript expression was observed in most neuroblastoma samples (Table 1) independent of stage, with 39 of 46 (85%) early-stage neuroblastomas (stages I, II, and IVS) expressing the gene versus 38 of 46 (83%) advanced-stage neuroblastomas (stages III and IV; $P = 1.00$, Fisher's exact test) at similar levels. As we observed in neuroblastoma cell lines, no relationship between *CDKN2A* and *ID2* expression was observed in a comparison of 92 samples coanalyzed for both genes. Thirty-four of 41 (83%) of the *CDKN2A*-positive samples expressed *ID2*, which

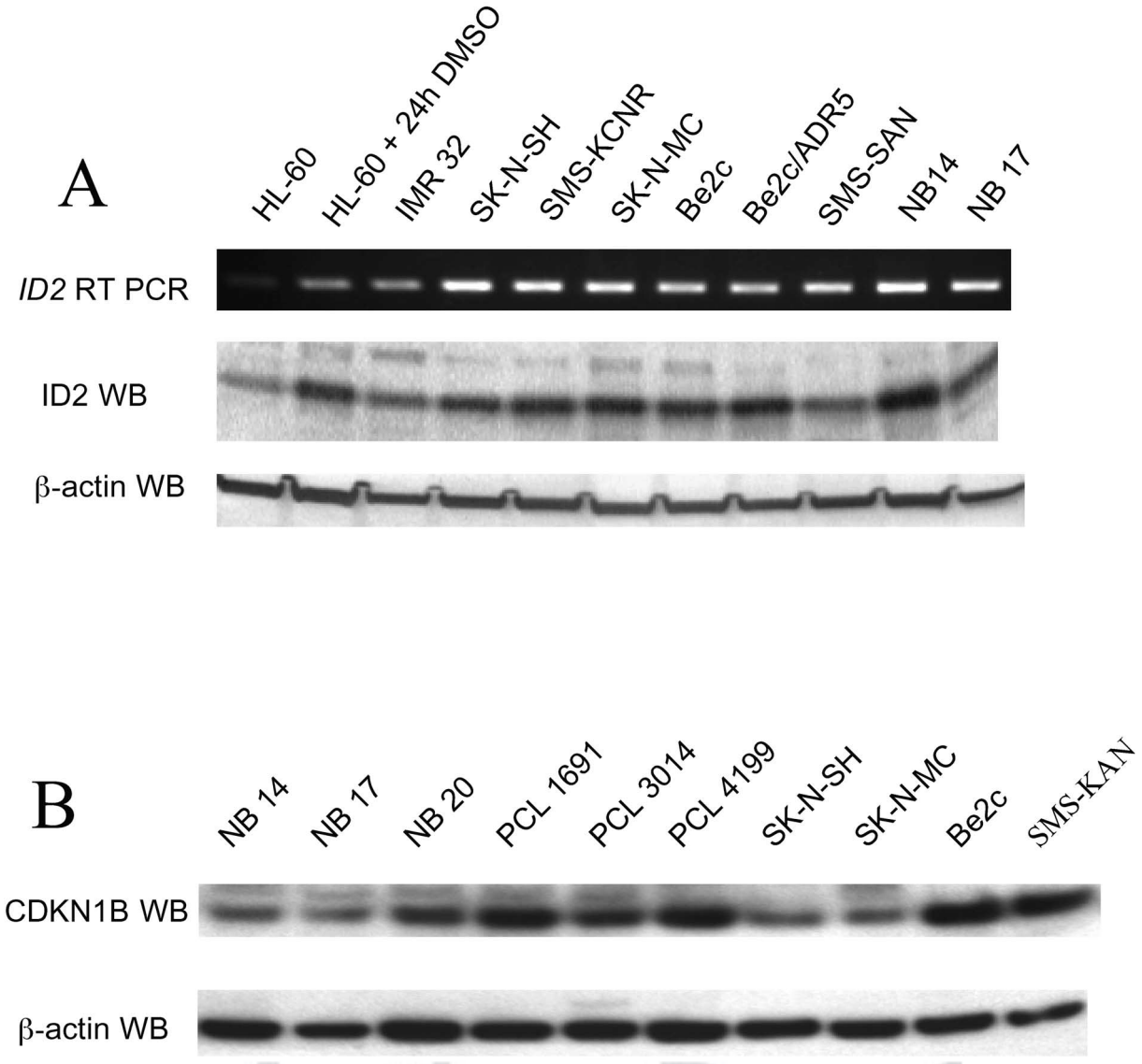

Figure 1. Expression of *ID2* transcript and protein and CDKN1B protein in neuroblastoma cell lines as determined by RT-PCR and Western blot analysis (WB = Western blot). (A) *ID2* transcript and protein were expressed in all neuroblastoma cell lines. These levels are comparable to those found in the HL-60 leukemia cell line after a 24-h induction of *ID2* by 1.25% DMSO. (B) All neuroblastoma cell lines expressed CDKN1B protein, with some expressing very high levels (NB20, PCL1691, PCL3014, PCL4199, Be2C, and SMS-KAN), and others (NB14, NB17, SK-N-SH, and the neuroepithelial cell line SK-N-MC) showing moderate expression levels. Expression of ACTB (β-actin) is shown as a control.

was not statistically different from the 43 of 51 (84%) of the *CDKN2A*-negative samples that expressed *ID2* ($P = 1.00$, Fisher's exact test). Identical results were obtained when only the 46 advanced-stage samples analyzed for both genes were compared. In these samples, 24 of 29 (83%) *CDKN2A*-positive samples expressed *ID2*, which was not statistically different from the 14 of 17 (82%) *CDKN2A*-negative samples that expressed *ID2* ($P = 1.00$, Fisher's exact test). F2

MYCN has been implicated in the regulation of *ID2* gene expression, with amplification of *MYCN* proposed as a mechanism involved in driving the expression of this gene in neuroblastoma (Lasorella et al., 2000). In primary neuroblastoma, none of the early-stage I, II, or IVS samples harbored *MYCN* gene amplification, although more than 80% expressed the *ID2* transcript. In advanced-stage neuroblastomas, 11 of 14 (79%) of the *MYCN*-amplified tumors highly expressed the *ID2* transcript. However, this was not significantly different from the 20 of 24 (83%) of the non-*MYCN*-amplified tumors ($P = 1.00$, Fisher's exact test) that expressed the *ID2* transcript.

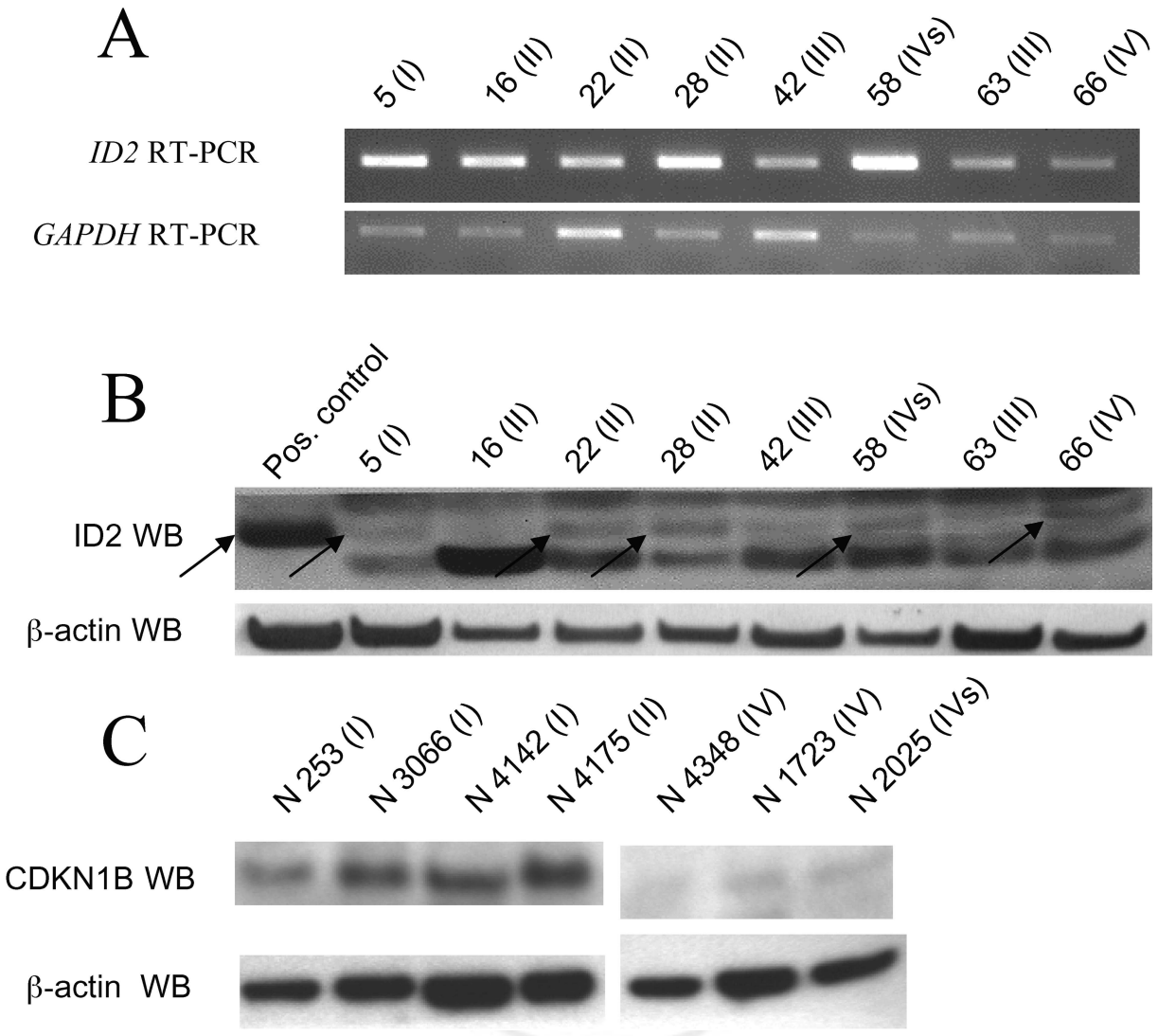

Figure 2. Expression of *ID2* transcript, *ID2* protein, and CDKN1B protein in primary neuroblastoma samples as determined by RT-PCR and Western blot analysis. (A) Expression of *ID2* transcript, which occurred in all primary neuroblastomas, with some expressing high levels of the transcript (i.e., samples 5, 16, 22, 28, and 58), and others having relatively moderate levels of the transcript (i.e., samples 42, 63, and 66). (B) ID2 protein was either not detectable (samples 16, 42, and 63) or barely discernable (samples 5, 22, 28, 58, and 66 as identified by arrows) in protein extracts from primary neuroblastomas. ID2 protein was readily identifiable by comparison against the migration profile of ID2 from cell lines, which expressed the nonspecific bands only weakly, and by the migration profile of ID2 visualized after induction by DMSO in HL60 cells (for example, see Fig. 1). Note that high transcript expression did not necessarily yield detectable ID2 protein. The osteosarcoma cell line SJ SA-1, determined in our laboratory to have high expression of *ID2*, is shown as a positive control. (C) CDKN1B protein expression was generally strong in lower-stage neuroblastoma samples (i.e., samples N3066, N4142, and N4175) but was weak or undetectable in upper-stage neuroblastoma samples (i.e., samples N4348 and N1723). Expression of *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) transcript and ACTB (β-actin) protein is shown as controls.

We sought to confirm the observations of frequent *ID2* expression in neuroblastoma at the protein level in primary neuroblastomas. In contrast to our observations of frequent *ID2* transcript and protein expression in neuroblastoma cell lines and *ID2* transcript expression in primary samples, ID2 protein was undetectable in the majority (35 of 52; 67%) of samples investigated (Table 1). Furthermore, in the 17 samples in which ID2 protein was detectable, the level of expression was very weak, with only a barely discernable band noted by Western blot analysis (Fig. 2B). Although the sample size was small, no correlation was evident between ID2 and CDKN2A protein expression in the 27 advanced-stage neuroblastoma samples examined for both proteins, consistent with our observations in neuroblastoma cell lines. Of the 6 samples with detectable ID2 protein, 3 (50%) also expressed CDKN2A protein, which was not significantly different from the 4 of 21 (24%) samples without

detectable ID2 protein that expressed CDKN2A ($P = 0.28$, Fisher's exact test). We noted that the primary neuroblastomas and, to a lesser extent, the neuroblastoma cell lines yielded highly immunoreactive bands at approximately 40, 21, and 12 kDa, in addition to ID2 at 14 kDa, which were generally expressed in most samples (Fig. 2B). Efforts to visualize ID2 better by increasing the amount of protein loaded on the gel only served to mask ID2 further by increasing the levels of these other proteins. Although the identities of these immunoreactive proteins are unknown, the possibility exists that the 12-kDa immunoreactive protein may be a degradation product of ID2.

CDKN1B in Neuroblastoma

Inactivation of *CDKN1B* is common in many cancers, occurring primarily during posttranslation. An examination of CDKN1B protein levels in the neuroblastoma cell lines revealed *CDKN1B* expression in all cell lines (Fig. 1B, Table 2). In the primary neuroblastomas, however, significantly fewer advanced-stage samples expressed CDKN1B protein than did those in the early stage [12 of 37 (32%) vs. 22 of 35 (63%); $P = 0.018$, Fisher's exact test; Table 1, Fig. 2C). Although the frequency of *CDKN2A* expression was significantly higher and the frequency of *CDKN1B* expression was significantly lower in advanced-stage neuroblastomas, an inverse expression relationship between the 2 proteins was not observed in the 35 advanced-stage samples that were compared for expression of both proteins. Of the 9 samples that expressed CDKN2A, 7 (78%) lacked significant CDKN1B expression. However, this was not significantly different from the 16 (62%) that failed to express CDKN1B of the 26 analyzed advanced-stage neuroblastomas that did not express CDKN2A ($P = 0.45$, Fisher's exact test). It is worth noting that the significant association in advanced-stage neuroblastoma between lack of *CDKN1B* expression and gain of *CDKN2A* expression appears to be entirely the result of the contribution of samples from those patients with the very worst prognoses, that is, those with stage IV malignancies, although protein expression was not quite significant. As can be seen in Table 1, analysis of both *CDKN2A* and *CDKN1B* expression revealed a strong association with stage IV. Whereas 7 of 13 (54%) stage III samples expressed CDKN1B, only 5 of 24 (21%) stage IV samples had high expression of CDKN1B protein ($P = 0.067$). Similarly, only 1 of 15 (7%) stage III samples expressed CDKN2A, whereas 9 of 27 (33%) stage IV samples expressed CDKN2A protein ($P = 0.068$). A similar association between *CDKN2A* expression and stage IV was observed at the transcript level, where 9 of 26 (35%) stage III and 26 of 41 (63%) stage IV samples expressed *CDKN2A* ($P = 0.026$).

DISCUSSION

Although the etiology of neuroblastoma is unknown, a number of genetic alterations have been associated with the disease, including deletions of 1p or 11q, unbalanced gain of 17q, and amplification of *MYCN*, each of which is a significant adverse prognostic factor in neuroblastoma (Brodeur, 2002). Of these and other genetic alterations associated with neuroblastoma, *MYCN* is the only oncogene known to be activated. In contrast to finding elevated oncogene levels, we (Diccianni et al., 1999; Omura-Minamisawa et al., 2001) and others (Easton et al., 1998) have reported that in many neuroblastoma cell lines and advanced-stage neuroblastomas, there paradoxically is very high expression of both mRNA and protein of the tumor-suppressor gene *CDKN2A*. Elevated *CDKN2A* expression also has been observed in other tumor types, such as ovarian cancer (Dong et al., 1997; Shigemasa et al., 1997) and prostate cancer (Lee et al., 1999; Halvorsen et al., 2000; Jarrard et al., 2002).

Downstream alterations of the cyclins, CDKs, or *RB1* are infrequent in neuroblastoma (Diccianni et al., 1999; Omura-Minamisawa et al., 2001). So in the current study, we investigated a possible mechanistic relationship in this disease of *CDKN2A* with other oncogenes and tumor-suppressor genes that might account for the inability of *CDKN2A* to be a tumor suppressor despite being highly expressed. Recently, *ID2* was reported to be very highly expressed in *MYCN*-amplified neuroblastoma (Lasorella et al., 2000, 2002). Because ID2 is an inhibitor of RB1 (Iavarone et al., 1994), we hypothesized that the growth-suppressive activity of CDKN2A might be antagonized by ID2 inactivation of RB1, which, in turn, leads to elevated *CDKN2A* expression in a feedback-loop mechanism. However, *ID2* transcript and protein expression occurred in all neuroblastoma cell lines investigated, whereas only one-third expressed CDKN2A protein. The *ID2* transcript was expressed in 83% of the primary neuroblastoma samples investigated, again harboring no relationship with *CDKN2A* expression. Taken together, these data suggest that ID2 inactivation of RB1 is not the mechanism by which neuroblastoma cells proliferate in the presence of high levels of *CDKN2A*.

Despite the irrefutable correlation between *MYCN* amplification and poor prognosis in neuroblastoma, the functional target of MYCN remains unknown. Given the role of ID2 as a regulator of RB1 and the cell cycle and given that *MYCN* amplification is significantly associated with an aggressive neuroblastoma phenotype, great interest was generated by reports that *ID2* protein levels are highly correlated with *MYCN* amplification in neuroblastoma cell lines, that *MYCN* binds and regulates the *ID2* promoter, that *ID2* level increases after *MYCN* induction in a *MYCN*-inducible cell line, and that *ID2* level has prognostic significance in primary neuroblastoma (Lasorella et al., 2000; 2002). Further support for this relationship was provided by a recent microarray analysis of neuroblastoma cell lines that also demonstrated a *MYCN*/*ID2* relationship, although quantitative RT-PCR analysis demonstrated only modest levels of *ID2*, which did not correlate with *MYCN* amplification (Raetz et al., 2003). In this study, we used RT-PCR and Western blot analysis to examine *ID2* expression in neuroblastoma cell lines and primary neuroblastomas. *ID2* was expressed in all neuroblastoma cell lines, including those without *MYCN* amplification. The majority of primary neuroblastoma samples also showed high expression of the *ID2* transcript, although ID2 protein expression was weak to undetectable. No relationship could be established between either *ID2* transcript or ID2 protein expression and either *MYCN* status or neuroblastoma stage.

These results, which we first reported in abstract form (Gebauer et al., 2002), are consistent with those of three other recent reports, all of which documented no correlation between *ID2* and *MYCN*. Vandescompele et al. (2003) and Wang et al. (2003), utilizing real-time RT-PCR, Northern blot, and Western blot analyses, also detected *ID2* in all the neuroblastoma cell lines they investigated, regardless of *MYCN* amplification status. Northern blot analysis of *ID2* in primary neuroblastoma samples by these investigators similarly failed to show a relationship with *MYCN* amplification or to have prognostic significance. Sato et al. (2003) also reported no significant relationship between *ID2* and *MYCN* mRNA expression in primary neuroblastoma, and they further documented that level of *ID2* mRNA did not differ significantly from that in normal ganglions. The results of at least 2 studies that examined *ID2* level in neuroblastoma cell lines were in general agreement with the *ID2* levels in *MYCN*-amplified but not in non-*MYCN*-amplified neuroblastoma cell lines. For example, in *MYCN*-amplified cell lines IMR32, LA-N-1, and NGP, both Lasorella et al. (2000) and Vandesompele et al. (2003) demonstrated moderate to high expression of ID2 protein. High levels of ID2 protein in *MYCN*-amplified cell lines NGP and SKNDZ was reported by both Wang et al. (2003) and Lasorella et al. (2000). In this study, we showed high levels of ID2 protein in the SMS-KCNR and IMR32 cell lines, in accord with the findings of Lasorella et al. (2000) and Vandesompele et al. (2003). Discrepant results appear when non-*MYCN*-amplified neuroblastoma cell lines are considered. We report here, as did Vandesompele et al. (2003) and Wang et al. (2003), finding high levels of both *ID2* transcript and protein in the non-*MYCN*-amplified SK-N-SH cell line, whereas Lasorella et al. (2000) reported very low levels of ID2 protein. Similarly, moderate to high levels of ID2 protein were found in the SK-N-AS and SK-N-F1 cell lines by Vandesompele et al. (2003) and Wang et al. (2003), whereas Lasorella et al. (2000) reported low to undetectable levels of the same protein.

The reasons for the differences in the reported *ID2* levels in the non-*MYCN*-amplified cells is unclear. It is possible that differences in *ID2* level reported by each group reflect the condition of the cells and/or media at the time the RNA extracts were made. *ID2* RNA level has been shown to decrease over time in culture (Jogi et al., 2002), with levels rebounding if the medium is freshened (Vandesompele et al., 2003). However, *ID2* levels increased whereas *MYCN* levels decreased when neuroblastoma cell lines were incubated under hypoxic conditions (Jogi et al., 2002). This increase in *ID2* was observed even in cell lines lacking detectable levels of *MYCN* RNA (Jogi et al., 2002). This result further exemplifies the absence of a relationship between *MYCN* and *ID2* expression. Culture differences also fail to account for the differences in the data on primary neuroblastoma presented by Lasorella et al. (2002) and other investigators, including ourselves, with regard to *ID2*, *MYCN*, and stage in primary neuroblastoma.

The current study, in addition to examining *ID2* mRNA in primary neuroblastomas, thus far has been the only one to semiquantitatively examine ID2 protein in primary samples by Western blot analysis. ID2 was only barely detectable in the primary tumors and was accompanied by several highly immunoreactive bands. Detection was not a technical problem, as we could easily detect ID2 in the cell lines and protein expression of the *ACTB* (β-actin) and *CDKN1B* genes, among others. Be-

cause Western blots are performed under denaturing conditions, it is unlikely that the higher-molecular-weight proteins seen on the blots represent complexes with ID2. On the other hand, if the 12-kDa protein is a degradation product of ID2, then ID2 protein is actually present in most primary neuroblastomas, which is consistent with the data we found about *ID2* transcripts in primary tumors, and transcript and protein expression in the cell lines. It is worth noting that this protein was detected independently of stage or *MYCN* status. Thus, if the 12kDa protein is a degradation product of ID2, it is further refutation of the purported *MYCN*/*ID2* relationship. The ability of this antibody to detect multiple immunoreactive proteins may be a confounding factor in the immunohistologic analysis of ID2 (Lasorella et al., 2002). It is possible that the reported relationship between *ID2* and prognosis observed in the immunohistochemical analysis of ID2 in primary neuroblastomas actually reflects a relationship with a non-ID2-immunoreactive protein. Together with the finding no concrete relationship of *ID2* with stage, prognosis, or *MYCN*, these data suggest that *ID2* is not a major player in neuroblastoma pathogenesis.

Overexpression of CDKN1B induces neuronal differentiation in mouse neuroblastoma cells (Kranenburg et al., 1995). CDKN1B levels increase in human neuroblastoma cell lines undergoing retinoic-acid-induced differentiation (Matsuo and Thiele, 1998; Borriello et al., 2000; Encinas et al., 2000). Differentiating primary neuroblastoma cells exhibited strong nuclear and cytoplasmic staining for CDKN1B, whereas undifferentiated neuroblasts showed only weak nuclear staining and no cytoplasmic staining (Shen et al., 2000). Most significantly, patients harboring CDKN1B-positive tumors survived significantly longer than did those with CDKN1B-negative tumors, with CDKN1B found to be an independent prognostic factor in neuroblastoma independent of *MYCN* status (Bergmann et al., 2001). Consistent with Bergmann et al. (2001), we also observed a significant association of low CDKN1B expression with advanced-stage tumors. Unfortunately, survival data were unavailable for a prognostic analysis of CDKN1B levels.

An inverse relationship between *MYCN* expression and *CDKN1B* levels also has been suggested. For example, it was found that a retinoic-acid-induced decrease in *MYCN* was associated with increased *CDKN1B* expression (Matsuo and Thiele, 1998) and also that adenovirus-mediated constitutive expression of *MYCN* led to decreased *CDKN1B* (Matsuo et al., 2001). Furthermore, when the *MYCN* locus was disrupted in mouse neuronal progenitor cells, these cells demonstrated increased *CDKN1B* expression (Knoepfler et al., 2002). However, in our analysis of *CDKN1B* in primary neuroblastoma, no relationship with *MYCN* amplification was observed, suggesting this is not the mechanism by which *MYCN* amplification is tumorigenic and by which *CDKN1B* levels are decreased in neuroblastoma cells. Nevertheless, our data, together with those of Bergman et al. (2001), add neuroblastoma to the long list of tumors in which a loss of CDKN1B and a poor prognosis can be documented.

CDKN1B works in concert with CDKN2A to regulate cell-cycle progression, with CDKN1B stabilizing the active CDK4/CCND1 complex. CDKN2A acts to inhibit G1 progression, not only by directly inhibiting the CDK4/CCND1 complex, but also by its binding releasing CDKN1B, which then inhibits the CDK2/CCNA1 complex and blocks late-G1– and G2–M progression of the cell cycle (Sherr and Roberts, 1999). We considered that there may be an inverse relationship between *CDKN1B* and *CDKN2A* levels in primary neuroblastoma, with the loss of *CDKN1B* offset by an increase in *CDKN2A* expression. However, despite the apparent inverse relationship between the expression of *CDKN1B* and the expression of *CDKN2A*, this association could not be established. Although loss of *CDKN1B* and expression of *CDKN2A* were both characteristic of advanced-stage neuroblastoma, each was expressed independent of the other. Furthermore, even though both *CDKN1B* and *ID2* are TGF-β-regulated genes (Polyak et al., 1994; Hacker et al., 2003), no relationship was found in the expression of protein by these genes.

Our efforts to understand the mechanism of growth deregulation in neuroblastoma, how *MYCN* acts to drive cellular proliferation, and how neuroblastoma can proliferate despite high expression of the *CDKN2A* tumor-suppressor gene prompted us to investigate the roles of *ID2* and *CDKN1B*. Our data failed to link either gene to the paradoxical expression of *CDKN2A* in neuroblastoma. Similarly, our data refuted the proposed relationship between *ID2*, *MYCN*, and neuroblastoma aggressiveness, suggesting that alternative mechanisms must be responsible for the actions of *MYCN* amplification in the pathogenesis of neuroblastoma. However, our results did support a role for *CDKN1B* in neuroblastoma biology independent of *ID2*, *CDKN2A*, and *MYCN*. Elevated *CDKN2A* expression may be

characteristic of an aggressive phenotype of human tumors, and if so, it is likely to reflect a compensatory response within the cell that is yet to be identified.

ACKNOWLEDGMENTS

Special thanks go to the Children's Oncology Group (COG) neuroblastoma tumor bank, the Cooperative Human Tissue Network, and the many members of the COG for providing the neuroblastoma samples used in this study.

REFERENCES

Beltinger CP, White PS, Sulman EP, Maris JM, Brodeur GM. 1995. No CDKN2 mutations in neuroblastoma. Cancer Res 55:2053–2055.

Benezra R, Davis RL, Lassar A, Tapscott S, Thayer M, Lockshon D, Weintraub H. 1990. Id: a negative regulator of helix–loop–helix DNA binding proteins. Control of terminal myogenic differentiation. Ann NY Acad Sci 599:1–11.

Bergmann E, Wanzel M, Weber A, Shin I, Christiansen H, Eilers M. 2001. Expression of P27(KIP1) is prognostic and independent of *MYCN* amplification in human neuroblastoma. Int J Cancer 95: 176–183.

Borriello A, Pietra VD, Criscuolo M, Oliva A, Tonini GP, Iolascon A, Zappia V, Ragione FD. 2000. p27(Kip1) accumulation is associated with retinoic-induced neuroblastoma differentiation: evidence of a decreased proteasome-dependent degradation. Oncogene 19:51–60.

Brodeur GM. 2002. Significance of intratumoral genetic heterogeneity in neuroblastomas. Med Pediatr Oncol 38:112–113.

Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM. 1984. Amplification of *MYCN* in untreated human neuroblastomas correlates with advanced disease stage. Science 224:1121–1124.

Diccianni MB, Chau LS, Batova A, Vu TQ, Yu AL. 1996. The p16 and p18 tumor suppressor genes in neuroblastoma: implications for drug resistance. Cancer Letters 104:183–192.

Diccianni MB, Omura-Minamisawa M, Batova A, Le T, Bridgeman L, Yu AL. 1999. Frequent deregulation of p16 and the p16/G1 cell cycle-regulatory pathway in neuroblastoma. Int J Cancer 80:145–154.

Dong Y, Walsh MD, McGuckin MA, Gabrielli BG, Cummings MC, Wright RG, Hurst T, Khoo SK, Parsons PG. 1997. Increased expression of cyclin-dependent kinase inhibitor 2 (CDKN2A) gene product P16INK4A in ovarian cancer is associated with progression and unfavourable prognosis. Int J Cancer 74:57–63.

Easton J, Wei T, Lahti JM, Kidd VJ. 1998. Disruption of the cyclin D/cyclin-dependent kinase/INK4/retinoblastoma protein regulatory pathway in human neuroblastoma. Cancer Res 58:2624–2632.

Ellenberger T, Fass D, Arnaud M, Harrison SC. 1994. Crystal structure of transcription factor E47: E-box recognition by a basic region helix–loop–helix dimer. Genes Dev 8:970–980.

Encinas M, Iglesias M, Liu Y, Wang H, Muhaisen A, Cena V, Gallego C, Comella JX. 2000. Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells. J Neurochem 75:991–1003.

Gebauer S, Diccianni M, Gebauer G, Yu A. 2002. P16 expression is not correlated with expression of Id2 or the loss of p27kip1 at protein level in advanced stage neuroblastoma (abstract). Proc Am Assoc Cancer Res 43:624.

Hacker C, Kirsch RD, Ju XS, Hieronymus T, Gust TC, Kuhl C, Jorgas T, Kurz SM, Rose-John S, Yokota Y, Zenke M. 2003. Transcriptional profiling identifies Id2 function in dendritic cell development. Nat Immunol 4:380–386.

Halvorsen OJ, Hostmark J, Haukaas S, Hoisaeter PA, Akslen LA. 2000. Prognostic significance of p16 and CDK4 proteins in localized prostate carcinoma. Cancer 88:416–424.

Iavarone A, Garg P, Lasorella A, Hsu J, Israel MA. 1994. The helix–loop–helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. Genes Dev 8:1270–1284.

Ishiguro A, Spirin KS, Shiohara M, Tobler A, Gombart AF, Israel MA, Norton JD, Koeffler HP. 1996. Id2 expression increases with differentiation of human myeloid cells. Blood 87:5225–5231.

Jan YN, Jan LY. 1993. HLH proteins, fly neurogenesis, and vertebrate myogenesis. Cell 75:827–830.

Jarrard DF, Modder J, Fadden P, Fu V, Sebree L, Heisey D, Schwarze SR, Friedl A. 2002. Alterations in the p16/pRb cell cycle checkpoint occur commonly in primary and metastatic human prostate cancer. Cancer Lett 185:191–199.

Jogi A, Ora I, Nilsson H, Lindeheim A, Makino Y, Poellinger L, Axelson H, Pahlman S. 2002. Hypoxia alters gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype. Proc Natl Acad Sci USA 99:7021–7026.

Kawamata N, Seriu T, Koeffler HP, Bartram CR. 1996. Molecular analysis of the cyclin-dependent kinase inhibitor family: p16(CDKN2/MTS1/INK4A), p18(INK4C) and p27(Kip1) genes in neuroblastomas. Cancer 77:570–575.

Knoepfler PS, Cheng PF, Eisenman RN. 2002. *MYCN* is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. Genes Dev 16:2699–2712.

Kranenburg O, Scharnhorst V, Van der Eb AJ, Zantema A. 1995. Inhibition of cyclin-dependent kinase activity triggers neuronal differentiation of mouse neuroblastoma cells. J Cell Biol 131:227–234.

Kurabayashi M, Jeyaseelan R, Kedes L. 1993. Two distinct cDNA sequences encoding the human helix–loop–helix protein Id2. Gene 133:305–306.

Lasorella A, Iavarone A, Israel MA. 1996. Id2 specifically alters regulation of the cell cycle by tumor suppressor proteins. Mol Cell Biol 16:2570–2578.

Lasorella A, Noseda M, Beyna M, Yokota Y, Iavarone A. 2000. Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. Nature 407:592–598.

Lasorella A, Boldrini R, Dominici C, Donfrancesco A, Yokota Y, Inserra A, Iavarone A. 2002. Id2 is critical for cellular proliferation and is the oncogenic effector of *MYCN* in human neuroblastoma. Cancer Res 62:301–306.

Lassar AB, Davis RL, Wright WE, Kadesch T, Murre C, Voronova A, Baltimore D, Weintraub H. 1991. Functional activity of myogenic HLH proteins requires hetero- oligomerization with E12/E47-like proteins in vivo. Cell 66:305–315.

Lee CT, Capodieci P, Osman I, Fazzari M, Ferrara J, Scher HI, Cordon-Cardo C. 1999. Overexpression of the cyclin-dependent kinase inhibitor p16 is associated with tumor recurrence in human prostate cancer. Clin Cancer Res 5:977–983.

Matsuo T, Thiele CJ. 1998. p27Kip1: a key mediator of retinoic acid induced growth arrest in the SMS-KCNR human neuroblastoma cell line. Oncogene 16:3337–3343.

Matsuo T, Seth P, Thiele CJ. 2001. Increased expression of p27Kip1 arrests neuroblastoma cell growth. Med Pediatr Oncol 36:97–99.

Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB, Weintraub H, Baltimore D. 1989. Interactions between heterologous helix–loop–helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58:537–544.

Nho RS, Sheaff RJ. 2003. p27kip1 contributions to cancer. Prog Cell Cycle Res 5:249–259.

Olson EN, Klein WH. 1994. bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. Genes Dev 8:1–8.

Omura-Minamisawa M, Diccianni MB, Chang RC, Batova A, Bridgeman LJ, Schiff J, Cohn SL, London WB, Yu AL. 2001. p16/p14(ARF) cell cycle regulatory pathways in primary neuroblastoma: p16 expression is associated with advanced stage disease. Clin Cancer Res 7:3481–3490.

Philipp-Staheli J, Payne SR, Kemp CJ. 2001. p27(Kip1): regulation and function of a haploinsufficient tumor suppressor and its misregulation in cancer. Exp Cell Res 264:148–168.

Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A. 1994. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev 8:9–22.

Raetz EA, Kim MK, Moos P, Carlson M, Bruggers C, Hooper DK, Foot L, Liu T, Seeger R, Carroll WL. 2003. Identification of genes that are regulated transcriptionally by Myc in childhood tumors. Cancer 98:841–853.

Rocco JW, Sidransky D. 2001. p16(MTS-1)/CDKN2/INK4a) in cancer progression. Exp. Cell Res 264:42–55.

Sato Y, Kobayashi Y, Sasaki H, Toyama T, Kondo S, Kiriyama M, Fujii Y. 2003. Expression of Id2 mRNA in neuroblastoma and normal ganglion. Eur J Surg Oncol 29:284–287.

Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY,

Hammond D. 1985. Association of multiple copies of the *MYCN* oncogene with rapid progression of neuroblastomas. N Engl J Med 313:1111–1116.

Sgambato A, Cittadini A, Faraglia B, Weinstein IB. 2000. Multiple functions of p27(Kip1) and its alterations in tumor cells: a review. J Cell Physiol 183:18–27.

Shen L, Tsuchida R, Miyauchi J, Saeki M, Honna T, Tsunematsu Y, Kato J, Mizutani S. 2000. Differentiation-associated expression and intracellular localization of cyclin-dependent kinase inhibitor p27KIP1 and c-Jun co-activator JAB1 in neuroblastoma. Int J Oncol 17:749–754.

Sherr CJ. 2004. Principles of tumor suppression. Cell 116:235–246.

Sherr CJ, Roberts JM. 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 13:1501–1512.

Shigemasa K, Hu C, West CM, Clarke J, Parham GP, Parmley TH, Korourian S, Baker VV, O'Brien TJ. 1997. p16 overexpression: a potential early indicator of transformation in ovarian carcinoma. J Soc Gynecol Investig 4:95–102.

Sun XH, Copeland NG, Jenkins NA, Baltimore D. 1991. Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix–loop–helix proteins. Mol Cell Biol 11:5603–5611.

Tanaka T, Slamon DJ, Shimoda H, Waki C, Kawaguchi Y, Tanaka Y, Ida N. 1988. Expression of Ha-ras oncogene products in human neuroblastomas and the significant correlation with a patient's prognosis. Cancer Res 48:1030–1034.

Tweddle DA, Pearson AD, Haber M, Norris MD, Xue C, Flemming C, Lunec J. 2003. The p53 pathway and its inactivation in neuroblastoma. Cancer Lett 197:93–98.

Vandesompele J, Edsjo A, De Preter K, Axelson H, Speleman F, Pahlman S. 2003. Id2 expression in neuroblastoma does not correlate to *MYCN* levels and lacks prognostic value. Oncogene 22:456–460.

Viglietto G, Fusco A. 2002. Understanding p27(kip1) deregulation in cancer: down-regulation or mislocalization? Cell Cycle 1:394–400.

Wang Q, Hii G, Shusterman S, Mosse Y, Winter CL, Guo C, Zhao H, Rappaport E, Hogarty MD, Maris JM. 2003. ID2 expression is not associated with *MYCN* amplification or expression in human neuroblastomas. Cancer Res 63:1631–1635.

Weintraub H. 1993. The MyoD family and myogenesis: redundancy, networks, and thresholds. Cell 75:1241–1244.

AQ1: Note that human genes are designated with capital letters in italics (including numbers) and that the symbols should only contain Latin letters and Arabic numerals (i.e., no Greek letters or Roman numerals) and that there are no dashes or punctuations within the gene designation. All genes should be italicized. Proteins are designated as genes but are not italicized.

AQ2: confusing. just said stage IV is advanced and has poor prognosis.

AQ3: No closing square bracket seen.