Tubedown Expression Correlates with the Differentiation Status and Aggressiveness of Neuroblastic Tumors

Darryl T. Martin,1 Robert L. Gendron,1 Jason A. Jarzemkowski,2 Arie Perry,3 Margaret H. Collins,4 Chitra Pushpanathan,1 Ewa Miskiewicz,1 Valerie P. Castle,2 and Hélène Paradis1

Abstract

Purpose: The discovery and validation of new prognostic factors and further refinement of risk group stratification are needed to improve clinical interpretation of neuroblastoma. Our laboratory isolated and characterized a developmentally regulated gene named TUBEDOWN against which we have raised a monoclonal antibody (OE5). Tubedown becomes down-regulated postnatally yet remains strongly expressed in some neuroblastos. The purpose of this study is to define the utility of Tubedown expression as a new measure of the differentiation status and aggressiveness of neuroblastic tumors.

Experimental Design: Tubedown protein expression was quantitatively assessed in neuroblastic tumors (neuroblastomas, ganglioneuroblastomas, and ganglioneuromas) and normal adrenal tissues using Western blot and OE5 immunohistochemistry. Regulation of Tubedown expression during retinoic acid–induced neuronal differentiation in neuroblastoma cell lines was assessed by Western blotting.

Results: High levels of Tubedown expression are observed in tumors with significant neuroblastic component, unfavorable histopathology, advanced stage, high-risk group, and poor outcome. In contrast, more differentiated subsets of neuroblastic tumors, ganglioneuroblastomas with favorable histopathology and ganglioneuromas, express low levels of Tubedown. In vitro, marked retinoic acid–induced neuronal differentiation responses of neuroblastoma cells are associated with a significant decrease in Tubedown expression, whereas limited neuronal differentiation responses to retinoic acid were associated with little or no decrease in Tubedown expression.

Conclusions: Our results indicate that the levels of Tubedown expression are linked to the differentiation status and aggressiveness of neuroblastic tumors and represent an independent prognostic factor for neuroblastoma. Tubedown expression may be useful to more accurately define different neuroblastic tumor subsets and ultimately provide more adequate assessment and treatment for neuroblastoma patients.

Neuroblastoma is one of the most common solid tumors occurring in children (1). Although the etiology of neuroblastoma is still not clear, evidence suggests that these neuroectodermal tumors arise in the sympathetic nervous system from a defect occurring during normal developmental processes (2–5). Neuroblastomas exhibit heterogeneity with respect to differentiation and tumor progression, making diagnosis and treatment a challenge (3, 6). In young infants, localized and stage IVS neuroblastoma can spontaneously undergo complete regression or differentiate into benign ganglioneuroma requiring minimal treatment (3). Conversely, children with advanced metastatic neuroblastoma have poor outcomes despite intensive multimodality therapies (1, 7). In recent years, choice of treatment for neuroblastoma has relied on a range of prognostic factors [age at diagnosis, International Neuroblastoma Staging System, histopathologic features (International Neuroblastoma Pathology Classification), DNA ploidy, and MYCN amplification status] that have been used to stratify these tumors into low-, intermediate-, or high-risk categories (4, 5). However, both the validation of these prognostic factors and further refinement of risk group stratification are needed to improve clinical interpretation (4, 5). Despite advancements in treatment, neuroblastoma still accounts for 15% of cancer deaths in children (4). Therefore, more effective diagnostic tools and treatments are needed to improve cure rates and to reduce toxicity and long-term side-effects of current therapies (1, 3, 4).

Authors’ Affiliations: 1Department of Medicine, Memorial University of Newfoundland, St. John’s, Newfoundland, Canada; 2Department of Pathology, University of Michigan Hospitals and Clinics, Ann Arbor, Michigan; 3Division of Neuropathology, Washington University School of Medicine, St. Louis, Missouri; and 4Department of Pathology, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio

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Requests for reprints: Hélène Paradis, Department of Medicine, Memorial University of Newfoundland, 300 Prince Philip Drive, St. John’s, Newfoundland, Canada A1B 3V6. Phone: 709-777-8556; Fax: 709-777-8619; E-mail: hparadis@mun.ca.

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Neuroblastoma differentiation is likely an important biological process that can affect tumor outcome (8, 9). The use of differentiating agents is effective in improving the survival rate of high-risk neuroblastoma patients while limiting toxicity in normal host cells (1). One such commonly used differentiation agent is retinoic acid. Retinoic acid is one of the most powerful in vitro differentiating agents of neuroblastoma cells (10). The monitoring of neuroblastoma differentiation using specific differentiation markers could be useful in predicting neuroblastoma outcome.

Our laboratory isolated and characterized a developmentally regulated gene named TUBEDOWN (11). Tubedown-1 has been defined as a protein of 69 kDa with homology to yeast Nat1, a subunit of the yeast acetyltransferase NatA (11). A longer 100-kDa variant of Tubedown-1, Tubedown-100, seems to be derived from the same transcript (12–14). Tubedown is transiently expressed during embryogenesis in several tissues, whereas in adults high levels are found in only a few tissues, including bone marrow, ocular endothelial cells, atrial endocardium, and blood vessels of regressing ovarian follicles (11, 15). TUBEDOWN transcript is also expressed in neuronal cells during brain development but suppressed during neuronal maturation (14). TUBEDOWN is also highly expressed in papillary thyroid carcinoma cells, whereas adult tissues except testis express low levels (12, 16). We have extensively characterized Tubedown as a negative regulator of angiogenesis in endothelial cells (17, 18). However, Tubedown could have different functions in other cellular contexts (13, 19). Because TUBEDOWN expression is tightly down-regulated following embryonic neural development, we hypothesized that the persistence of Tubedown may be an important factor in pediatric tumors, such as neuroblastoma, which are thought to result from derangements in normal differentiation processes. The present study addresses a hypothesis that levels of Tubedown expression are linked to the differentiation status and aggressiveness of neuroblastic tumors and could represent an independent prognostic factor for neuroblastomas.

Materials and Methods

Tumor specimens. Snap-frozen tissues and paraffin-embedded sections from neuroblastoma, ganglioneuroblastoma, and ganglioneuroma were obtained from the Cincinnati Children’s Hospital Medical Center (Cincinnati, OH), the Brain Tumor Tissue Bank (London, Ontario, Canada), and the Janeway Child Health Centre (St. John’s, Newfoundland, Canada). Two tissue microarrays were constructed using triplicate 1.0-mm cores taken from 45 paraffin-embedded, formalin-fixed neuroblastic tumors (30 neuroblastoma, 7 ganglioneuroblastoma, 7 ganglioneuroma, and 5 composite/mixed histology tumors), 5 normal adrenal glands, and 20 other neural crest–derived and unrelated neoplasms obtained from archival material at the University of Michigan and the Cooperative Human Tissue Network (National Cancer Institute, Bethesda, MD). Specimens from the tissue microarrays had previously been classified according to International Neuroblastoma Pathology Classification criteria (8, 9), and clinical data (age at diagnosis, sex, stage, treatment protocol, initial response to treatment, event-free survival, time to relapse, and time to death) were made available. All human specimens were obtained and studied under the approval of the Institutional Review Boards of the author’s institutions.

MYCN fluorescence in situ hybridization. Analyses were done on representative sections (20). A commercial probe cocktail was used, consisting of a Spectrum Orange–labeled centromere enumerating probe 2 paired with a Spectrum Green–labeled MYCN probe on 2p24 (Vysis, Inc., Downers Grove, IL). The probes were diluted 1:50 with tDenHyb hybridization buffer (Insitius Biotechnologies, Albuquerque, NM). For each hybridization, 100 nonoverlapping nuclei were enumerated for MYCN and centromere enumerating probe 2 signals. Cells with MYCN to centromere enumerating probe 2 ratios >4 or innumerable MYCN signals were considered amplified. In nonamplified cases, those with >10% cells containing more than two copies of centromere enumerating probe 2 were defined as having polysomy 2.

Cell cultures. LA-N-5 (21) and IMR-32 (22) cell lines were gifts from Dr. Thomas Inge (Cincinnati Children’s Hospital Medical Center). The LA-N-5 and IMR-32 cells were respectively maintained in RPMI 1640 and in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mmol/L glutamine. The SK-N-DZ and RF/6A (rhesus macaque choroid-retina endothelial) cell lines were acquired from the American Type Culture Collection (ATCC, Manassas, VA) and maintained, respectively, in RPMI 1640 and in DMEM supplemented with 10% FBS, 0.1 mmol/L nonessential amino acids, and 4 mmol/L glutamine. The EWS-96 cell line was derived from a tumor specimen obtained from a patient with Ewing’s sarcoma and will be described elsewhere. Exponentially growing cells were used for every experiment.

All-trans-retinoic acid (Sigma, Oakville, Ontario, Canada) was dissolved in 5% DMSO and added to cells at an optimal concentration of 4 μmol/L. The medium (with or without retinoic acid) was changed every 3 days. Cells were photographed using a Leica DMIRE2 microscope system (Leica, Richmond Hill, Ontario, Canada) with a QImaging RETIGA Exi camera and Openlab software. Viability was assessed using trypan blue dye exclusion. All cell growth assays were done in triplicate.

Western blot. Cell lysates were prepared from snap-frozen tumor specimens and cell lines as described previously (11). Protein lysates were quantified using albumin as standard and analyzed by SDS-PAGE. Western blotting was done by standard procedures using enhanced chemiluminescence Plus chemiluminescence detection reagent (Amer-sham Biosciences, Piscataway, NJ) for anti-NE-M (Zymed Laboratories, San Francisco, CA), vimentin (Ab-1; Oncogene Research Products, San Diego, CA), c-Myb (C-19; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-MycN (EMD Biosciences, San Diego, CA) antibodies. AB1272 anti-Tubedown Western blot analyses were done as described (11, 15). All Western blots were stripped and reprobed with either extracellular signal-regulated kinase (ERK)-1 (Santa Cruz Biotechnology) or α-tubulin (Sigma) antibodies.

Turbedown immunohistochemistry. Sections from paraffin-embedded tissues were deparaffinized, postfixed in 4% paraformaldehyde, and incubated overnight with anti-Tubedown mouse monoclonal OE5 supernatant or negative control supernatant supplemented with control

![Fig. 1. Western blot analysis of Tubedown-1 expression in cell lines and primary neuroblastic tumors. Western blot analysis using Ab1272 anti-Tubedown (Tbdn-1) antibody of LA-N-5 and RF/6A cell lines and neuroblastoma (NB) and ganglioneuroblastoma (GNB) pathology specimens. Bottom, ERK Western blot analysis was used to show loading equivalency and protein integrity. Representative experiment.](image-url)
isotype-matched IgG2a antibody (DakoCytomation, Mississauga, Ontario, Canada) in 3% nonfat milk in TBS with 0.05% Tween 20. The mouse monoclonal OE5 hybridoma (18) recognizes Tubedown recombinant protein. Sections were developed using alkaline phosphatase and Vector Red Substrate kit (Vector Laboratories, Burlingame, CA) and photographed in triplicate using a Leica DMIRE2 microscope system with a QImaging RETIGA Exi camera and Openlab software.

**Northern blotting and real-time reverse transcription-PCR.** Northern blotting was done as described previously (11). Blots were hybridized with a 693-bp 32P-labeled MYCN cDNA probe (Image clone ID 5502743; ATCC) and reprobed with a mouse 18S ribosomal cDNA fragment to confirm loading equivalency and RNA integrity. Densitometric measurements of band intensity were completed using OptiQuant software.

For reverse transcription-PCR (RT-PCR) analysis, cDNA was prepared from total RNA using Roche Reverse Transcription kit (Roche, Branchburg, NJ). The cDNA was then amplified by real-time PCR with primers 5’-AACCCCAATGATGATGGAAA-3’ and 5’-CCAAAGCAATA-GATGGCTGA-3’ specific for human Tubedown (Genbank accession no. BC039818) using a 7000 sequence detector system (Applied Biosystems, Foster City, CA). EWS-96 cells were used as a standard for the experiments. All experiments were done in at least triplicate.

**Data and statistical analyses.** To stratify neuroblastoma patients into low-, intermediate-, and high-risk groups, prognosis variables, including age at diagnosis, International Neuroblastoma Staging System, histopathology (International Neuroblastoma Pathology Classification), and MYCN amplification status, were used as described previously (4).

Tubedown immunostaining was quantified as described previously (23). Tubedown staining levels for each specimen were averaged and the average background levels were subtracted. To standardize single tumors and tumors from the tissue microarrays, paraffin-embedded sections from a Ewing’s sarcoma xenograft tumor were used as a control in every experiment. Tubedown relative level of expression was calculated from a ratio of expression of each specimen over that of the Ewing’s sarcoma tumor.

Quantitative analyses were compared using the two-tailed Student’s t test with a Microsoft Excel program. Tests for homogeneity of variance were done as described. The data were considered to be statistically significant if the P value was ≤0.05.

**Results**

High levels of Tubedown expression in neuroblastic tumors correlate with advanced stages and neuroblastic unfavorable histopathology. Western blotting showed that Tubedown is expressed in the LA-N-5 human neuroblastoma cell line and in several neuroblastoma tumor specimens (Fig. 1). Much higher levels of Tubedown were detected in the stage II and III neuroblastoma specimens compared with stage I neuroblastoma and ganglioneuroblastoma specimens (Fig. 1). Additional neuroblastic tumor specimens were next analyzed for Tubedown expression by quantitative immunohistochemistry using anti-Tubedown OE5 monoclonal antibody (Fig. 2; ref. 18). In neuroblastoma, the cells that stained most intensely for Tubedown were of neuroblastic appearance (small round cells; Fig. 2). Tubedown staining was not detected in blood cells and blood vessels (Fig. 2). Normal adrenal tissues contained significantly lower Tubedown staining (0.62 ± 0.11 relative units) than stage II, III, and IV neuroblastoma tumors.

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5 http://helios.bto.ed.ac.uk/bto/statistics/table8.html#Test%20for%20homogeneity
Moreover, stage I and II neuroblastoma exhibited significantly lower levels of Tubedown immunostaining (0.97 ± 0.10 and 1.04 ± 0.13 relative units, respectively) than the advanced stage III and IV neuroblastoma tumors (1.34 ± 0.26 and 1.41 ± 0.05, respectively; \( P = 6.8 \times 10^{-14} \); Figs. 2 and 3A).

In the more differentiated neuroblastoma subtypes (ganglioneuroblastoma and ganglioneuroma), both ganglion-like and stromal (Schwann-like) cells faintly stained for Tubedown at levels lower than the neuroblastic-like cells observed in neuroblastoma specimens (Figs. 2 and 3A). Interestingly, two of two nodular ganglioneuroblastoma specimens with unfavorable histopathology and significant neuroblastic component expressed much higher levels of Tubedown in neuroblastic-like cells (1.57 ± 0.06 relative units) than all favorable ganglioneuroblastoma specimens with minimal neuroblastic component (0.65 ± 0.10; Figs. 2 and 3A). Significantly lower levels of Tubedown expression in the ganglioneuroma specimens (0.72 ± 0.11 relative units) and ganglioneuroblastoma specimens with favorable histopathology and minimal

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**Fig. 3.** Quantitative analysis of Tubedown expression in neuroblastic tumors. A, levels of Tubedown (\( Tbdn \)) expression in normal adrenal tissues (\( Normal, n = 4 \)) and neuroblastic tumors (ganglioneuroma (GN), \( n = 6 \); ganglioneuroblastoma with favorable histopathology: ganglioneuroblastoma (\( GNB \)), \( n = 6 \); ganglioneuroblastoma with unfavorable histopathology and significant neuroblastic component: ganglioneuroblastoma (\( GNBn \)), \( n = 2 \); neuroblastoma tumor stage I (\( S1 \)), \( n = 9 \); neuroblastoma tumor stage II (\( S2 \)), \( n = 5 \); neuroblastoma tumor stage III (\( S3 \)), \( n = 2 \); and neuroblastoma tumor stage IV (\( S4 \)), \( n = 11 \)). \( B \), significantly higher levels of Tubedown expression were present in the unfavorable neuroblastoma specimens (\( n = 13 \)) compared with the favorable neuroblastoma (\( n = 17 \)). \( C \), Kaplan-Meier analysis displaying the correlation between low Tubedown expression in neuroblastoma specimens and overall survival (\( P = 0.038 \) at 3 y). Low expression levels (\(< 1.3 \) relative units, \( n = 9 \)) versus high expression levels (\( \geq 1.3 \) relative units, \( n = 13 \)). \( D \), significantly lower levels of Tubedown expression were present in the low-risk group neuroblastoma specimens (\( n = 14 \)) compared with the high-risk group neuroblastoma specimens (\( n = 11 \)). A, B, and D, \( \ast \), relative Tubedown expression units for each specimen; \( \mid \), averages of Tubedown levels for each category. Bars, SE.
neuroblastic component were observed compared with the advanced stage IV neuroblastoma tumors ($P = 6.0 \times 10^{-6}$ and 0.0011, respectively; Fig. 3A). Although the more differentiated ganglioneuroma and ganglioneuroblastoma tumors with minimal neuroblastic component express lower levels of Tubedown than neuroblastoma specimens, the differentiation status within neuroblastoma specimens, categorized according to the International Neuroblastoma Pathology Classification system, was not associated with a significant difference in Tubedown levels. The undifferentiated neuroblastoma specimens expressed 1.18 ± 0.13 relative units of Tubedown, whereas the differentiated and poorly differentiated neuroblastoma specimens expressed, respectively, 1.12 ± 0.09 and 1.18 ± 0.13 relative units of Tubedown.

There was a direct correlation between unfavorable histopathology of neuroblastoma specimens and a high level of Tubedown expression. High levels of Tubedown expression (1.38 ± 0.08 relative units) were present in the unfavorable tumors, whereas low levels of Tubedown expression (1.09 ± 0.07 relative units) were observed in the favorable tumors ($P = 0.009$; Fig. 3B). High levels of Tubedown ($\geq 1.3$ relative units) were observed in 77% of unfavorable neuroblastoma tumors compared with 24% of favorable tumors. There was no correlation between the levels of Tubedown expression and

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**Fig. 4.** Morphologic differentiation of neuroblastoma cells on retinoic acid treatment. LA-N-5, SK-N-DZ, and IMR-32 neuroblastoma cell lines grown for 3 or 6 d in the absence (A and C, respectively) or presence (B and D, respectively) of retinoic acid (RA). LA-N-5 cells (D) exhibit an increase in the length and the thickness of neurites (white arrows) and cellular clustering (black arrows) in response to retinoic acid treatment compared with control (C). Magnification, ×400.
whether the patient was diagnosed at an early age (less than 12 or 18 months of age). Tubedown expression did not correlate with MYCN amplification status of neuroblastoma tumors. Similarly, the treatment status of the neuroblastic tumors did not show significant correlation with Tubedown staining.

**High levels of Tubedown in neuroblastoma tumors correlate with high-risk and low survival rate.** Classification of our neuroblastoma cohort into three risk groups, low, intermediate, and high, revealed a significant positive correlation between high Tubedown expression and the high-risk neuroblastoma patients (Fig. 3D). Lower levels of Tubedown expression (1.00 ± 0.08 relative units) were detected in the low-risk neuroblastoma patients compared with the intermediate-risk (1.32 ± 0.12) and high-risk neuroblastoma patients (1.41 ± 0.06; \( P = 0.0004 \)). High levels of Tubedown expression (≥1.3 relative units) were observed in 73% of high-risk neuroblastoma cases compared with 21% in low-risk cases. Kaplan-Meier analysis (24) revealed significantly better outcomes for patients with low Tubedown expression compared with high expression (3-year overall survival; \( P = 0.038 \); Fig. 3C). Patients with low Tubedown expression had no notable events, whereas 46% of patients with high Tubedown expression relapsed or died. Moreover, high Tubedown expression (1.50 ± 0.05 relative units) was found in the patients who relapsed or succumbed to the disease (\( n = 8 \)), whereas a low level of Tubedown expression (1.01 ± 0.10 relative units) was observed in patients who survived without relapse (\( n = 14 \)).

Analysis of our neuroblastoma cohort revealed that the event-free survival rate of patients within the cohort depended on the International Neuroblastoma Staging System (stage IV: relative risk, 6.1), patient’s age at diagnosis (≥1 year: relative risk, 2.6), undifferentiated status (relative risk, 2.5), and MYCN amplification (relative risk, 1.1). The relative risk factors for our neuroblastoma cohort were comparable with other published cohorts (25–27). The median age of the patient at diagnosis was 24 months (range, 0.1–288 months), and the percentage of MYCN amplified tumors was 29%.

**Tubedown is down-regulated in neuroblastoma cells undergoing marked neuronal differentiation.** Because Tubedown levels in the differentiated neuroblastic tumor subtypes (ganglioneurotumors and ganglioneuroblastoma with minimal neuroblastic component) are low and because the state of neuroblastoma tumor cell differentiation is likely an important biological factor for the clinical outcome (8, 9), we examined Tubedown expression as a function of the differentiation of neuroblastoma cell lines in vitro. Tubedown expression was analyzed in neuroblastoma cell lines induced to undergo differentiation by treatment with retinoic acid. The extent of neuronal differentiation in response to retinoic acid treatment was first analyzed in three neuroblastic-type neuroblastoma cell lines (LA-N-5, IMR-32, and SK-N-DZ). On treatment with retinoic acid, LA-N-5 cells developed significantly more neurite-like processes (increased length and thickness) than controls (Fig. 4). Retinoic acid treatment of SK-N-DZ and IMR-32 cells did not significantly induce process development compared with controls (Fig. 4). Retinoic acid treatment resulted in moderate inhibition of cell growth in LA-N-5, whereas more significant growth inhibition was observed in IMR-32 and SK-N-DZ cells (Fig. 5). The retinoic acid–induced differentiation in LA-N-5 cells was accompanied with a marked up-regulation of neurofilament 160 kDa (NF-M) expression (Fig. 6A). The SK-N-DZ and IMR-32 cells displayed a modest or no increase in NF-M expression on retinoic acid treatment (Fig. 6A). All three neuroblastoma lines treated with retinoic acid showed down-regulation of vimentin (Fig. 6B) and c-Myb expression (Fig. 6C). LA-N-5 and IMR-32 lines expressed high levels of MYCN transcript and protein. However, only LA-N-5 cells significantly down-regulated MYCN transcript (data not shown) and MycN protein (Fig. 6D) expression on retinoic acid treatment.

Analysis of Tubedown levels on retinoic acid–induced differentiation in these neuroblastoma cell lines revealed a marked reduction of expression only in LA-N-5 cells (Fig. 6E). The limited retinoic acid–induced differentiation of SK-N-DZ and IMR-32 cells was associated with a modest or no decrease of Tubedown expression, respectively (Fig. 6E). Real-time

![Figure 5](https://www.aacrjournals.org/clinres/2007/13/5/1485/1485{-fig5.png})

**Fig. 5.** Cell growth of neuroblastoma cells on retinoic acid–induced differentiation. Decreases in viable cell numbers are observed for LA-N-5 (A), SK-N-DZ (B), and IMR-32 (C) cell lines when treated with retinoic acid for 3 d (3R) and 6 d (6R) compared with controls for 3 d (3C) and 6 d (6C). Columns, mean percentage of the control; bars, SE. Results represent the average of at least three experiments.
RT-PCR analysis of TUBEDOWN transcript expression during retinoic acid–induced differentiation of LA-N-5 and IMR-32 cells revealed the same pattern of down-regulation observed for the protein expression (data not shown). These results indicate that significant reduction in Tubedown expression only takes place on induction of extensive neuronal differentiation in LA-N-5 cells.

Discussion

Tubedown is highly expressed in neuroblastoma cell lines and in certain subsets of neuroblastic tumors. In normal adrenal tissues, Tubedown expression is almost undetected. High Tubedown levels are detected in tumors presenting significant neuroblast component and unfavorable histopathology, such as advanced-stage neuroblastoma and some nodular ganglioneuroblastoma. Conversely, low levels were detected in low-stage neuroblastoma, ganglioneuroblastoma with favorable histopathology, and ganglioneuroma. High levels of Tubedown expression also correlated with high-risk group and significantly reduced overall survival rate. These results suggest that Tubedown may be a useful marker for unfavorable neuroblastic histopathology of neuroblastic tumors. This may be especially true for the ganglioneuroblastoma subsets, as a marked difference was observed in the levels of expression of Tubedown in ganglioneuroblastoma with favorable versus unfavorable histopathology. Tubedown may offer a means to assess the state of differentiation in cases of ganglioneuroblastoma, which are difficult to assess. The state of differentiation of ganglioneuroblastoma may be critical to clinical outcome of the disease (28).

Our results also indicate that Tubedown is a useful marker for neuroblastoma tumors within the high-risk group and advanced stages as well as for predicting poor outcome. However, because the range of Tubedown expression overlapped to a certain degree in low-risk versus high-risk patients and in tumors with favorable versus unfavorable histologic features, the determination of Tubedown levels in neuroblastic tumors may be more useful in multivariate analyses with other variables (age at diagnosis, International Neuroblastoma Staging System, histopathologic features, DNA ploidy, and MYCN amplification status).

The more differentiated subset of neuroblastic tumors (ganglioneuroblastoma with favorable histopathology and ganglioneuroma) expresses lower levels of Tubedown. However, the differentiation status within neuroblastoma specimens was not associated with significant differences in Tubedown expression. Because the International Neuroblastoma Pathology Classification system (8, 9) categorizes neuroblastoma tumors as differentiating if $\geq 5\%$ of the cells seem differentiated, whereas poorly differentiated if $\leq 5\%$ of cells seems differentiated (28), a 5% more or less of differentiated cells would not affect the overall levels of Tubedown expression within a given specimen. Tubedown levels may only be reduced if the tumor specimen presents significant levels of differentiation as seen in ganglioneuroblastoma and ganglioneuroma. Moreover, in vitro, reduction in Tubedown levels only occurs on extensive differentiation of neuroblastoma cells. In vitro, extensive neuronal differentiation was characterized by Tubedown suppression, increases in neurite-like formation and NF-M expression, but a marked decrease in MycN expression. Reductions in growth, vimentin, and c-Myb expression do not seem to be sufficient to lead to Tubedown suppression. Our in vitro analyses agree with the pattern of expression of Tubedown in neuroblastic tumors and indicate that Tubedown expression correlates with the differentiation status of neuroblastic tumors.
Tubedown levels did not correlate with MYCN amplification status of neuroblastoma tumors. MYCN amplification is a poor prognosis factor that is observed in ~25% of neuroblastoma tumors (3). In our neuroblastoma cohort, MYCN was found amplified in 29% of neuroblastoma tumors, whereas high levels of Tubedown were found in 46% of neuroblastoma tumors. At least one other factor (the allelic loss of 11q chromosomal locus) associated with high-risk features, such as advanced stage, older age at diagnosis, and unfavorable histopathology, was reported not to correlate with MYCN amplification (4). Both MycN and Tubedown protein expression are reduced on marked retinoic-acid–induced differentiation of the LA-N-5 neuroblastoma cell line (Fig. 6). However, although several studies have established a link between MYCN RNA and/or protein overexpression and poor outcomes, there is still some controversy surrounding this issue (29, 30). The relative risk of our neuroblastoma cohort for MYCN amplification was slightly low compared with the cohorts presented by others (25, 26). However, it is still clear that, within our neuroblastoma cohort, amplification of MYCN leads to a higher relative risk compared with tumors that are non-MYCN amplified.

To date, very few markers have been proven to represent an independent prognostic value for neuroblastoma tumors (3, 4, 25). Higher levels of Tubedown expression in advanced-stage, histologically unfavorable, and high-risk group neuroblastoma could be the consequence of a more aggressive tumor phenotype or could indicate that Tubedown itself somehow drives the aggressiveness of neuroblastoma tumors. 

**References**


In vitro, only marked differentiation of neuroblastoma cell lines is associated with reduced expression of Tubedown. In primary tumors, lower levels of Tubedown are observed in the more differentiated subsets of neuroblastomatic tumors. These results could indicate that Tubedown is involved in blocking differentiation. At present, the role of Tubedown in aggressive neuroblastoma is not clear. However, in yeast, the Tubedown homologue NaT1 acts in a complex with the acetyltransferase Ada1 to regulate a wide range of cellular processes, including cell growth and differentiation (31–33). Similarly to the yeast system, Tubedown forms a complex with a mouse homologue of the yeast Adr1 acetyltransferase (mARD1; ref. 14) and is involved in cellular processes that regulate cell growth and differentiation in different cellular contexts (13, 18, 19).

Our results indicate that the levels of Tubedown expression are linked to the differentiation status and aggressiveness of neuroblastoma tumors. Tubedown expression may be useful to more accurately define different neuroblastoma tumor subsets and to provide a new independent prognostic indicator of neuroblastoma.

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