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Permalink
https://escholarship.org/uc/item/11c8v74f

Journal
Genes Chromosomes & Cancer, 41(4)

ISSN
1045-2257

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Publication Date
2004-12-01

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

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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-
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; Dicianni 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 (Dicianni 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 (Dicianni 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 non-functional 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 was also 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 S13-KAN, S13-SAN, and S13-KCNR cell lines were a gift from C. Patrick Reynolds (Naval Medical Research Institute, Bethesda, MD). The NMB7 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 μg/ml) were generously provided by J. Biedler (Memorial Sloan-Kettering Cancer Center, New York, NY). The osteosarcoma cell line SJSA-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 μM sodium fluoride, 1 μM 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 Immobilon-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, dH2O 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 fromNeo-markers 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).
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 MgCl₂, 0.04 mM dNTP mix, 0.2 pmol/reaction volume containing 1 μl of each primer (sense: ACTCGCATCCACTATTGGTCAG; antisense: AAAGGTCCATTCAACTTGAAATACGAATCGTTGGTAATG) 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 intron-less 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).

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, 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).

### 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 (85%) 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...
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).

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 1. Expression of ID2 transcript and protein and CDKN1B protein in neuroblastoma cell lines as determined by RT-PCR and Western blot analysis (WB). (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.](image-url)
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

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 66) 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 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, N1723, and N2025). Expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) transcript and ACTB (β-actin) protein is shown as controls.
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 posttranslational. 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 (La-sorella 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. Vandesompele 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 ganglia. 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 increased 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


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.