Id2 Is Critical for Cellular Proliferation and Is the Oncogenic Effector of N-Myc in Human Neuroblastoma

Anna Lasorella, Renata Boldrini, Carlo Dominici, Alberto Donfrancesco, Yoshifumi Yokota, Alessandro Inserra, and Antonio Iavarone

Departments of Neurology [A. L., A. I.] and Developmental and Molecular Biology [A. I.], Albert Einstein College of Medicine, Bronx, New York 10461; Departments of Pathology [R. B.], Pediatric Oncology [A. D., C. D.], and Surgery [A. I.], Bambino Gesù’ Children Hospital, Rome 00165, Italy; Department of Pediatrics, La Sapienza University, Rome 00161, Italy [C. D.]; and Department of Biochemistry, Fukui Medical University, Fukui 910-1193, Japan [Y. Y.]

ABSTRACT

Perturbation of the function of the retinoblastoma (Rb) protein is found in most human tumors. Id2 is a natural target of the Rb protein that is recruited by Myc oncoproteins to bypass the tumor suppressor function of Rb. Here we report that an “N-Myc-Id2 pathway” persists during late development of the nervous system and parallels the rising levels of active Rb in neuronal precursors withdrawing from the cell cycle. An immuno-histochemical analysis of primary neuroblastoma from 47 patients shows that expression of Id2 is strongly predictive of poor outcome, irrespective of other clinical and biological variables. Overexpression of Id2 mediates cellular transformation and is required to maintain the malignant behavior of neuroblastoma cells. Correspondingly, embryonic fibroblasts from Id2-null mice display impaired ability to proliferate. We suggest that Id2 overexpression may be a better prognostic indicator than N-myc gene amplification in neuroblastoma. Thus, disrupting Id2 function may lead to new and useful therapeutic strategies for cancer patients.

INTRODUCTION

Id proteins are “integrators” of positive and negative environmental stimuli to the transcriptional machinery that regulates differentiation (1, 2). One member of the Id family, Id2, coordinates inhibition of differentiation and stimulation of cell proliferation by inactivating the Rb3 tumor suppressor protein (3, 4). Id2 operates, at least in part, under control of Myc proto-oncogenes, which directly bind to and activate the Id2 promoter (5). By raising Id2 levels, Myc proteins circumvent the block on cell cycle progression imposed by the Rb pathway. Not surprisingly, tumor cells with oncogenic activation of Myc, such as the neuroblastoma cell lines carrying N-myc gene amplification, overexpress Id2 to constitutively bypass the cell cycle checkpoint imposed by Rb (5).

Previous studies proposed that the different biological properties of Rb and Rb family members result from their ability to regulate E2F transcription factors (6–8). Our recent results from genetic intercrosses of Rb and Id2 knockout mice indicated that negative control of Id2 activity is an additional requirement for Rb function in vivo (5). Here we analyzed the expression of N-Myc and Id2 during normal development and in primary neuroblastoma. Because of the crucial importance ascribed to loss of Rb function in human cancer, we investigated whether overexpression of Id2 induced cellular transformation and predicted clinical outcome in children with neuroblastoma. Having found that Id2 expression is a prognostic determinant in neuroblastoma, we asked whether inhibition of Id2 could serve as a point of intervention in cancer by studying the consequences of eliminating Id2 from primary and tumor cells.

MATERIALS AND METHODS

Patient Population and Statistical Methods. We analyzed 47 primary neuroblastoma samples from 47 patients diagnosed and treated between 1991 and 2000 at the Ospedale Pediatrico Bambino Gesù’ (Rome, Italy). Median follow-up was 46 months (range, 12–108 months). Staging was performed according to the International Neuroblastoma Staging System criteria. The patient population included 8 stage 1, 8 stage 2, 9 stage 3, 21 stage 4, and 1 stage 4S. Stages 1, 2, and 4S were classified as favorable, whereas stages 3 and 4 were unfavorable. Event-free survival time for each patient was defined as the time from the date of diagnosis to the date of earliest occurrence of recurrence, disease progression, or death resulting from any cause. The Pearson correlation coefficient was used to test the strength of association between the continuous variables. Associations among Id2 expression and other prognostic variables were examined by $\chi^2$ test. Survival analyses were performed according to the method of Kaplan and Meier, and comparisons of outcome between subgroups were performed by the log-rank test for univariate comparisons. For multivariate analysis, Cox’s proportional hazard regression model was applied. Statistical analyses were performed using StatView 4.1 (Abacus Concepts, Inc., Berkeley, CA).

Immunohistochemistry. Antibodies used for immunohistochemistry included a previously characterized anti-N-Myc monoclonal antibody at concentration of 10 $\mu$g/ml (Ref. 9; Oncogene Research Products, Boston, MA) and the anti-id2 polyclonal antibody C-20 at concentration of 2 $\mu$g/ml (Santa Cruz Biotechnology, Santa Cruz, CA). Specificity of Id2 immunostaining was assessed by preabsorption of the antibody with the peptide from which the antibody was raised. This, but not preabsorption with a nonrepetitive peptide, abolished Id2 staining. Controls using polyclonal rabbit immunoglobulin instead of primary antibody showed no evidence of staining. Also, tissues from Id2 null mice provided a negative control for Id2 antibody (Fig. 1c). Immunohistochemical results of N-Myc and Id2 staining in neuroblastoma were scored independently by two investigators (A. L. and A. I.). At least 600 cells from 5 to 10 high-power fields were scored for each tumor sample.

Immunoblot Analysis. Mouse embryonic tissues were collected on dry ice, and proteins were extracted with RIPA buffer in the presence of protease and phosphatase inhibitors. The antibodies used for immunoblotting were anti-Id2 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), anti-N-Myc (Oncogene Research Products), and anti-Rb (G3-245; Pharmingen, San Diego, CA).

Growth Experiments and Antisense Oligonucleotide Transfections. Primary MEFs were obtained from 13.5-day-old embryos and cultured as described (5). Passage 4 cells were plated at density of $3 \times 10^5$/35-mm dish and counted at the indicated days. Unless specified otherwise, cells were cultured in DMEM supplemented with 10% fetal bovine serum (Sigma Chemical Co.). Retroviral infection of NIH 3T3 was done as described (5). For saturation density experiments, cells were plated in DMEM containing 5% fetal bovine serum and cultivated for up to 7 days. Cells were counted daily with a hemocytometer. For growth in soft agar of NIH 3T3 derivatives and LAN1, 5 $\times 10^5$ and 1 $\times 10^6$ cells, respectively, were plated in 35-mm dishes in 0.33% agar solution (BiTek; Difco) in DMEM containing 5% fetal bovine serum. The bottom layer was prepared using 0.6% agar in growth medium.

Received 8/14/01; accepted 11/15/01.

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 by Grant RO1-CAB85628 from the NIH (to A. I.). A. I. is recipient of a Siminheimer Scholar award.

2 To whom requests for reprints should be addressed, at Department of Neurology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461.

3 The abbreviations used are: Rb, retinoblastoma; MEF, mouse embryonic fibroblast; BrdUrd, 5-bromodeoxyuridine; LI, labeling index; GFP, green fluorescent protein.
analyzed for the ability to incorporate BrdUrd and form colonies in soft agar.

RESULTS

N-Myc and Id2 Display an Overlapping Pattern of Expression during Development. We performed an immunohistochemical analysis of N-Myc and Id2 proteins in the mouse embryo at mid-gestation (embryonic day 15, E15). At this stage of development, Rb is essential for cell cycle withdrawal and differentiation of neural progenitors (10, 11). We found a striking overlap between the pattern of expression of N-Myc and Id2 in epithelial and neural tissues (Fig. 1). Lung is an example of abundant expression of N-Myc and Id2 proteins in the epithelium without any detectable signal in the adjacent mesenchyme (Fig. 1, a–c). A similar pattern of expression is present in the small intestine (data not shown). During early neurogenesis (before day E12), N-Myc and Id2 are expressed in the telencephalon throughout the proliferating ventricular zone (12–14). However, at E15, the two proteins appear in the intermediate zone, which predominantly contains postmitotic neurons (Fig. 1, d and e). Positive staining in the ventricular and subventricular layers is detectable only in small areas of the olfactory lobe and the hippocampus (data not shown). N-Myc and Id2 are also abundant in large, differentiated neurons of dorsal root and trigeminal ganglia, which fail to incorporate BrdUrd at this stage of development (Fig. 1, f and g, and data not shown). To determine at which time active Rb appears in the brain and how it relates with N-Myc and Id2, we compared expression of N-Myc, Id2, and Rb in embryonic and postnatal brains by Western immunoblot analysis (Fig. 1h). Although expression of N-Myc and Id2 decreases with development, significant amounts of the two proteins are found at E15, and they are still detectable after 9 days of postnatal life. Rb undergoes a change from hypophosphorylated (abundant at E10) to hypophosphorylated, active forms by E15. Thus, expression of N-Myc and Id2 is detectable at midgestation in predominantly postmitotic neuroectodermal tissues and is paralleled by a shift of Rb from inactive to active forms.

Id2 Expression Is Predictive of Poor Survival of Neuroblastoma Patients. To determine whether the “N-Myc-Id2 pathway” is activated in neuroblastoma cells before tissue culture and whether Id2 expression correlates with clinical behavior, we conducted an immunohistochemical analysis for the proteins N-Myc and Id2 in 47 primary neuroblastomas. N-Myc and Id2 were not expressed in postnatal adrenal medulla, the most common site of origin of neuroblastoma in humans (data not shown). N-Myc and Id2 were either absent (‘negative’ samples; Fig. 2, a and b) or detectable in the nuclei of 25% or more tumor cells (“positive” samples; Fig. 2, c–f). In our series there were 8 tumors with N-myc gene amplification (Table 1). All of them showed positive staining for N-Myc and Id2. Among the 37 tumors without N-myc gene amplification, 19 had N-Myc protein expression. These findings confirm results from previous studies, which indicated that mechanisms other than gene amplification lead to deregulated expression of N-Myc in a significant number of neuroblastomas (9, 15, 16). With the exception of one case (N-Myc negative/Id2 positive), we found an invariable correlation between the expression of N-Myc and Id2 (28 tumors were N-Myc positive/Id2 positive; 18 tumors were N-Myc negative/Id2 negative). We scored the percentage of neuroblastoma cells in each tumor that stained positive for N-Myc and Id2. The correlation was determined by the Pearson correlation coefficient (0.868; P < 0.0001; Fig. 2g). Next, we examined the correlation between Id2 expression and clinical behavior of neuroblastoma. On all patients, the cumulative overall and event-free survival of Id2-negative tumors were 0.877 ± 0.082 and 0.889 ± 0.074 versus 0.417 ± 0.103 and
0.472 ± 0.094 for Id2-positive tumors (Fig. 3, a and b). The Kaplan-Meier analysis showed that Id2 expression was predictive of increased mortality (Fig. 3, a and b; log-rank P = 0.0046 and 0.0065 for overall and event-free survival, respectively). Outcome of neuroblastoma patients of ages <1 year is good, regardless of other prognostic variables (17). Conversely, patients with N-myc gene amplification display an invariably poor prognosis (18). We therefore asked whether Id2 remained a predictive indicator for patients older than 1 year of age and/or lacking N-myc gene amplification. Kaplan-Meier plots of event-free survival showed that Id2 expression was associated with increased mortality in each subgroup (Fig. 3, c–e). χ² analysis demonstrated a positive correlation between Id2 expression and unfavorable clinical stages (Table 1; P = 0.0051). When outcome was evaluated in this subgroup of patients, there was a trend toward a correlation between Id2 expression and low event-free survival (P = 0.12; Fig. 3f). In a multivariate Cox proportional hazard model that included Id2 expression (positive), age (<1 year), and N-myc copy number (>3 copies), Id2 expression was the strongest independent predictor of disease-free survival (P = 0.0264; relative hazard, 10.996; Table 2).

Id2 Transforms NIH 3T3 and Is a Rate-limiting Factor for Proliferation of Fibroblasts and Neuroblastoma. To determine whether the large amounts of Id2 expressed by neuroblastoma cells are sufficient to induce transformation, we introduced Id2 in NIH 3T3
cells. NIH 3T3 transduced with LZRS-GFP-Id2 retrovirus (NIH 3T3-Id2) expressed levels of Id2 protein comparable with the endogenous levels of Id2 in neuroblastoma cells carrying amplification and overexpression of the N-myc oncogene (Fig. 4a). NIH 3T3-Id2 cells overcame the serum requirement of vector-infected NIH 3T3 for entry into S-phase and long-term proliferation (Fig. 4b and data not shown). In the presence of high levels of Id2, cells overgrew a monolayer doubling the saturation density of control cells (Fig. 4c). As a further sign of transformation, when cultured in soft agar, NIH 3T3-Id2 formed distinct foci (29 ± 5 colonies/well), which were absent in cultures of vector-infected NIH 3T3 (Fig. 4d). However, NIH 3T3-Id2 were not tumorigenic when injected in nude mice (data not shown).

To assess the consequences of loss of Id2 function on cell proliferation, we analyzed MEFs derived from Id2<sup>−/−</sup>/Id2<sup>−/−</sup> and Id2<sup>+/−</sup>/Id2<sup>+/−</sup> embryos. Id2<sup>−/−</sup> MEFs showed a noticeably lower rate of division (Fig. 4e). The defective ability to grow persisted when we compared 3T3 derivatives from Id2<sup>−/−</sup> MEFs with their wild-type counterparts, indicating that immortalization was not sufficient to overcome the requirement for Id2 (data not shown). We next sought to determine

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Kaplan-Meier curves of neuroblastoma patients according to Id2 status. Id2 expression is associated with decreased overall (a) and event-free survival (b) in the entire series of patients tested. Event-free survival for patients without N-myc amplification (c), age >1 year (d), age >1 year without N-myc amplification (e), and unfavorable stages (f).
whether opposing the uncontrolled expression of Id2 in neuroblastoma cells could affect their malignant behavior. For these experiments, we used the neuroblastoma cell line LAN1 that carries N-myc amplification and Id2 overexpression (5). Treatment of LAN1 with a phosphothioate Id2 antisense oligonucleotide for 24 h led to a decrease of endogenous Id2 by ~60% compared with a mismatched oligonucleotide (Fig. 4f). Consistent with reduction of Id2, BrdUrd incorporation showed decreased S-phase entry (15% versus 28% in mismatched treated cells; Fig. 4g). Interestingly, reduction of Id2 protein levels in LAN1 led to an even stronger effect on the ability of these cells to form colonies in soft agar (from 41 ± 3 colonies/well for mismatched treated LAN1 to 12 ± 2 colonies/well for anti-Id2-treated cells; Fig. 4h). These results suggest that lowering Id2 in neuroblastoma may generate antitumor mechanisms involving more than antiproliferative effects.

**DISCUSSION**

During development of the nervous system, Rb is not required for induction of neuronal determination and migration of neural progenitors from the ventricular zone to postmitotic areas of the brain, but it is essential for neurons to exit the cell cycle and survive (10). In the absence of Rb, neurons undergo ectopic proliferation and apoptosis (11). These defects are rescued by ablation of Id2 in vivo and are recapitulated in vitro by overexpressing Id2 in normal cortical progenitors (5, 19). Previous studies proposed that loss of Id proteins from the proliferating zones of the brain, which occurs during early neurogenesis, initiates neuronal gene expression and differentiation (13, 14, 20). We suggest that permanent elimination of Ids from the ventricular zone renders these early events insensitive to the genetic inactivation of Rb. However, persistent expression of the "N-Myc-Id2 pathway" in postmitotic areas of the central nervous system and peripheral nervous system may dictate the window of Rb requirement. In this model, Rb is essential during midgestation in postmitotic areas of the nervous system to control Id2 function and execute terminal cell cycle withdrawal, complete differentiation, and secure survival of neuronal cells.

To our knowledge, Id2 is the first transcriptional target of N-Myc, the expression of which correlates with the N-Myc protein during

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Relative hazard</th>
<th>Confidence interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Id2 expression (positive)</td>
<td>10.996</td>
<td>1.325–91286</td>
<td>0.0264</td>
</tr>
<tr>
<td>Age (&lt;1 year)</td>
<td>0.122</td>
<td>0.015–1.010</td>
<td>0.0511</td>
</tr>
<tr>
<td>N-myc amplification (≥3 copies)</td>
<td>1.733</td>
<td>0.583–5.155</td>
<td>0.3288</td>
</tr>
</tbody>
</table>

* n = 47.
development and in neuroblastoma. The immunohistochemical analysis of Id2 in neuroblastoma shows that tumor aggressiveness depends, at least in part, on activation of the N-Myc-Id2 pathway. In our series, among the 17 patients who relapsed and/or died, 15 (88%) overexpressed Id2 and 2 (12%) did not. Therefore, the vast majority of prognostically unfavorable neuroblastoma deregulates Id2 expression. Although N-myc gene amplification is a well-established adverse prognostic indicator in neuroblastoma, the significance of N-Myc protein expression is still debated (9, 15, 16, 21). In our series, expression of Id2 correlates with N-Myc (Fig. 2g). However, the unique feature of assaysing Id2 in neuroblastoma is that its expression integrates the effects of N-myc activation and possibly other upstream signals to overcome the crucial tumor suppressor function of the Rb pathway. A number of studies suggested that inactivation of the Rb pathway is a determinant of poor prognosis for cancer patients (22–24). We propose that neuroblastoma with favorable outcome may retain a functional Rb pathway, whereas Id2-independent mechanisms for Rb inactivation may be present in the small subgroup of unfavorable, Id2-negative neuroblastoma. The analysis of Id2 expression has considerable potential to be of practical use in the routine assessment of neuroblastoma patients. This is strengthened by the fact that immunohistochemical tests for Id2 expression are inexpensive and could easily be available to most medical centers.

Our studies on the role of Id2 in cellular proliferation show that loss of Id2 significantly reduces the rate of cellular proliferation of primary and immortalized embryonic fibroblasts. These data are consistent with previous reports showing impaired proliferation rate of other cell types from Id2−/− mice (25, 26). It is likely that a critical threshold of Id2 determines the rate of proliferation in primary cells. Speculation of these findings are our results showing that overexpression of Id2 at levels comparable with those found in neuroblastoma cells renders cells insensitive to extracellular antimitogenic signals such as serum deprivation and contact inhibition, thus conferring oncogenic potential. The role of Id2 as an oncogenic factor in neuroblastoma is specifically supported by reduced entry into S-phase and severely compromised anchorage-independent growth of neuroblastoma cells, where Id2 expression has been forcibly reduced by antisense oligonucleotides.

In human tumors, Rb is functionally inactivated by genetic alterations of the “Rb pathway” or by constitutive activation of the “N-Myc-Id2 pathway.” We have now shown that Id2 expression determines the rate of proliferation of primary, immortalized, and tumor cell lines. These observations suggest that Id2 will make a major contribution to the inappropriate cell proliferation that results from loss of the negative control of Rb upon Id2 in tumors with genetic alterations of the “Rb pathway.” Anti-Id2 therapeutic approaches might be attractive new tools, even in tumors where inactivation of Rb results from mechanisms different from the activation of the “N-Myc-Id2 pathway.”

ACKNOWLEDGMENTS

We thank M. Noseda for help with immunohistochemistry. We also thank R. Russell for invaluable support in the preparation of pathological specimens and histological consultation.

REFERENCES

20. Lyden, D., Young, A. Z., Zaggag, D., Yan, W., Gerald, W., O’Reilly, R., Bader, B. L., Hynes, R. O., Zhuang, Y., Manova, K., and Benezra, R. Id1 and Id3 are required for neurogenesis, angiogenesis and vasculization of tumour xenografts [see comments]. Nature (Lond.), 401: 670–677, 1999.
Id2 Is Critical for Cellular Proliferation and Is the Oncogenic Effector of N-Myc in Human Neuroblastoma

Anna Lasorella, Renata Boldrini, Carlo Dominici, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/1/301

Cited articles
This article cites 26 articles, 13 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/1/301.full#ref-list-1

Citing articles
This article has been cited by 24 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/62/1/301.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.