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Abundant but Inactive-state gp140$^{proto\text{-}trk}$ Is Expressed in Neuroblastomas of Patients with Good Prognosis

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Steady-state levels of gp140$^{proto\text{-}trk}$ in cell lines and tumor tissues of neuroblastoma were examined by immunoblotting with anti-gp140$^{proto\text{-}trk}$. The level of gp140$^{proto\text{-}trk}$ varied but showed good correlations with the stage of the tumor and the age of the patients at the time of diagnosis. Moreover, patients with higher expression of gp140$^{proto\text{-}trk}$ clearly had a far better survival rate than those with lower expression, suggesting that suppression of gp140$^{proto\text{-}trk}$ strongly correlates with the malignant conversion of the tumor. However, we found that neither autophosphorylation of gp140$^{proto\text{-}trk}$ nor tyrosine phosphorylation of cellular proteins was elevated in tumors of the higher expression group. These results suggest that gp140$^{proto\text{-}trk}$ does not actively participate in the process of transformation or the suppression of malignant conversion. Rather, the higher level of gp140$^{proto\text{-}trk}$ may reflect the greater level of differentiation of tumor cells.

Key words: *trk* — gp140$^{proto\text{-}trk}$ — Tyrosine kinase — Neuroblastoma — Prognosis marker

Neuroblastoma is a childhood tumor derived from the sympathoadrenal progenitors of the neural crest. An interesting feature of this tumor is the wide range of prognoses that closely correlate with the extent of tumor spread at diagnosis and the age of the patient.[1] Based on the clinical manifestations, Evans *et al.*[2] subclassified neuroblastoma into 5 clinical stages from I to IVs. Tumors of localized distribution are grouped as stages I and II, whereas those with remote metastasis are grouped as stages III and IV. The stage IVs tumors are apparently metastatic but show better prognostic outcome than stage IV tumors and can spontaneously regress.[3,4] A clear correlation is observed between this staging and the prognosis of the patients. In patients over 1 year of age, there is an almost 100% survival rate in localized tumors as compared to poor prognostic outcomes in non-localized, disseminated tumors.[5,6] The age of the patients is another important factor that correlates with the prognosis. The majority of neuroblastomas occurring under 1 year of age are localized or stage IVs tumors and have a favorable prognostic outcome.[5] On the other hand, patients over 1 year of age often have advanced tumors[5] and their survival rates have not improved much, despite highly aggressive modern therapy.[7] In these advanced tumors, amplification of N-*myc* is specifically observed,[8,9] suggesting the involvement of as-yet-unidentified molecular events leading to the aggressive, adverse phenotype of the tumor.

Neuroblastoma cells appear to be arrested at various stages of neuronal differentiation, and the grade of differentiation correlates to the clinical course.[1,6] Differentiation of sympathetic neuroblasts requires nerve growth factor (NGF), and this NGF stimulation is mediated by a transmembrane kinase, gp140$^{proto\text{-}trk}$, which is encoded by *trk*.[10,11] However, despite the importance of gp140$^{proto\text{-}trk}$, its role in neuroblastoma cells is largely unclear.

By use of an image analyzer for the assay of the relative level of immunologically detected gp140$^{proto\text{-}trk}$, we studied the steady-state levels of gp140$^{proto\text{-}trk}$ expression in various neuroblastoma tissues surgically resected from patients. During the preparation of this manuscript, Suzuki *et al.*[12] and Nakagawara *et al.*[13] reported the absence of *trk* mRNA expression in agressive neuroblastoma by Northern-blot analysis. In these reports, however, it remained unclear whether the *trk* mRNA is translated normally and whether its product, gp140$^{proto\text{-}trk}$, is functional in the neuroblastoma tissues. Confirming and extending the above reports, we show here that abundant but inactive-state gp140$^{proto\text{-}trk}$ is expressed in early stages of neuroblastoma but the level is strongly depressed in advanced, malignant stages. Our results suggest that suppression of gp140$^{proto\text{-}trk}$ expression strongly correlates with malignant conversion of the tumor.

MATERIALS AND METHODS

Cells and tumor tissues Cell lines derived from human neuroblastoma, Nagai, TGW, GOTO, IMR-32, NB-1,

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SK-N-SH, and SK-N-MC were supplied by the Japanese Cancer Research Resources Bank. Cell lines derived from human glioma, T98, U231, and MG198 were kindly supplied by Dr. J. Yoshida (Nagoya University School of Medicine, Department of Neurosurgery). Tumor tissues surgically resected from patients were immediately frozen in liquid nitrogen and stored at −80°C until use.

Preparation of anti-gp140$^{proto\text{-}trk}$ antibody and anti-phosphotyrosine antibody Polyclonal antibody raised in rabbits against a synthetic peptide corresponding to the 14 carboxy-terminal residues of the deduced proto-*trk* sequence was prepared as described previously.[14, 15] Polyclonal anti-phosphotyrosine antibody was prepared with v-*abl*-encoded protein expressed in bacteria as described previously.[16, 17]

Preparation of surgically resected tumor tissues Tumor tissues surgically resected were immediately frozen in liquid nitrogen and stored at −80°C. The frozen tissues were then homogenized in a buffer containing 2% SDS and 5% mercaptoethanol. The protein concentration of each sample was determined by use of a protein assay kit (Bio-Rad Laboratories).

Immunoblotting and immunoprecipitation Analysis of gp140$^{proto\text{-}trk}$ and phosphotyrosine-containing proteins by immunoblotting and immunoprecipitation has been described previously.[15, 18, 19] Immunoblotting of the same filter with anti-phosphotyrosine antibody and with anti-gp140$^{proto\text{-}trk}$ antibody was conducted as described previously.[20] Briefly, anti-phosphotyrosine antibodies were stripped from the filter after the autoradiography by incubation with 5 *M* sodium iodide and 1 m*M* sodium thiosulfate, followed by a brief rinse in distilled water. After autoradiography to confirm that all radioactivity had been removed, the stripped filter was reprobed with anti-gp140$^{proto\text{-}trk}$ antibody.

Assay of relative amount of gp140$^{proto\text{-}trk}$ The relative amount of gp140$^{proto\text{-}trk}$ visualized by immunoblotting and autoradiography was analyzed with a BAS-2000 image analyzer (Fujix Co. Ltd., Tokyo).

RESULTS

Expression of gp140$^{proto\text{-}trk}$ in human neuroblastoma and glioma cell lines To detect gp140$^{proto\text{-}trk}$, we prepared an antibody against 14 carboxy-terminal residues of *trk* as previously reported.[14] This antibody recognized a 140 kDa polypeptide in PC12 cells that showed NGF-dependent kinase activity (Fig. 1A), specific for tyrosine (data not shown). With this antibody, we examined the expression of gp140$^{proto\text{-}trk}$ in 7 neuroblastoma cell lines and 3 glioma cell lines (Fig. 1B). While gp140$^{proto\text{-}trk}$ was undetectable in glioma cells, various levels of gp140$^{proto\text{-}trk}$ expression were observed in neuroblastoma cells. Of the 7 cell lines we tested, NB-1 showed the highest level of gp140$^{proto\text{-}trk}$ expression. Therefore, we used NB-1 as a control in the following experiments.

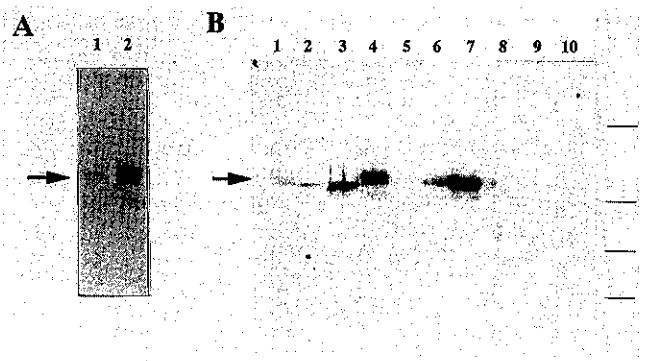

Fig. 1. Expression of gp140$^{proto\text{-}trk}$ in PC12 cells and neuroblastoma cell lines. (A) Kinase assay of gp140$^{proto\text{-}trk}$ immunoprecipitated from NGF-untreated (1) and -treated (2) PC12 cells. (B) Immunoblotting of gp140$^{proto\text{-}trk}$ in neuroblastoma cell lines. Lanes 1–7, neuroblastoma cell lines, lanes 8–10 glioma cell lines. Lane 1, SK-N-MC; 2, SK-N-SH; 3, IMR-32; 4, GOTO; 5, TGW; 6, Nagai; 7, NB-1; 8, MG178; 9, U231; 10, T98. Arrow, gp140$^{proto\text{-}trk}$.

Expression of gp140$^{proto\text{-}trk}$ in neuroblastoma tissues and its correlation with staging We next examined the expression of gp140$^{proto\text{-}trk}$ in surgically resected neuroblastoma tissues by immunoblotting with anti-gp140$^{proto\text{-}trk}$ antibody, and compared the level of expression with clinical manifestations. Forty-one neuroblastomas, 9 ganglioneuro-blastomas, 1 ganglioneuroma, and 2 normal adrenal glands as listed in Table I were used for the study. The relative level of gp140$^{proto\text{-}trk}$ expression compared with that of NB-1 cells was estimated in each tumor by using an image analyzer. As shown in Table I and Fig. 2, diverse levels of gp140$^{proto\text{-}trk}$ expression were observed in these tumor tissues, ranging from 0.4% to 269% of the levels in NB-1 cells. No clear difference was observed between neuroblastoma and ganglioneuroblastoma in the level of gp140$^{proto\text{-}trk}$ expression (data not shown). However, we found a good correlation between the level of gp140$^{proto\text{-}trk}$ and stages of tumor tissues (Fig. 3). While stage I showed the highest level of gp140$^{proto\text{-}trk}$ expression, stage IV tumors expressed the lowest levels. Tumor tissues of stages II and III showed intermediate levels of gp140$^{proto\text{-}trk}$ expression. Notably, in tumor tissues of stage IVs, which have a good prognostic outcome despite the extensive metastasis, the level of gp140$^{proto\text{-}trk}$ was higher than that of stage IV. Differences of gp140$^{proto\text{-}trk}$ levels between stage I and II, I and III or I and IV were found to be statistically significant ($P<0.05$) using Bonferroni's method. Thus, these results suggest that malignant conversion of neuroblastoma cells correlates with the suppression of gp140$^{proto\text{-}trk}$ expression.

Table I. Clinical Features and gp140$^{proto\text{-}trk}$ Levels of Surgically Resected Specimens

Stage	No.	Month	Pathology	gp140$^{proto\text{-}trk}$	Prognosis
I	1	12	NB	91.8	80
	2	7	NB	41.1	51
	3	8	NB	218.8	27
	4	9	NB	71.1	25
	5	28	GNB	59.9	24
	6	8	NB	268.8	16
	7	6	NB	174.4	15
	8	8	NB	137.8	13
	9	11	NB	133.0	13
	10	9	NB	103.9	7
	11	8	NB	89.4	6
II	12	49	NB	47.8	118
	13	13	GNB	10.2	114
	14	7	GNB	134.4	94
	15	9	NB	88.7	62
	16	0	NB	5.3	46
	17	7	NB	62.9	41
	18	3	NB	84.2	33
	19	9	NB	121.6	25
	20	0	NB	25.3	17
	21	10	NB	58.4	13
	22	13	NB	33.0	9
	23	8	NB	29.5	6
III	24	2	NB	60.0	122
	25	1	NB	191.4	113
	26	32	NB	51.2	47-D
	27	36	NB	9.5	37-D
	28	43	NB	43.6	6-D
	29	162	NB	15.2	6-D
	30	12	NB	99.4	82
	31	7	GNB	59.0	74
	32	14	NB	49.8	45
IV	33	52	NB	14.7	14-D
	34	33	NB	21.3	18-D
	35	44	NB	25.5	9-D
	36	2	NB	8.7	12-D
	37	42	NB	58.6	54
	38	10	NB	14.3	46
	39	33	GNB	84.0	41
	40	106	NB	28.1	2-D
	41	27	GNB	45.3	28
	42	12	NB	101.2	22
	43	39	GNB	92.1	10
	44	21	NB	0.4	9
	45	23	NB	13.0	6
IVs	46	8	NB	24.9	82
	47	8	GNB	103.4	74
	48	7	NB	47.6	73
	49	1	NB	87.2	48
	50	8	GNB	128.6	15
GN		60	—	ND	—
AG10		9	—	ND	—
AG45		23	—	ND	—

Stage, Evans' staging system; Month, the age of first diagnosis; NB, neuroblastoma; GNB, ganglioneuroblastoma; GN, ganglioneuroma; gp140$^{proto\text{-}trk}$, relative radioactivity compared with that of NB-1 as a control (NB-1=100); ND, not detectable; AG10 and AG45, normal adrenal gland tissue of case no. 10 and 45, respectively; Prognosis, survival length (month) as at Jan. 1st 1993; x-D, died after x months.

Correlation of gp140$^{proto\text{-}trk}$ levels in neuroblastoma with age and prognosis of patients Since the age of the patient is known to be prognostically of great importance, we compared the levels of gp140$^{proto\text{-}trk}$ expression with patients' age at the time of first diagnosis. As shown in Fig. 4, we found that the level of gp140$^{proto\text{-}trk}$ roughly correlated with the patients' age. Most patients under 1 year old showed a higher level of gp140$^{proto\text{-}trk}$ expression than those over 1 year old. All the cases which showed gp140$^{proto\text{-}trk}$ expression higher than NB-1 cell expression were found to be under 1 year old, and these cases were mostly stage I. On the other hand, over 1 year old, most cases showed weak gp140$^{proto\text{-}trk}$ expression (lower than 50% of NB-1 cell expression).

We next compared the levels of gp140$^{proto\text{-}trk}$ expression with the prognostic outcome of these cases. For this purpose, we tentatively chose a 50% level of gp140$^{proto\text{-}trk}$ expression in NB-1 cells as a borderline. At this level, we separated the cases into a higher expression group (28 cases) and a lower expression group (22 cases). The survival curve in each group was studied by the method of Kaplan and Meier. As shown in Fig. 5A, we found that the prognostic outcome of the higher expression group was clearly better than that of the lower expression group, and this difference was significant ($P<0.05$). It is possible, however, that these results merely reflect the favorable prognosis of stage I, since most of the stage I cases were grouped into the higher expression group. To eliminate this possibility, we limited the cases to the advanced stages and compared the levels of gp140$^{proto\text{-}trk}$ expression with the prognostic outcome. With the advent of modern combined therapy, the survival rate of the advanced stages has been greatly improved,[21] yet advanced neuroblastoma can still be fatal, as shown in Table I. In our study, 9 cases of stage III and 13 cases of stage IV were analyzed. In these cases, 3 of 4 of the lower expression group in stage III have died, but all the patients of the higher expression group in stage IV are still alive, in spite of multiple remote metastasis at the time of diagnosis. Although only 1 patient in the higher

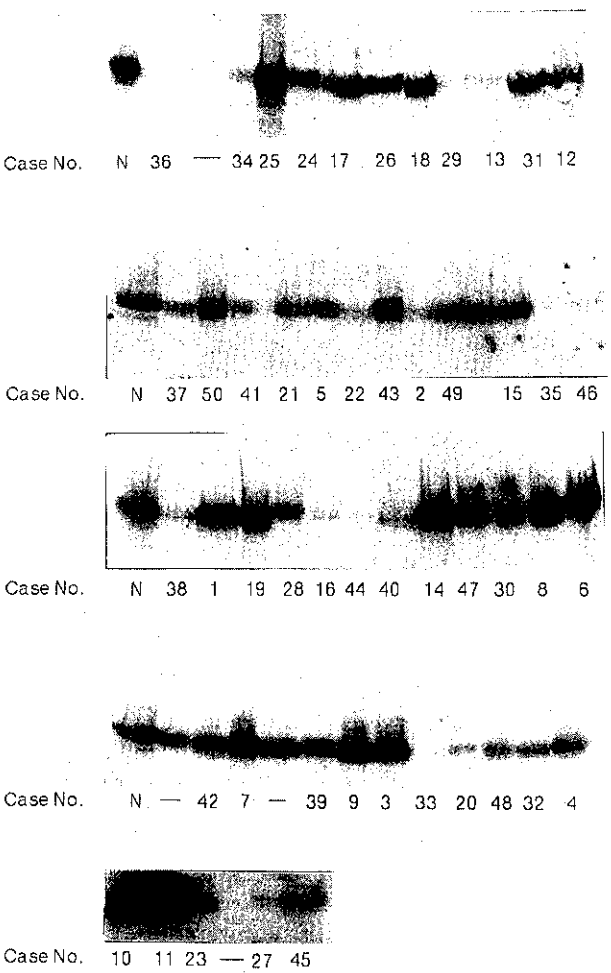

Fig. 2. Levels of gp140$^{proto\text{-}trk}$ expression in neuroblastoma tissues used for the assay. gp140$^{proto\text{-}trk}$ in tumor tissues was detected by immunoblotting. The numbers correspond to the case numbers in Table I. N, NB-1 cells. Unnumbered cases (bars) have unclear prognostic outcome.

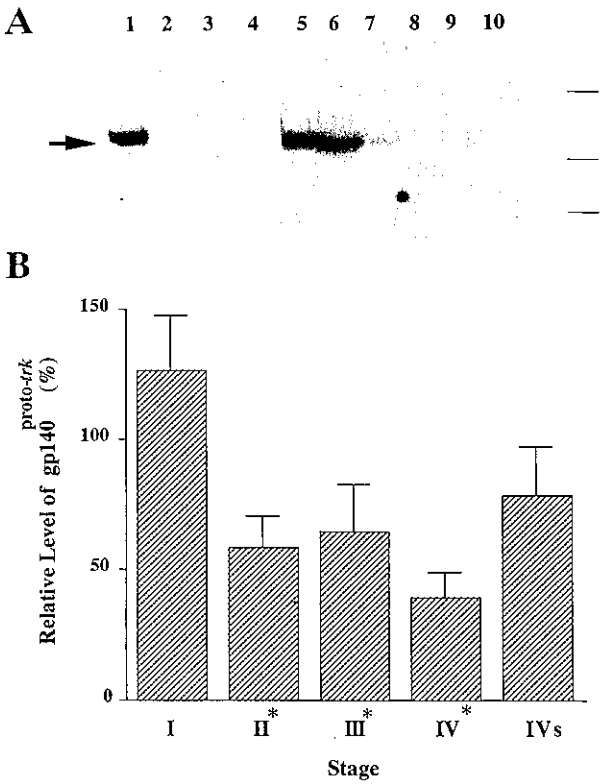

Fig. 3. Expression of gp140$^{proto\text{-}trk}$ in neuroblastoma tissues and its correlation with Evans' staging. (A) Immunoblotting of gp140$^{proto\text{-}trk}$ in NB-1 cells (lane 1), normal adrenal glands (lane 2, case No. AG10 in Table I; lane 3, case No. AG45), ganglioneuroma (lane 4, case No. GN), stage I neuroblastoma (lane 5, case No. 10; lane 6, case No. 11), stage II neuroblastoma (lane 7, case No. 23), stage III neuroblastoma (lane 8, case No. 27), and stage IV neuroblastoma (lane 9, case No. 44; lane 10, case No. 45). Arrow, gp140$^{proto\text{-}trk}$. (B) Correlation of gp140$^{proto\text{-}trk}$ levels with Evans' staging. Relative levels of gp140$^{proto\text{-}trk}$ in various neuroblastoma tissues were assayed as described in "Materials and Methods." 100%, relative level of NB-1 cells. *, differences from stage I were statistically significant ($P<0.05$). Number of patients; stage I, 11; stage II, 12; stage III, 9; stage IV, 13; stage IVs, 5.

expression group of stage III has died, this case was found to have a borderline-level expression of gp140$^{proto\text{-}trk}$ (51.2%), had the longest survival period among the lower expression group of advanced stages. As shown in Fig. 5B, the difference in prognostic outcome between the higher expression group and the lower expression group became much more prominent in advanced stages, and this difference was statistically significant ($P<0.05$). While 85% of the higher expression group is still alive, 75% of the lower expression group died within 4 years. These results suggest that the tumors of the higher expression group respond well to the combined modern therapy, and that the level of gp140$^{proto\text{-}trk}$ expression in advanced stages of neuroblastoma strongly correlates with the prognostic outcome in our cases.

Autophosphorylation of gp140$^{proto\text{-}trk}$ and cellular protein phosphorylation in neuroblastoma tissues of the higher expression group As shown in Fig. 2, a high level of gp140$^{proto\text{-}trk}$ was expressed in many cases of tumor tissues, yet the tumor cells did not stop growing or differentiate. These results raise the possibility that the differentiation signal mediated by gp140$^{proto\text{-}trk}$ is blocked in certain steps of the signaling pathway, or that transformation in these neuroblastoma cells with a high level of gp140$^{proto\text{-}trk}$ may be caused by aberrant activation of gp140$^{proto\text{-}trk}$. It is,

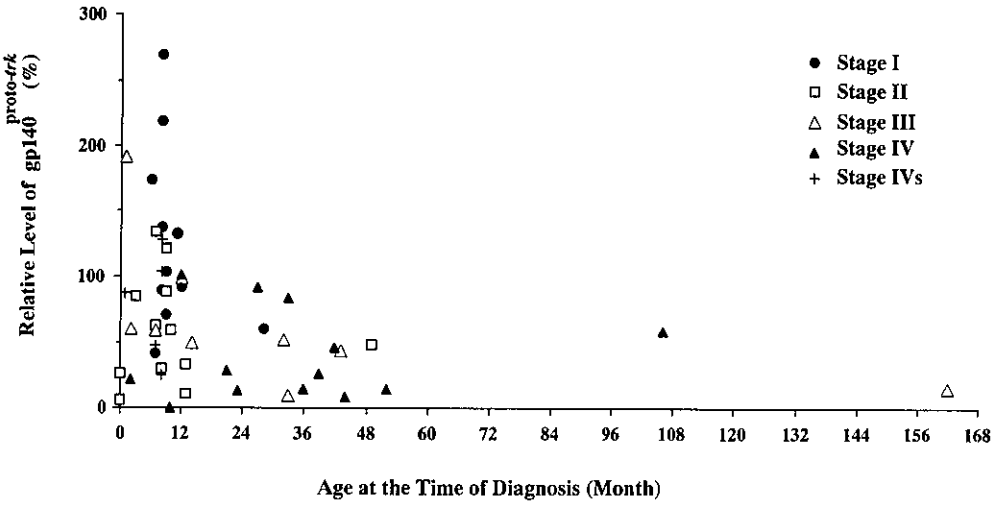

Fig. 4. Correlation of gp140$^{proto\text{-}trk}$ with patients' age. Relative levels of gp140$^{proto\text{-}trk}$ were compared with patients' age at first diagnosis. 100%, relative level of NB-1 cells.

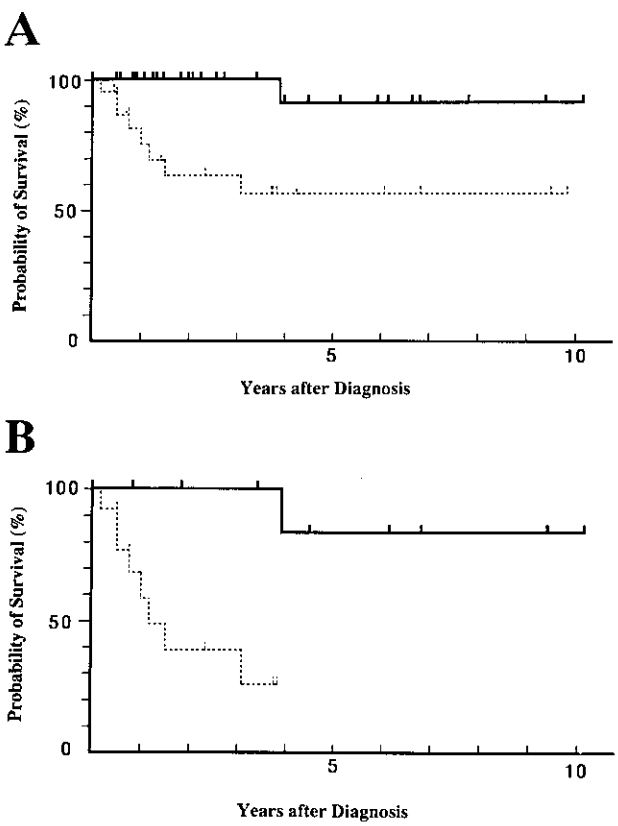

Fig. 5. Correlation of gp140$^{proto\text{-}trk}$ level with survival. Survival curves of the higher expression group (solid lines) and the lower expression group (broken lines) were compared. (A) all cases. (B) Stage III and IV cases.

therefore, of interest to examine whether gp140$^{proto\text{-}trk}$ of the higher expression group is enzymatically active, and whether the level of tyrosine phosphorylation in these neuroblastoma cells is elevated. We assayed autophosphorylation levels of gp140$^{proto\text{-}trk}$ in tissues of the higher expression group and compared these levels with the levels of NGF-treated and untreated NB-1 cells. The same amounts of tumor tissues were lyzed, immunoprecipitated with anti-gp140$^{proto\text{-}trk}$, and subjected to SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody or anti-gp140$^{proto\text{-}trk}$ (Fig. 6 A, B). Although similar amounts of gp140$^{proto\text{-}trk}$ were immunoprecipitated from tumor tissues and NB-1 cells, autophosphorylation of gp140$^{proto\text{-}trk}$ was undetectable in all cases of neuroblastoma tissue. On the other hand, gp140$^{proto\text{-}trk}$ of NB-1 cells assayed as a control showed clearly that autophosphorylation responded to NGF-treatment. Thus, it is likely that gp140$^{proto\text{-}trk}$ remains inactive in these tumor tissues, at least at the time of operation, although it is expressed at high levels.

Tyrosine-phosphorylation of cellular proteins in these tumor tissues was next examined and compared with that of normal adrenal gland cells from the patients. As shown in Fig. 6C, no clear difference was observed between tumor tissues and adrenal glands in the profiles or levels of tyrosine phosphorylation of cellular proteins. These results are consistent with the above findings and suggest that transformation of neuroblastoma cells is not accompanied with aberrant activation of tyrosine kinases.

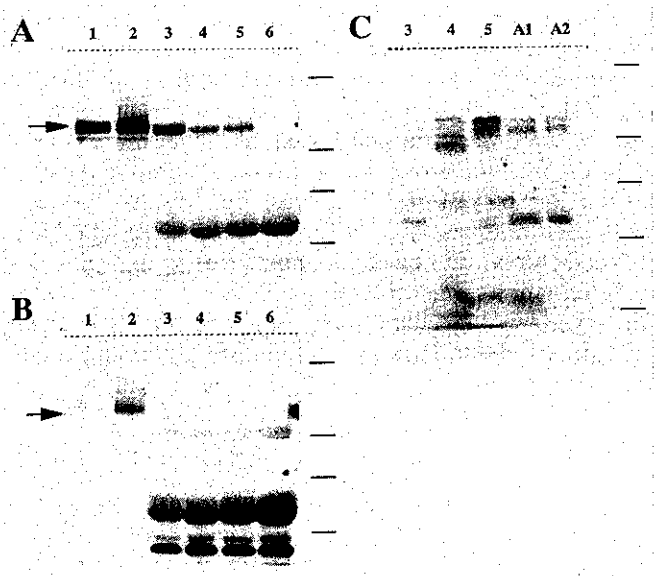

Fig. 6. Autophosphorylation of gp140$^{proto\text{-}trk}$ and cellular protein phosphorylation in neuroblastoma tissues of the higher expression group. (A) gp140$^{proto\text{-}trk}$ was immunoprecipitated with anti-gp140$^{proto\text{-}trk}$ from NGF-untreated (1) and -treated (2) NB-1 cells, or tumor tissues of the higher expression group freshly prepared by surgical disection (3, case No. 23; 4, case No. 11; 5 and 6, case No. 10). In lane 6, anti-TRK antibody was omitted. Then, immunoprecipitated samples were subjected to immunoblotting with anti-gp140$^{proto\text{-}trk}$. (B) Anti-TRK antibody on the filter in (A) was removed and the same filter was again probed with anti-phosphotyrosine antibody as described in Materials and Methods. (C) Immunoblotting of phosphotyrosine-containing proteins in cell lysates of neuroblastoma tissue (3–5) and normal adrenal glands (A1 and A2) with anti-phosphotyrosine antibody. Arrows in (A) and (B), gp140$^{proto\text{-}trk}$. Bars, positions of molecular mass standards (200, 97.4, 68 and 43 kDa).

DISCUSSION

By Northern- and dot-blot hybridization analysis, an inverse relationship between *trk* expression and N-*myc* amplification in neuroblastoma cells has been reported.[22, 23] Since the amplification of N-*myc* is found in about 50% of the advanced stages of neuroblastoma, and strongly correlates with rapid progression of the disease and poor prognosis,[9, 24] these results indicated that the level of *trk* in neuroblastoma may correlate with a favorable prognostic outcome. Despite these findings, the expression level and the kinase activity of gp140$^{proto\text{-}trk}$ in neuroblastoma tissues have remained unclear. Confirming and extending the previous observations, we have shown here that the levels of gp140$^{proto\text{-}trk}$ expression in neuroblastoma cells are diverse but closely correlate with staging, age and prognostic outcome. While the levels of gp140$^{proto\text{-}trk}$ expression in tumors of stage I and under 1 year of age were substantially high, those of advanced stages and after infancy were clearly low. However, our results do not mean that tumors with high gp140$^{proto\text{-}trk}$ expression simply represent those of stage I. Even in advanced stages (III and IV), a certain number of tumors have relatively high levels of gp140$^{proto\text{-}trk}$ expression, and these tumors are estimated to have a good prognostic outcome despite multiple remote metastasis at the time of diagnosis. On the contrary, all the fatal tumors in advanced stages except one case in stage III turned out to have lower expression. Thus, these results suggest a clear correlation between the suppression of gp140$^{proto\text{-}trk}$ expression and malignant conversion, and strongly support the clinical applicability of gp140$^{proto\text{-}trk}$ as a prognostic marker for neuroblastoma. Recently, Suzuki *et al.*[12] and Nakagawara *et al.*[13] independently reported the absence of gp140$^{proto\text{-}trk}$ mRNA expression in aggressive neuroblastoma by Northern-blot analysis. Our results are consistent with their findings and suggeat that the level of gp140$^{proto\text{-}trk}$ reflects the level of its mRNA production in neuroblastoma cells. It is noteworthy that, compared with previous studies, the method used in this study, including immunoblotting with anti-gp140$^{proto\text{-}trk}$ and application of an image analyzer for the estimation of gp140$^{proto\text{-}trk}$ level, has practical advantages in terms of easiness, rapidity and stability of assay.

The proto-*trk* was originally identified as a proto-oncogene of *trk*, a human oncogene isolated from a colon carcinoma biopsy.[25] The *trk* oncogene was generated by a genomic rearrangement that fused a tropomyosin gene with the *trk* proto-oncogene. Since most neuroblastomas of stage I in patients under 1 year of age expressed high levels of p140$^{proto\text{-}trk}$, the possibility must be considered that gp140$^{proto\text{-}trk}$ might be aberrantly activated and stimulate the cell growth in an early stage of tumor development. However, this is not the case. So far as we examined, neither tyrosine phosphorylation of gp140$^{proto\text{-}trk}$ nor aberrant elevation of cellular protein phosphorylation was observed in neuroblastoma tissues of the high expression group, suggesting that gp140$^{proto\text{-}trk}$ in these tissues remains inactive as a kinase.

Several lines of evidence suggest that generation of neuroblastoma involves a block in the differentiation of neural crest precursor cells. For example, the stage IVs neuroblastomas can spontaneously differentiate to mature nonproliferative cells resembling ganglion cells.[4] Primary neuroblastoma cells exhibit different maturation states *in vivo*, resembling certain stages of neuronal cell development. In a series of infant autopsies, a high frequency of adrenal lesions histologically identical to neuroblastomas was observed,[26] but the majority of these lesions probably differentiate during normal growth and

development. Undifferentiated neuroblastoma cells can be induced to differentiate *in vitro* by a variety of morphogens such as retinoic acid.[27] Despite these findings, it has been shown that most neuroblastoma cell lines could not differentiate in response to NGF treatment.[28] Since the cell lines used were derived from tumors of advanced stages, the inability of these cells to differentiate in response to NGF-treatment may depend on the insufficient expression of gp140$^{proto\text{-}trk}$.

Although the name, NGF, means a growth factor, NGF has a growth-suppressive effect on cells. Upon treatment with NGF, PC12 cells stop growing, develop excitable membranes and differentiate into a sympathetic neuronal phenotype.[29] These differentiated cells become NGF-dependent for their survival. Given these findings, the result that high levels of gp140$^{proto\text{-}trk}$ were specifically expressed in neuroblastoma of good prognostic outcome, raised the possibility that gp140$^{proto\text{-}trk}$ might function to inhibit the malignant conversion of the tumors. However, the low phosphotyrosine content in neuroblastoma tissues of the higher expression group indicates that the better prognosis is probably not due to the growth-suppressive effect of gp140$^{proto\text{-}trk}$ via kinase activation. Rather, it is likely that the high expression of gp140$^{proto\text{-}trk}$ correlates with greater differentiation of tumor cells. Nakagawara *et al.*[13] reported that primary cultures of neuroblastoma of the higher expression group responded to NGF treatment *in vitro*, and required NGF for their survival. However, in neuroblastoma tissues, the cancer cells did not stop growing or differentiate despite the high expression of gp140$^{proto\text{-}trk}$. Thus, our results, together with their findings, suggest the presence of some unidentified factor(s) that causes immortalization and prevents differentiation of neuroblastoma cells *in vivo*. Studies of the NGF level and the responsiveness to NGF treatment of neuroblastoma tissues of the higher expression group are in progress.

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