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Expression of the neurotrophin receptor TrkC is linked to a favorable outcome in medulloblastoma

(brain tumor/receptor tyrosine kinase/cerebellum)

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ABSTRACT **Medulloblastoma, the most common malignant brain tumor of childhood, has a variable prognosis. Although half of the children and young adults with the disease survive longer than 10 years after diagnosis, the others relapse and die despite identical therapy. We have examined the expression of neurotrophins and their receptors in medulloblastoma samples snap frozen in the operating room to preserve RNA integrity. All tumors (n = 12) were found to express mRNA encoding neurotrophin 3 and its receptor TrkC. The level of *trkC* expression was highly variable, with a more than 50-fold difference between the highest and lowest values. By Kaplan–Meier analysis, patients with tumors expressing high levels of *trkC* mRNA had significantly longer intervals without disease progression than those with low levels (log-rank, P = 0.03) and a more favorable overall survival (log-rank, P = 0.05). Thus, *trkC* expression is a prognostic indicator for patients with medulloblastoma.**

For children less than 15 years old, medulloblastoma accounts for 20% of all central nervous system neoplasms. The tumor arises in the posterior fossa and is made up of small round cells with little cytological differentiation. Immunohistochemical studies have demonstrated the expression of neuronal and/or glial markers, as well as other molecules typical of the developing nervous system (1–3). Because of these characteristics, it has been proposed that medulloblastoma may arise as a derivative of primitive neuroectodermal cells, possibly from the external granule cell layer of the developing cerebellum (4, 5).

Cerebellar granule cell growth and differentiation occurs in coordination with the expression of a family of growth factors known collectively as neurotrophins. The members of this homologous family of molecules include nerve growth factor, brain-derived neurotrophic factor (BDNF) (6, 7), neurotrophin 3 (NT-3) (8–11), and neurotrophin-4/5 (NT-4/5) (12, 13). Both BDNF and NT-3 are found in the immature cerebellum (10). Early granule cells in the external granule cell layer respond to BDNF, whereas more mature granule cells respond to NT-3 but not BDNF (14).

Two classes of transmembrane glycoproteins serve as receptors for the neurotrophins. The first class, represented by the p75 low-affinity nerve growth factor receptor, has no tyrosine kinase activity. It binds nerve growth factor, BDNF, and NT-3 (15–18). The second class of receptor is represented by members of the Trk family of tyrosine kinases. These glycoproteins function as selective high-affinity neurotrophin receptors whose tyrosine kinase is activated by neurotrophin binding. Activated kinases, in turn, autophosphorylate tyrosine residues in the cytoplasmic domain of the receptors. The first member of this family, TrkA, preferentially binds nerve growth factor (19, 20). Another member, TrkB, specifically binds BDNF and NT-4/5 (12, 21–23), whereas TrkC binds NT-3 alone (24).

In this study, we examined whether neurotrophins and receptors implicated in cerebellar granule cell development are expressed within medulloblastomas. To this end, we froze tumor samples from 12 patients in the operating room by rapid immersion in liquid nitrogen in order to preserve RNA and protein integrity. mRNA transcripts for NT-3 and TrkC were found in all of the tumor samples. The level of *trkC* mRNA expression was variable, but it was highest in tumors from patients with less aggressive disease. Using Kaplan–Meier analysis, patients with tumors expressing high levels of *trkC* were found to have significantly longer intervals without disease progression and a more favorable overall survival than those with lower levels of expression.

METHODS

Tumor samples from 12 patients treated at Boston Children's Hospital and the Brigham and Women's Hospital between 1984 and 1994 were snap frozen in liquid nitrogen in the operating room and then stored at −70°C until further analysis. A total of 63 patients were treated for medulloblastoma at these institutions over this interval. All adequately frozen samples were analyzed. The clinical characteristics at the time of diagnosis for the patients tested in this study (mean age = 11 years, 5 total resection/7 subtotal resection, 4 metastatic/8 not metastatic) were comparable to those not included (mean age = 9 years, 19 total resection/32 subtotal resection, 19 metastatic/32 not metastatic). Medulloblastoma cell lines were obtained from the American Type Culture Collection, SY5Y and trkPC12 cells were obtained from David Kaplan (National Cancer Institute), and HiB5 was obtained from Ron McKay (National Institutes of Health). All were maintained in Dulbecco's modified Eagle's medium with 10% (vol/vol) heat-inactivated fetal calf serum.

Total cellular RNA was isolated on a CsCl gradient after tissue disruption in guanidine isothiocyanate by Polytron homogenization of tumor samples and trituration of cultured cells. To prepare protein extracts, the solid tumors were minced and then solubilized with three strokes of a Dounce homogenizer in RIPA solution containing sodium vanadate, aprotinin, and phenylmethylsulfonyl fluoride. Insoluble ma-

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Abbreviations: NT-3, neurotrophin 3; NT-4/5, neurotrophin 4/5; BDNF, brain-derived neurotrophic factor; GAPDH, glyceraldehyde phosphate dehydrogenase.

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terials were removed by centrifugation (14,000 × *g* for 10 min), leaving solubilized proteins in the supernatant.

RNA samples (20 μg per lane) were fractionated by electrophoresis in a 1% agarose gel. The RNA was transferred onto GeneScreen*Plus* (New England Nuclear) nylon membranes. Northern hybridization with complementary ^{32}P-labeled DNA probes, generated by the random hexamer method, was done according to the protocol of Bartel *et al.* (25). A low-stringency wash [1× standard saline citrate (SSC)/0.1% SDS at room temperature] was followed by two 15-min high-stringency washes (0.2× SSC/0.1% SDS at 55°C). Differences in loading the lanes were assessed by reprobing the blots for glyceraldehyde phosphate dehydrogenase (GAPDH).

For receptor analysis, protein samples (500 μg of extract) were immunoprecipitated with an antibody that recognizes the tyrosine kinase-positive forms of TrkA, TrkB, and TrkC (26). The immunoprecipitates were washed extensively, fractionated by SDS/PAGE, and transferred to Immobilon. The blots were probed with an antibody to phosphotyrosine (27) or an antibody that we prepared to a synthetic peptide corresponding to the carboxyl-terminal portion of TrkB (residues 812–821). This antibody recognizes all of the Trk receptors and does not cross-react with other receptor tyrosine kinases (R.A.S. and C.D.S., unpublished data).

The relationship of neurotrophin receptor expression with disease progression or death was assessed by Kaplan–Meier plots compared by a log-rank test. A Wilcoxon rank-sum test was used to compare the level of *trkC* expression of medulloblastomas with that of other tumors.

RESULTS

A summary of the clinical data for all patients is given in Table 1. All patients with medulloblastoma were treated with surgery followed by craniospinal radiation and chemotherapy.

Expression of mRNA for Neurotrophins and Their Receptors in Medulloblastoma. Northern analysis identified transcripts encoding the TrkC receptor in RNA derived from all of the medulloblastoma samples and one cell line. Tumors from patients 1 and 3, shown as representative examples, were found to express high levels of the 14.0-kb transcript, which encodes a full-length TrkC receptor protein, as well as the 5.5-kb form, which encodes a TrkC receptor lacking tyrosine kinase (Fig. 1) (28, 29). Patient 2 had these forms but at a much lower level of expression. The cell line Daoy had a low level of the 5.5-kb variant, whereas D283 had little to no expression of either transcript.

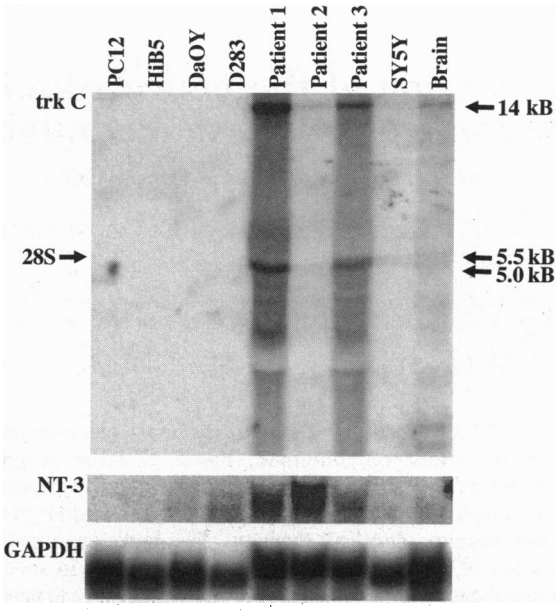

FIG. 1. Expression of NT-3 and its receptor TrkC. Northern blot analysis was done using probes for *trkC* (*Top*) and NT-3 (*Middle*). To control for unequal loading, the blots were stripped and reprobed for the GAPDH gene (*Bottom*). The samples are from PC12 cells transfected with TrkA receptor, the HiB5 rat hippocampal cell line, medulloblastoma cell lines Daoy and D283, medulloblastoma samples from patients 1, 2, and 3, human neuroblastoma cell line SY5Y, and adult mouse brain.

The 14.0-kb bands were quantified by measurement on the PhosphorImager. These measurements were corrected for differences in loading by dividing with the signal from a constitutively expressed gene (obtained by reprobing the blots for GAPDH). An index of expression was calculated by dividing the corrected *trkC* measurement with that from mouse brain RNA (also corrected for loading). To allow comparison of medulloblastoma samples analyzed on different Northern blots, a single preparation of mouse brain RNA was used as a standard reference for all of the Northern blots

Table 1. Patient summary

Patient no.	Initial age, yr	Diagnosis	Extent of disease	Current status (months after diagnosis)*	Extent of surgery	Treatment		*trkC* index	*trkB* index 9 kb/7 kb
						Chemo	XRT		
1	5	Medullo	Local	Alive (11)	GTR	CVP	CSI	14.6	0.9/1.4
2	9	Medullo	Meta	Dead (9)	STR	CVP	CSI	0.8	3.1/12.9
3	27	Medullo	Local	Alive (14)	GTR	VP	CSI	11.5	0/2.8
4	6	Medullo	Local	Dead (14)	GTR	CVP	CSI	0.4	19.1/20
5	11	Medullo	Meta	Relapsed (41)	STR	CVP	CSI	2.6	0/0
6	9	Medullo	Local	Alive (59)	STR	VP	CSI	12.1	0/10.3
7	21	Medullo	Meta	Relapsed (90)	STR	VP	CSI	12.7	16.8/17.3
8	23	Medullo	Local	Dead (102)	GTR	VP	CSI	23.3	86.9/130
9	9	Medullo	Local	Relapsed (6)	STR	CVP	CSI	7.6	35.7/21.7
10	1	Medullo	Local	Relapsed (7)	GTR	CVP	CSI	16.8	10.8/3.3
11	0.7	Medullo	Meta	Alive (5)	STR	CVP	CSI	1.1	0/15.1
12	12	Medullo	Local	Alive (1)	STR	CVP	CSI	24.8	0/0
13	1	Ependym	Local		GTR			0.2	0/323.8
14	0.6	Mal rhab	Local		STR			0.4	0/21.4
15	3	Mal rhab	Local		GTR			0.0	0/0
16	6	Pil astro	Local		GTR			3.6	0/288

*Interval to the present time except for patients who have died for whom the interval is time until death. Medullo, medulloblastoma; Ependym, ependymoma; Mal rhab, malignant rhabdoid tumor; Pil astro, pilocytic astrocytoma; Meta, metastatic; GTR, gross total resection; STR, subtotal resection; CVP, cytoxan/vincristine/*cis*-platinum; VP, vincristine/*cis*-platinum; Chemo, chemotherapy; XRT, radiation therapy; CSI, craniospinal irradiation.

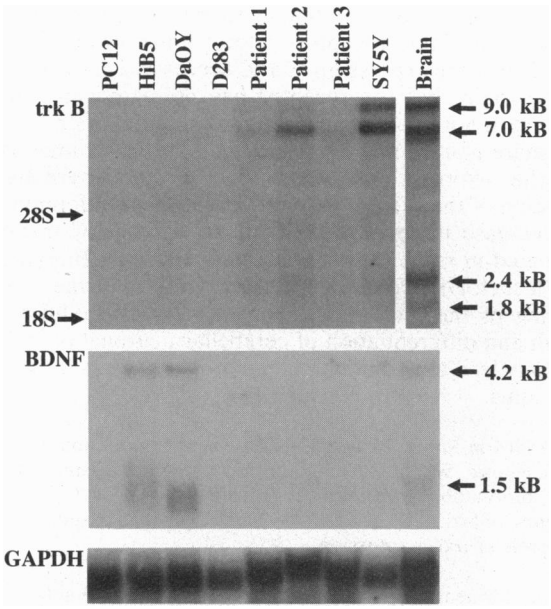

FIG. 2. Expression of BDNF and its receptor TrkB. Northern analysis was done using probes for the extracellular domain of *trkB* (*Top*) and for BDNF (*Middle*). The blots were stripped and reprobed for GAPDH (*Bottom*). Samples are as described in Fig. 1. Since the level of expression in mouse brain was considerably higher than that in the tumors, the lane was printed with a sensitivity 10-fold less than that of the other lanes.

used in this study. The range of expression indices is shown in Table 1.

To determine whether the pattern of *trkC* expression in medulloblastoma differs from other tumors, samples were obtained from four patients with other diagnoses (see Table 1). Expression of high levels of trkC mRNA was only seen in medulloblastoma samples. The levels of expression in the other samples were significantly lower than those found in medulloblastoma (Wilcoxon rank-sum test, $P < 0.05$) (30). Furthermore, these data argue against the possibility that the high levels of neurotrophin receptors that we have detected are derived from normal or reactive cerebellar tissue within the tumor sample.

In addition to transcripts encoding the full-length TrkC receptor, all 12 of the medulloblastoma samples expressed transcripts for NT-3 (see Fig. 1), which is the preferred ligand for TrkC (28). Both of the medulloblastoma cell lines expressed NT-3.

Northern hybridization revealed a different pattern of expression for transcripts encoding TrkB (examples shown in Fig. 2). The predominant alternative splice variant expressed in these tumors was 7.0 kb, which encodes a truncated receptor lacking a tyrosine kinase domain (31, 32). Considering all patients, 7 with medulloblastoma had detectable expression of the 9.0-kb *trkB* transcript, which encodes the full-length receptor, whereas 10 had expression of the kinase-negative variant. Because the level of *trkB* expression in most tumors was considerably below that of our reference tissue (mouse brain), all *trkB* indices were multiplied by 100 for ease of manipulation. The Daoy cell line was found to express a very low level of the 7.0-kb transcript alone, whereas the D283 line had no apparent *trkB* expression.

For the other tumor types, no patients were found to express the full-length variant of *trkB*, and three expressed the kinase-negative form. The specimens from patients with ependymoma and astrocytomas had higher levels of expression of the 7.0-kb splice variant than any of the other tumors. This is consistent with their ependymal and astrocytic origin, since these tissues have been found to express predominantly kinase-negative variants of neurotrophin receptors (32).

Only one of the medulloblastomas expressed BDNF, which is the principal central nervous system ligand for the TrkB receptor, although BDNF transcripts were found in RNA derived from both cell lines (Fig. 2). The low-affinity nerve growth factor receptor (p75) was present in four of the medulloblastomas. No detectable expression of *trkA* was found in any of the tumors or tumor cell lines (data not shown).

Relationship of High *trkC* Expression with Progression and Survival. The range of *trkC* expression indices was quite large, with a >50-fold difference between the lowest and highest values (Table 1). We dichotomized the 12 patients with medulloblastoma using an expression index of 10.7, the mean of our group of patients, as the point of division. Patients with high tumor *trkC* expression indices were found to have a significantly longer interval of freedom from disease progression than those with low indices (Fig. 3*A*). In contrast, levels of *trkB* expression were not associated with differences in disease progression (Fig. 3*B*). A significant improvement in overall survival also was found for patients with high levels of trkC ($P = 0.05$) but not for trkB ($P = 0.86$). The level of expression of the low-affinity nerve growth factor receptor (p75) was not associated significantly with either rate of disease progression or overall survival, consistent with previous studies (33).

Expression of Trk Receptor Proteins in Medulloblastoma. Since the tumor samples were found to express mRNAs encoding the full-length TrkC receptor, we tested for the

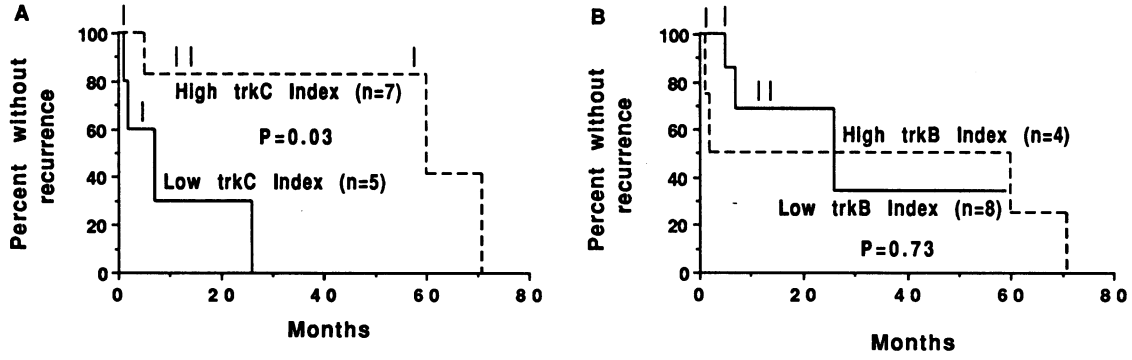

FIG. 3. Kaplan–Meier plots indicating the percentage of children remaining free of disease progression after the initial diagnosis of their tumor. The patients were divided into groups according to the levels of expression of *trkC* (*A*) or *trkB* (*B*) mRNA within their tumor. Tick marks above each line indicate the length of follow-up for patients in each group without disease progression. Patients expressing high levels of trkC (index greater than 10.7; see Table 1) had longer intervals without disease progression than those with low levels. Comparisons are by the log-rank test. See note added in proof.

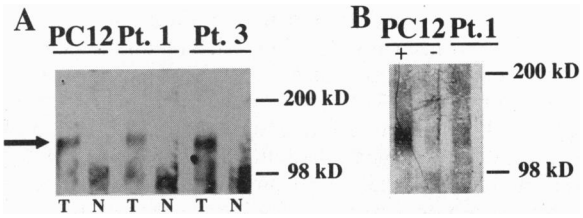

FIG. 4. Trk proteins are present in medulloblastomas. (*A*) Protein extracts from patients 1 and 3 were immunoprecipitated with an anti-Trk antibody (T) (26) or with a nonimmune sera (N) and then probed by Western analysis using a second antibody to Trk. As a control, PC12 cells overexpressing *trkA* (trkPC12) were analyzed in the same manner. The arrow indicates the Trk protein. Molecular mass standards are as indicated. (*B*) Protein extracts from patient 1 and nerve growth factor-stimulated (+) or control unstimulated trkPC12 (−) cells were immunoprecipitated with an anti-Trk antibody (26) and then probed by Western analysis using an antibody to phosphotyrosine (27).

presence of receptor proteins. Due to limited quantities of tumor samples, we were able to test only two patients (patients 1 and 3) with high levels of *trkC*. By using an antibody that detects but cannot distinguish between all Trk receptors, these medulloblastoma samples were found to have receptor proteins (Fig. 4*A*). It is likely that the proteins detected are TrkC since only the full-length *trkC* mRNA is expressed in significant quantity in these samples (Figs. 1 and 2). Since the TrkC ligand NT-3 is also expressed in the tumor samples, we tested for the presence of receptor activation *in vivo*. The sample from patient 1 was found to have tyrosine-phosphorylated receptors (Fig. 4*B*), indicating that Trk receptors within the tumor are endogenously activated. Ambiguous results were obtained when the protein sample from patient 3 was tested for tyrosine-phosphorylated receptor.

DISCUSSION

In this study, we have demonstrated the expression of NT-3 and its receptor, TrkC, in medulloblastoma. The level of *trkC* mRNA expression was variable, but it was higher in medulloblastoma samples from patients with less aggressive disease.

The expression of both ligand and receptor within medulloblastoma is significant in that it implies the autonomous activation of tumor cell neurotrophin receptors. There are several possible functional consequences of this activation. Neurotrophins most commonly have been found to promote either differentiation or survival of responsive cells. Infrequently, neurotrophins have been found to have mitogenic activity (34, 35). We found that patients with the least aggressive medulloblastomas had high levels of *trkC* mRNA and that tumor cell lines, which can be maintained *in vitro*, had no detectable full-length receptors. These results suggest that NT-3 promotes differentiation of tumor cells with functional TrkC receptors. Endogenous activation of neurotrophin receptors in medulloblastoma does not appear to promote increased tumor growth. This is in contrast to astrocytomas, in which autonomous activation of a different receptor tyrosine kinase by platelet-derived growth factor is correlated with aggressive tumor growth (36, 37).

Patients with tumors expressing high levels of *trkC* expression had longer intervals of progression-free survival as well as a more favorable overall survival. In neuroblastoma, another neuronal precursor tumor, high levels of *trkA* expression are correlated strongly with a favorable outcome (38, 39). Neuroblastomas with low levels of *trkA* often are histologically undifferentiated. These more aggressive tumors may arise from less mature sympathoadrenal precursor cells than the neurotrophin receptor-expressing tumors (38).

For medulloblastoma, it remains to be determined whether activation of TrkC receptors alters tumor behavior. It is possible that the expression of *trkC* serves as a marker for the cell of origin, but the receptor has no significant role in dictating tumor behavior. Less aggressive tumors may arise from more mature and differentiated external granule layer cells that express TrkC receptors (14, 40). Alternatively, activation of the NT-3 receptor may promote differentiation and decrease tumor growth. Activation of TrkC might be modulated in some medulloblastomas by low-affinity nerve growth factor receptor (41, 42) or TrkB. A further understanding of the molecular mechanisms that modulate the growth and differentiation of cerebellar neuronal progenitor cells may lead to additional therapeutic strategies for medulloblastoma.

Note Added in Proof. Patient 11 had disease progression 6 months after diagnosis. When Kaplan–Meier plots were calculated with this added information, increased progression-free survival ($P < 0.01$) and improved overall survival ($P < 0.05$) remained associated with high levels of *trkC* expression.

Probes for neurotrophins were generated from full-length cDNAs of rat NT-3 (courtesy of Dr. W. Friedman, Robert Wood Johnson Medical School) and human BDNF (courtesy of Dr. G. Yankopoulos, Regeneron Pharmaceuticals, Tarrytown, NY). The *trkA* cDNA was obtained courtesy of Dr. R. McKay (National Institutes of Health). A 994-base *Eco*RI/*Hin*dIII fragment of the murine *trkB* cDNA was used, which corresponds to the extracellular domain of the protein (courtesy of Drs. F. Lamballe and M. Barbacid, Squibb). The full-length 2526-base porcine *trkC* cDNA was used (courtesy of Drs. F. Lamballe and M. Barbacid). A 270-base fragment of rat p75 low-affinity nerve growth factor receptor was courtesy of Dr. M. Chao (Cornell University Medical College). Rat GAPDH cDNA was courtesy of Dr. M. Greenberg (Harvard Medical School). We thank Mr. S. Khoxayo and Ms. L. Rua for technical assistance; Ms. T. Leong for help with statistical analysis; Drs. P. Black, J. Madsen, and R. M. Scott for tumor specimens; and Dr. D. Anthony for interpreting the neuropathology specimens. The work was supported by a Clinical Investigator Development Award (R.A.S.) and grants from the National Institutes of Health (S.L.P., NS27773; and C.D.S., GM31489) and the Brain Tumor Society.

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