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

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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 oncogene 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 G1-S cell cycle checkpoint. This is often through direct functional inactivation of negative regulators of this transition such as the Rb3 tumor suppressor gene RB1. However, mutations of RB1 or other genes that negatively regulate passage through G1-S such as CDKN2A are extraordinarily rare in neuroblastos (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 manufacturer’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 32P-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 available upon request.

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3 The abbreviations used are: Rb, retinoblastoma protein; COG, Children’s Oncology Group; RT-PCR, reverse transcription-PCR; 4-OHT, 4-hydroxy-tamoxifen.
able online. 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 β-actin antibody (Santa Cruz Biotechnology) at 1:5000 dilution and detected using the Id2 rabbit polyclonal antibody C-20 (Santa Cruz Biotechnology, Santa Clara, CA). After scanning, probe cell intensity files were generated with microarray software and gene expression levels were normalized to the mean expression value of all 12,625 probe sets represented on the chip and reported 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).

**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. 2A). Meanwhile, we confirmed these findings with real-time quantitative RT-PCR to have an assay useful for analysis of the primary tumor samples. Fig. 2B 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. 2C). Lastly, Id2 protein was detected in all cell lines lysates studied, and there was 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.

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* Internet address: egenome.chop.edu/oncology/maris/Oligonucleotide.html.
no apparent correlation with MYCN gene amplification status (Fig. 2D).

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. 2D). 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. 3A 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. 3B 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

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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.
MYCN mRNA expression was a stage 2A tumor with focal MYCN/H11005 (fluorescence cation 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 G1-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 posttranscriptional 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


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