[CANCER RESEARCH 63, 1631–1635, April 1, 2003]

ID2 Expression Is not Associated with *MYCN* Amplification or Expression in Human Neuroblastomas[1]

Qun Wang, George Hii, Suzanne Shusterman, Yael Mosse, Cynthia L. Winter, Chun Guo, Huaqing Zhao, Eric Rappaport, Michael D. Hogarty, and John M. Maris[2]

Division of Oncology, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104 [Q. W., G. H., S. S., Y. M., C. L. W., C. G., M. D. H., J. M. M.]; Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 [S. S., E. R., M. D. H., J. M. M.]; and Division of Biostatistics, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104 [H. Z.]

ABSTRACT

***MYCN* is a biologically and clinically important oncogene in human neuroblastoma as genomic amplification reliably predicts for aggressive tumor behavior and a poor prognosis. However, the mechanism by which *MYCN* amplification and overexpression contributes to a highly malignant phenotype remains obscure. *ID2* is a dominant inhibitor of the *RB1* tumor suppressor gene product and recently was suggested to be a direct transcriptional target of *MYCN*. Overexpression of Id2 protein has thus been postulated to result in functional inactivation of retinoblastoma in *MYCN*-amplified neuroblastomas, offering a potential explanation for the undifferentiated and highly proliferative nature of most *MYCN*-amplified neuroblastomas, as well as the paucity of retinoblastoma pathway mutations observed in clinical samples. We therefore sought to determine the likelihood that *ID2* overexpression is associated with *MYCN* amplification and overexpression in human neuroblastoma. *ID2* was not differentially expressed in 39 primary neuroblastoma specimens analyzed by oligonucleotide array-based expression analysis, and there was no correlation with *MYCN* expression levels. *ID2* mRNA and protein expression was highly variable and independent of *MYCN* amplification status and mRNA expression in 10 human-derived neuroblastoma cell lines. In addition, *ID2* mRNA expression was not associated with *MYCN* gene amplification status ($P = 0.15$) or *MYCN* expression ($r = 0.22$) in 131 separate diagnostic primary neuroblastoma samples analyzed by real-time quantitative RT-PCR. These data suggest that transcriptional regulation of *ID2* by the MycN oncoprotein is unlikely to be a seminal molecular event resulting in a highly malignant neuroblastoma phenotype.**

INTRODUCTION

Neuroblastoma is a clinically heterogeneous pediatric malignancy that remains responsible for a significant proportion of overall childhood cancer mortality (1). *MYCN* proto-oncogene amplification is the hallmark of the most aggressive subset of human neuroblastomas (2). This genomic alteration is highly associated with metastatic disease at diagnosis and an increased probability of treatment failure. Despite the clear clinical significance of *MYCN* amplification in neuroblastoma (3–5), there is very little information available on how this acquired genetic alteration translates into a more malignant tumor phenotype.

The vast majority of human cancers show cell cycle deregulation through disruption of the G_1-S cell cycle checkpoint. This is often through direct functional inactivation of negative regulators of this transition such as the Rb[3] tumor suppressor gene *RB1*. However, mutations of *RB1* or other genes that negatively regulate passage through G_1-S such as *CDKN2A* are extraordinarily rare in neuroblastomas (6, 7). Id2 is a protein that binds the Rb tumor suppressor and is thought to sequester it from interacting with E2F transcription factors (8). Thus, overexpression of Id2 theoretically has the same functional consequence as underexpression of Rb. Lasorella *et al.* (8) recently showed that all *MYCN*-amplified neuroblastoma cell lines they tested also dramatically overexpressed Id2 protein, whereas Id2 protein was not detectable in the *MYCN* single copy cell lines. In addition, they showed data consistent with Myc proteins directly interacting with the *ID2* promoter and that overexpression of Id2 caused loss of Rb tumor suppressor function and increased cellular proliferation. Lastly, the same investigators recently showed that Id2 protein expression was the most powerful predictor of adverse outcome in a relatively small group of 47 neuroblastoma cases (9). These data suggest that *ID2* is a direct transcriptional target of MycN and that disruption of Id2-Rb interaction might be a focus for future therapeutic intervention in human neuroblastoma.

As part of an ongoing experiment analyzing the neuroblastoma transcriptome in diagnostic primary tumor samples, we noticed that there appeared to be no correlation between *ID2* and *MYCN* mRNA expression levels. Because of the potential clinical significance of the published results, we therefore sought to additionally investigate the relationship between *MYCN* amplification and overexpression with *ID2* expression in human neuroblastoma.

MATERIALS AND METHODS

Samples and RNA. Ten well-characterized human neuroblastoma cell lines (5 with *MYCN* amplification) and 170 primary neuroblastoma samples (27 with *MYCN* amplification) from the COG were available for this study. RNA was extracted from exponentially growing cell lines (before confluence in a T75 flask) or from 50–100 mg of snap-frozen tumor sample using the Total RNA Isolation Kit (Ambion, Houston, TX) following the manufacture's instructions. All RNA samples were subjected to DNase I (Ambion) digestion.

Northern Blot. Thirty μg of total RNA were fractionated on a 1.2% formaldehyde-agarose gel, transferred onto Hybond-N membrane (Amersham Pharmacia Biotech) and UV cross-linked at 120,000 μJ. Equal loading and transfer were verified via ethidium bromide staining. The membrane was then hybridized to a 700-bp *ID2* cDNA probe (isolated from IMAGE clone pT7T3D) that was random labeled with ^{32}P-labeled. Quantitation of Northern data was performed by densitometric analysis of *ID2* expression normalized to 28S rRNA on the ethidium bromide-stained gel (NIH Image v1.62).

Real-Time Quantitative RT-PCR. Two μg of total RNA were used for cDNA synthesis with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time quantitative RT-PCR was performed using an ABI Prism 7700 (Applied Biosystems, Foster City, CA) thermal cycler in a duplex PCR reaction with differentially labeled *ID2*- and 18S RNase-specific primers and probes picked using the Primer Express software v1.5 (Applied Biosystems). Oligonucleotide sequences for *ID2* and *MYCN* cDNA amplification are avail-

Received 11/8/02; accepted 2/3/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported, in part, by the Hope Street Kids Cancer Research Foundation Grant (to Q. W. and J. M. M.) and NIH Grants R01-CA78545 (to J. M. M.), R01-CA87847 (J. M. M.) and U01-CA30969 (Children's Oncology Group), and the Leonard Madelyn Abramson Endowed Chair.

[2] To whom requests for reprints should be addressed, at Division of Oncology, Children's Hospital of Philadelphia, Abramson Research Center, Room 902A, 34th and Civic Center Boulevard, Philadelphia, PA 19104-4318. Phone: (215) 590-2821; Fax: (215) 590-3770; Email: maris@email.chop.edu.

[3] The abbreviations used are: Rb, retinoblastoma protein; COG, Children's Oncology Group; RT-PCR, reverse transcription-PCR; 4-OHT, 4-hydroxy-tamoxifen.

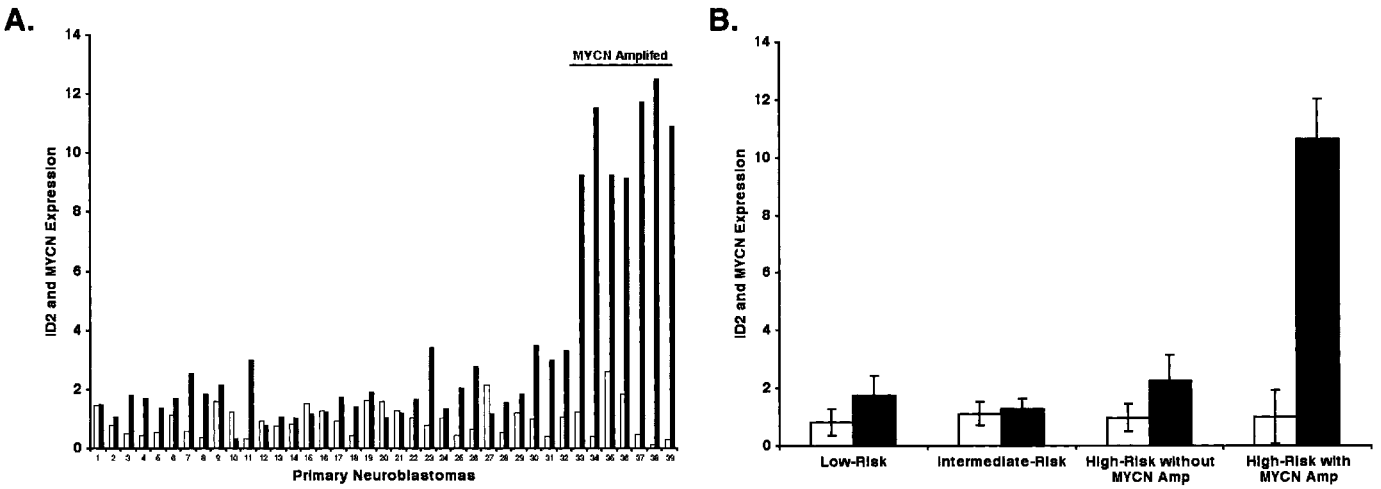

Fig. 1. Relative *ID2* and *MYCN* mRNA expression values derived from oligonucleotide expression analysis of 39 primary neuroblastomas. Expression values for *ID2* (□) and *MYCN* (■) were normalized to the mean expression value of all 12,625 probe sets represented on the chip and represented as relative expression values. Normalization to housekeeping genes such as *glyceraldehyde-3-phosphate dehydrogenase* gave identical results (data not shown). There was no evidence for differential expression of *ID2* in the 39 primary neuroblastomas and no association with *MYCN* expression for individual tumors (*A*) or when summarized by four clinicobiological subsets (*B*): low risk (nos. 1–11), intermediate risk (nos. 12–20), high risk with single copy *MYCN* (nos. 21–32), and high risk with *MYCN* amplification (nos. 33–39).

able online.[4] Relative expression of *ID2* was determined by normalization to both the 18S RNase expression and the *ID2*/18S ratio in normal human brain using the comparative C_T method (10). All real-time quantitative RT-PCR experiments included a no template control and were performed in triplicate.

Western Blot. Cells from 10 exponentially growing neuroblastoma cell lines were harvested at ~75% confluence. Pellets were lysed, and protein concentration was estimated using Coomassie plus-200 Protein Assay Reagent (Bio-Rad, Hercules, CA). Sixty μg of protein were separated by SDS-PAGE in a 15% gel according to standard protocols and transferred onto polyvinylidene difluoride membranes (Bio-Rad). Id2 expression pattern was analyzed using the Id2 rabbit polyclonal antibody C-20 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilution and goat antirabbit horseradish peroxidase second antibody (Santa Cruz Biotechnology) at 1:5000 dilution and detected by enhanced chemiluminescence as described by the manufacturer (Amersham Biosciences, Piscataway, NJ). As a control, a hTERT-immortalized retinal pigment epithelium cell line, hTERT-RPE1 (Clontech, Palo Alto, CA), was retrovirally infected with a *MYCN:ER* expression construct (a kind gift from Dr. William A. Weiss). This construct includes the entire *MYCN*-coding sequence (altered at its 3′ end by changing the stop codon to Leu and the preceding sense codon from Cys to Asp) in frame with the *ER*α subunit, as described for *MYC:ER* (11, 12). This sequence was cloned into the pBabe puro retroviral vector (13), resulting in constitutive expression of MycN:ER. Chaperonins sequester this chimeric protein in the cytosol unless the estrogen receptor ligand, 4-OHT, is present in the culture media, whereby chaperonins are displaced and MycN:ER translocates to the nucleus where it is transcriptionally active. Each blot was stripped and reprobed with an actin-specific antibody (Santa Cruz Biotechnology) and densitometric analysis of the Id2/actin ratio determined with NIH Image v1.62.

Expression Profiling on High-Density Oligonucleotide Arrays. Thirty-nine diagnostic primary tumor samples were histologically examined to confirm >80% tumor content and characterized for allelic status at 1p36 (14), 2p24 (*MYCN*), 11q14-23 (15) and 17q23-25 (16), as well as DNA index (17) and clinical variables, including age at diagnosis, stage, and Shimada histopathology (18). On the basis of these data, 11 samples were designated low risk, 9 samples intermediate risk, 12 samples high risk with single copy *MYCN*, and 7 samples high risk with amplified *MYCN*. Five to 20 μg of total RNA were used to generate cRNA, which was hybridized to Affymetrix HG_U95Av2 oligonucleotide arrays according to manufacturer instructions (Affymetrix, Santa Clara, CA). After scanning, probe cell intensity files were generated with Affymetrix MAS 5.0. Statistical modeling of probe set behavior was conducted using Probe Profiler software (Corimbia, Berkeley, CA). The model weights probe pairs based on the consistency of performance and is intended to decrease the contribution of nonspecific cross-hybridization effects to signal calculation. Probe Profiler also facilitates visualization of several quality control metrics, including overall chip intensity and background noise, as well as hybridization controls and housekeeping genes controls. The resulting gene-specific noise reduction and masking of outliers in the data set improves the overall quality of data extraction before any additional analyses.

We first validated the reproducibility of the Affymetrix U95Av2 platform by examining gene expression profiles from four human neuroblastoma-derived cell lines. Each cell line was analyzed in duplicate with the repeat hybridizations using RNA from the same harvest divided into separate aliquots. Correlation coefficients for each comparison was >0.99. Replicate analysis was not possible for the primary tumor samples, but the use of multiple samples within each risk group permitted consistent trends in the data to be evaluated and no outliers were observed. Expression values for *ID2* and *MYCN* were normalized to the mean expression value of all 12,625 probe sets represented on the chip and reported as relative expression values.

RESULTS AND DISCUSSION

As part of an ongoing study examining gene expression profiles in human neuroblastoma clinicobiological subsets, we first noted that *ID2* expression did not correlate with *MYCN* amplification status or expression. *ID2* and *MYCN* expression data were obtained from high-density oligonucleotide arrays in a set of 39 diagnostic primary neuroblastoma samples. Fig. 1 shows that there was no association between *ID2* mRNA expression and *MYCN* amplification status, *MYCN* mRNA expression, or clinicobiological risk group.

We next examined *ID2* and *MYCN* expression at the mRNA and protein levels in 10 human neuroblastoma-derived cell lines. Both RNA and protein were harvested during the exponential phase of cell growth at 70–80% confluence for all cell lines to avoid any influence of culture conditions on gene expression. Northern blotting showed *ID2* to be differentially expressed with no apparent correlation to *MYCN* gene amplification status (Fig. 2*A*). Meanwhile, we confirmed these finding with real-time quantitative RT-PCR to have an assay useful for analysis of the primary tumor samples. Fig. 2*B* shows that the real-time quantitative RT-PCR data were clearly associated with the level of expression detected by Northern blotting. Importantly, and as shown by others, high *MYCN* expression was only observed in the neuroblastoma cell lines with genomic amplification (Fig. 2*C*). Lastly, Id2 protein was detected in all cell line lysates studied, and there was

[4] Internet address: egenome.chop.edu/oncology/maris/Oligonucleotide.html.

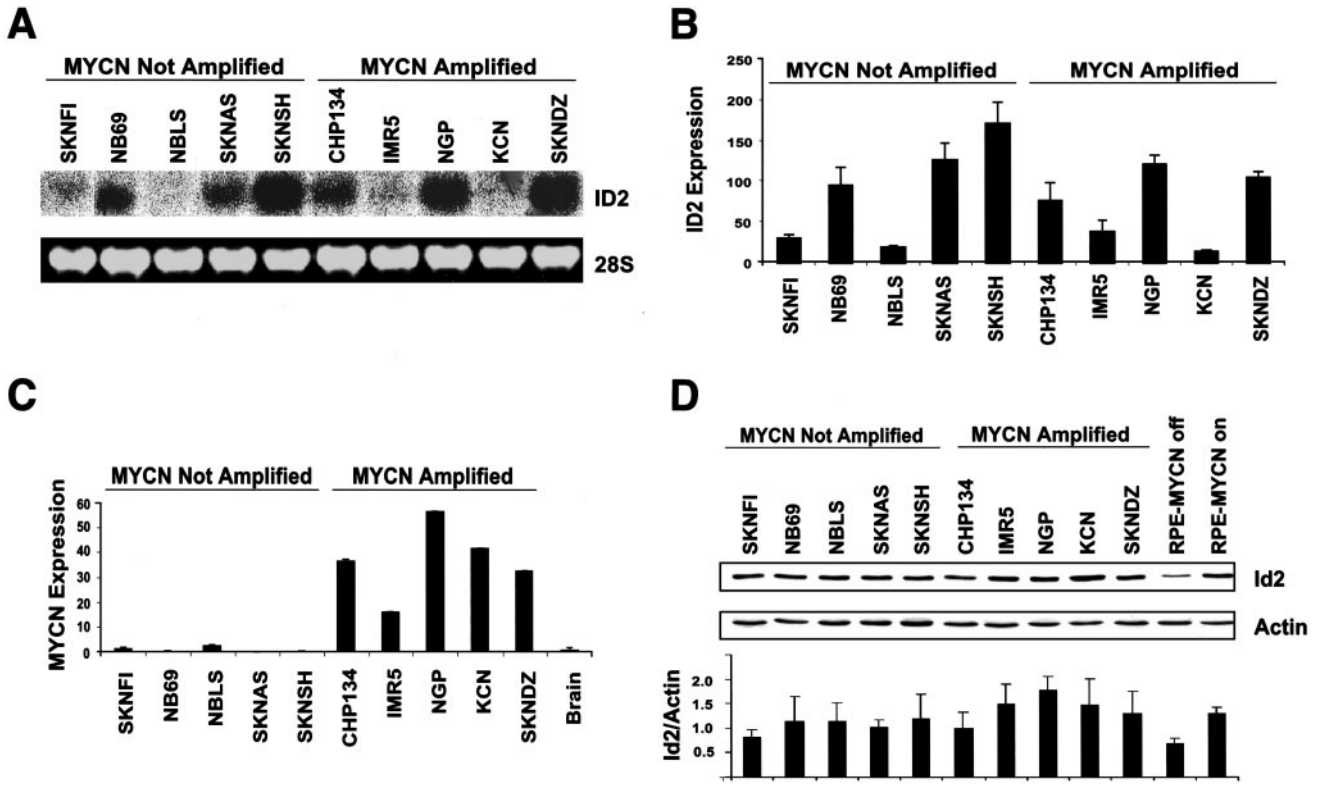

Fig. 2. *ID2* expression in relation to *MYCN* amplification and expression in ten human neuroblastoma-derived cell lines. *A*, Northern blot showing differential expression of *ID2* but no association with *MYCN* amplification status. Quantitation of Northern data were performed by densitometric analysis of *ID2* expression normalized to 28S rRNA on the ethidium bromide stained gel (NIH Image v1.62, data not shown). *B*, *ID2* expression as measured by real-time quantitative RT-PCR shows outstanding concordance with Northern data. *C*, *MYCN* mRNA expression as measured by real-time quantitative RT-PCR is strictly associated with *MYCN* amplification status. *D*, Id2 protein expression is present in all neuroblastoma cell lines tested and is induced by forced expression of *MYCN* (RPE1 cell line) and did not appear strictly correlated to either *ID2* mRNA expression or *MYCN* amplification status. The retinal epithelial cell line RPE1 was used as a control. Functional *MYCN* expression (RPE-MYCN on) was associated with an increase in Id2 expression. Densitometric analysis of the Id2/actin ratio is shown.

no apparent correlation with *MYCN* gene amplification status (Fig. 2*D*).

As a control, we used a functionally inducible *MYCN* system to assess Id2 expression in nontransformed human cells of neuroectoderm origin (see "Materials and Methods"). RPE-*MYCN* cells are derived from human retinal pigment epithelium and constitutively express a chimeric MycN:ER protein that is transcriptionally active only after ER ligand binding. The addition of 4-OHT to the culture media results in the rapid translocation of the chimera to the nucleus and induction of *bona fide MYCN* target genes such as *ODC1* (data not shown). Protein lysates were harvested in the absence (off) and the presence (on) of 400 nM of 4-OHT for 12 h. Id2 expression was relatively low in the absence of functional *MYCN* transcripts, and there was a modest reproducible increase in Id2 mRNA and protein level after induction of functional *MYCN* expression (Fig. 2*D*). These data are consistent with previous observations that *ID2* may be a direct *MYCN* target, although we did not perform additional studies such as chromatin immunoprecipitation to unequivocally demonstrate a protein-DNA interaction at the *ID2* promoter.

Additional studies were performed in 131 separate diagnostic human neuroblastoma samples obtained from patients enrolled on COG Neuroblastoma Biology Studies designed to examine potential new prognostic markers (Table 1). Each specimen was immediately snap frozen and RNA integrity assured by spectrophotometry and gel electrophoresis. In addition, each specimen had *MYCN* gene copy number determined by fluorescence *in situ* hybridization (19). Fig. 3*A* shows that there was no correlation between *ID2* mRNA expression and *MYCN* gene copy status. Indeed, there was a trend toward *ID2* expression being higher in the *MYCN* single copy cohort of cases ($P = 0.15$). We also compared *MYCN* expression as measured by real-time quantitative RT-PCR to *ID2* expression levels. Fig. 3*B* shows that as expected, the majority of *MYCN*-amplified tumors showed high *MYCN* mRNA expression. There was significant overlap

Table 1 *Demographics of 131 neuroblastoma cases*

	No.	Percentage
Median age diagnosis (days)	19.5 months	
Stage		
1	20	19%
2A	8	8%
2B	17	16%
3	21	20%
4	36	34%
4S	4	4%
Not available	25	
MYCN		
Not amplified	111	85%
Amplified	20	15%
DNA index		
Not diploid	77	64%
Diploid	43	36%
Not available	11	
Shimada pathology		
Favorable	55	60%
Unfavorable	37	40%
Not available	39	
1p36 allelic status		
No LOH	68	73%
LOH	25	27%
Not available	37	
11q23 allelic status		
No LOH	69	73%
LOH	26	27%
Not available	36	

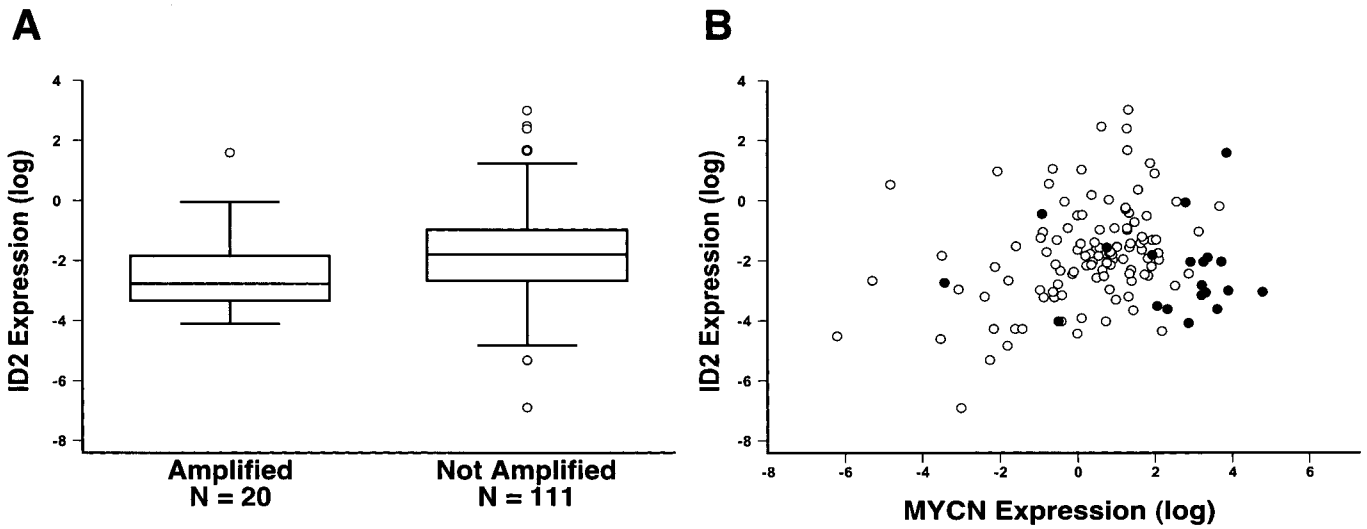

Fig. 3. *ID2* mRNA expression is not associated with *MYCN* amplification status or mRNA expression. *A*, quantitative *ID2* mRNA expression in amplified ($N = 20$) *versus* nonamplified ($N = 111$) primary neuroblastomas. Log-transformed real-time PCR data are represented as *box plots*: *box* represents 25th percentile through the 75th percentile (median value or 50th percentile denoted by *line through box*), and *whiskers* above and below extend to the 90th and 10th percentiles, respectively. There is no association between *ID2* mRNA expression and *MYCN* amplification status, and there is a trend toward *ID2* values being higher in the *MYCN*-amplified tumors ($P = 0.15$). *B*, *scatter plot* of log-transformed *ID2* and *MYCN* mRNA expression values showing no detectable association ($r = 0.22$). Twenty *MYCN*-amplified tumors denoted by ●.

in *MYCN* mRNA expression between the amplified and nonamplified samples when mRNA expression was measured by this technique, but there was no correlation between *MYCN* and *ID2* mRNA expression ($r = 0.22$). The one *MYCN*-amplified tumor with very low *MYCN* mRNA expression was a stage 2A tumor with focal *MYCN* amplification and significant intratumoral heterogeneity demonstrated by fluorescence *in situ* hybridization (data not shown). Therefore, both the *MYCN* and *ID2* expression levels in this one sample likely do not represent the *MYCN*-amplified component. Taken together, these primary tumor data are consistent with *ID2* mRNA expression levels that are independent of both *MYCN* gene copy number and mRNA expression level. Because the specimens for this study were ascertained in the last 18 months, outcome analyses were not performed.

The mechanism by which *MYCN* amplification and overexpression contribute to a highly malignant tumor phenotype in human neuroblastoma remains an enigma. The fact that targeted expression of *MYCN* to the murine neural crest results in a tumor phenotype that closely recapitulates human neuroblastoma (20) strongly suggests that aberrant *MYCN* expression is an intrinsic and fundamental molecular genetic alteration in a subset of these embryonal malignancies. However, the molecular effectors of *MYCN* overexpression in neural crest precursor cells have not been clearly defined. The hypothesis that *ID2* is a *MYCN* target gene that results in functional inactivation of Rb through sequestration (8) is therefore of both biological and clinical relevance. This hypothesis provides an appealing explanation for the apparent lack of mutations or aberrant expression discovered in G_1-S checkpoint control genes such as *RB1*, *CCND1* (cyclin D), *CDK4*, *CDK6*, and *CDKN2A* in the majority of human neuroblastoma samples (6, 7, 21, 22). It would also provide a biochemical link between the observations of *MYCN* amplification associated with an undifferentiated and highly proliferative subset of malignancies. Lastly and perhaps most importantly, the recent observation that the *ID2* expression was the most powerful predictor of survival in a relatively small cohort of 47 neuroblastoma cases (9) suggested that these observations could provide prognostic information and perhaps a target for rationally designed therapeutics.

Data from the RPE-*MYCN* cell line suggest that forced expression of *MYCN* results in an increase in *ID2* mRNA and protein expression, but these experiments were not designed to demonstrate unequivocally that *ID2* is indeed a direct *MYCN* target gene. Because *MYC* and *MYCN* are rather promiscuous, albeit relatively weak, transactivators, and additional studies such as chromatin immunoprecipitation assays will be required to additionally support *ID2* as a direct transcriptional target of the *MYCN* protein. Our data simply show a temporal correlation of *MYCN* protein expression and increased ID2 levels in a neuroepithelial cell line system that was used as a positive control for our neuroblastoma cell line protein expression experiments.

Despite the apparent association of forced *MYCN* expression with increased *ID2* protein levels, our data clearly demonstrate no association between *MYCN* amplification status and *ID2* mRNA expression in human neuroblastoma-derived cell lines or primary tumors. *ID2* expression at the mRNA level was present in all cell lines (by real-time quantitative RT-PCR) but was highly variable. Indeed, the highest expression levels were observed in the SKNAS and SKNSH cell lines, both of which do not harbor *MYCN* amplification or overexpression. Likewise, *ID2* protein was detectable in each cell line studied but again with no apparent correlation with *MYCN* amplification status. The primary tumor data from both real-time quantitative RT-PCR and oligonucleotide arrays confirmed these observations. It is important to emphasize that this study focused on the transcriptional regulation of *ID2* and that Id2 protein levels were only examined in the human neuroblastoma-derived cell lines. It is possible that post-transcriptional regulation of Id2 is different depending on *MYCN* amplification status and that an immunohistochemical study of Id2 protein expression would have provided different results, but this seems unlikely based on our cell line data showing no evidence for differential Id2 expression by Western blotting.

Taken together, our data demonstrate that *ID2* mRNA expression is not associated with *MYCN* amplification status or expression levels in human neuroblastoma. It therefore seems unlikely that *ID2* expression would serve as an independent prognostic marker for disease outcome, although we were unable to address this directly with our sample set. Although the Id protein are clearly critical regulators of normal human development, we could find no direct evidence that aberrant *ID2* expression was the result of *MYCN* overexpression or part of the downstream effector arm of this genomic alteration.

REFERENCES

1. Brodeur, G. M., and Maris, J. M. Neuroblastoma. *In:* P. A. Pizzo and D. G. Poplack (eds.), Principles and Practice of Pediatric Oncology, Ed. 4, pp. 895–938. Philadelphia: J. B. Lippincott Company, 2002.
2. Maris, J. M., and Matthay, K. K. Molecular biology of neuroblastoma. J. Clin. Oncol., *17:* 2264–2279, 1999.
3. Schmidt, M. L., Lukens, J. N., Seeger, R. C., Brodeur, G. M., Shimada, H., Gerbing, R. B., Stram, D. O., Perez, C., Haase, G. M., and Matthay, K. K. Biologic factors determine prognosis in infants with stage IV neuroblastoma: a prospective Children's Cancer Group study. J. Clin. Oncol., *18:* 1260–1268, 2000.
4. Katzenstein, H. M., Bowman, L. C., Brodeur, G. M., Thorner, P. S., Joshi, V. V., Smith, E. I., Look, A. T., Rowe, S. T., Nash, M. B., Holbrook, T., Alvarado, C., Rao, P. V., Castleberry, R. P., and Cohn, S. L. Prognostic significance of age. MYCN oncogene amplification, tumor cell ploidy, and histology in 110 infants with stage D(S) neuroblastoma: the pediatric oncology group experience: a pediatric oncology group study. J. Clin. Oncol., *16:* 2007–2017, 1998.
5. Rubie, H., Hartmann, O., Michon, J., Frappaz, D., Coze, C., Chastagner, P., Baranzelli, M. C., Plantaz, D., Avet-Loiseau, H., Benard, J., Delattre, O., Favrot, M., Peyroulet, M. C., Thyss, A., Perel, Y., Bergeron, C., Courbon-Collet, B., Vannier, J. P., Lemerle, J., and Sommelet, D. N-Myc gene amplification is a major prognostic factor in localized neuroblastoma: results of the French NBL 90 study. Neuroblastoma Study Group of the Societe Francaise d'Oncologie Pediatrique. J. Clin. Oncol., *15:* 1171–1182, 1997.
6. Beltinger, C. P., White, P. S., Sulman, E. P., Maris, J. M., and Brodeur, G. M. No CDKN2 mutations in neuroblastomas. Cancer Res., *55:* 2053–2055, 1995.
7. Thompson, P. M., Maris, J. M., Hogarty, M. D., Seeger, R. C., Reynolds, C. P., Brodeur, G. M., and White, P. S. Homozygous deletion of CDKN2A (p16INK4a/p14ARF) but not within 1p36 or at other tumor suppressor loci in neuroblastoma. Cancer Res., 679–686, 2001.
8. Lasorella, A., Noseda, M., Beyna, M., Yokota, Y., and Iavarone, A. Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. Nature (Lond.), *407:* 592–598, 2000.
9. Lasorella, A., Boldrini, R., Dominici, C., Donfrancesco, A., Yokota, Y., Inserra, A., and Iavarone, A. Id2 is critical for cellular proliferation and is the oncogenic effector of N-myc in human neuroblastoma. Cancer Res, *62:* 301–306, 2002.
10. De Preter, K., Speleman, F., Combaret, V., Lunec, J., Laureys, G., Eussen, B. H., Francotte, N., Board, J., Pearson, A. D., De Paepe, A., Van Roy, N., and Vandesompele, J. Quantification of MYCN, DDX1, and NAG gene copy number in neuroblastoma using a real-time quantitative PCR assay. Mod. Pathol., *15:* 159–166, 2002.
11. Jiang, X. R., Jimenez, G., Chang, E., Frolkis, M., Kusler, B., Sage, M., Beeche, M., Bodnar, A. G., Wahl, G. M., Tlsty, T. D., and Chiu, C. P. Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. Nat. Genet., *21:* 111–114, 1999.
12. Eilers, M., Picard, D., Yamamoto, K. R., and Bishop, J. M. Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. Nature (Lond.), *340:* 66–68, 1989.
13. Morgenstern, J. P., and Land, H. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res., *18:* 3587–3596, 1990.
14. Maris, J. M., Weiss, M. J., Guo, C., Gerbing, R. B., Stram, D. O., White, P. S., Hogarty, M. H., Sulman, E. P., Thompson, P. M., Lukens, J. N., Matthay, K. K., Seeger, R. C., and Brodeur, G. M. Loss of heterozygosity at 1p36 independently predicts for disease progression, but not decreased overall survival probability, in neuroblastoma patients: a Children's Cancer Group Study. J. Clin. Oncol., *18:* 1888–1899, 2000.
15. Guo, C., White, P. S., Weiss, M. J., Hogarty, M. D., Thompson, P. M., Stram, D. O., Gerbing, R., Matthay, K. K., Seeger, R. C., Brodeur, G. M., and Maris, J. M. Allelic deletion at 11q23 is common in *MYCN* single copy neuroblastomas. Oncogene, *18:* 4948–4957, 1999.
16. Bown, N., Cotterill, S., Lastowska, M., O'Neill, S., Pearson, A. D., Plantaz, D., Meddeb, M., Danglot, G., Brinkschmidt, C., Christiansen, H., Laureys, G., and Speleman, F. Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. N. Eng. J. Med., *340:* 1954–1961, 1999.
17. Look, A. T., Hayes, F. A., Shuster, J. J., Douglass, E. C., Castleberry, R. P., Bowman, L. C., Smith, E. I., and Brodeur, G. M. Clinical relevance of tumor cell ploidy and n-myc gene amplification in childhood neuroblastoma: a Pediatric Oncology Group Study. J. Clin. Oncol., *9:* 581–591, 1991.
18. Shimada, H., Ambros, I. M., Dehner, L. P., Hata, J., Joshi, V. V., and Roald, B. The international neuroblastoma pathology classification (Shimada) system. Cancer (Phila.), *86:* 364–372, 1999.
19. Mathew, P., Valentine, M. B., Bowman, L. C., Rowe, S. T., Nash, M. B., Valentine, V. A., Cohn, S. L., Castleberry, R. P., Brodeur, G. M., and Look, A. T. Detection of MYCN gene amplification in neuroblastoma by fluorescence in situ hybridization: a pediatric oncology group study. Neoplasia, *3:* 105–109, 2001.
20. Weiss, W. A., Aldape, K., Mohapatra, G., Feuerstein, B. G., and Bishop, J. M. Targeted expression of MYCN causes neuroblastoma in transgenic mice. EMBO J., *16:* 2985–2995, 1997.
21. Kawamata, N., Seriu, T., Koeffler, H. P., and Bartram, C. R. Molecular analysis of the cyclin-dependent kinase inhibitor family: p16(CDKN2/MTS1/INK4A), p18(INK4C) and p27(Kip1) genes in neuroblastomas. Cancer (Phila.), *77:* 570–575, 1996.
22. Omura-Minamisawa, M., Diccianni, M. B., Chang, R. C., Batova, A., Bridgeman, L. J., Schiff, J., Cohn, S. L., London, W. B., and Yu, A. L. p16/p14(ARF) cell cycle regulatory pathways in primary neuroblastoma: p16 expression is associated with advanced stage disease. Clin. Cancer Res., *7:* 3481–3490, 2001.

ID2 Expression Is not Associated with *MYCN* Amplification or Expression in Human Neuroblastomas

Qun Wang, George Hii, Suzanne Shusterman, et al.

Cancer Res 2003;63:1631-1635.