Implications of *EPHB6*, *EFNB2*, and *EFNB3* expressions in human neuroblastoma

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Neuroblastoma (NB) is a common pediatric tumor that exhibits a wide range of biological and clinical heterogeneity. EPH (erythropoietin-producing hepatoma amplified sequence) family receptor tyrosine kinases and ligand ephrins play pivotal roles in neural and cardiovascular development. High-level expression of transcripts encoding EPHB6 receptors (*EPHB6*) and its ligands ephrin-B2 and ephrin-B3 (*EFNB2*, *EFNB3*) is associated with low-stage NB (stages 1, 2, and 4S) and high *TrkA* expression. In this study, we showed that *EFNB2* and *TrkA* expressions were associated with both tumor stage and age, whereas *EPHB6* and *EFNB3* expressions were solely associated with tumor stage, suggesting that these genes were expressed in distinct subsets of NB. Kaplan-Meier and Cox regression analyses revealed that high-level expression of *EPHB6*, *EFNB2*, and *EFNB3* predicted favorable NB outcome ($P < 0.005$), and their expression combined with *TrkA* expression predicted the disease outcome more accurately than each variable alone ($P < 0.00005$). Interestingly, if any one of the four genes (*EPHB6*, *EFNB2*, *EFNB3*, or *TrkA*) was expressed at high levels in NB, the patient survival was excellent (>90%). To address whether a good disease outcome of NB was a consequence of high-level expression of a ''favorable NB gene,'' we examined the effect of *EPHB6* on NB cell lines. Transfection of *EPHB6* cDNA into IMR5 and SY5Y expressing little endogenous *EPHB6* resulted in inhibition of their clonogenicity in culture. Furthermore, transfection of *EPHB6* suppressed the tumorigenicity of SY5Y in a mouse xenograft model, demonstrating that high-level expressions of favorable NB genes, such as *EPHB6*, can in fact suppress malignant phenotype of unfavorable NB.

Neuroblastoma (NB) is a common pediatric solid tumor of neural crest origin. The tumor occurs frequently in infants and young children and originates in the adrenal glands or the sympathetic chain. NB exhibits a wide range of clinical heterogeneity, ranging from cases that are curable without treatment to those that progress relentlessly despite the most aggressive treatment. Several markers have been described that can predict disease outcome of NB, including patient age at diagnosis, tumor stage, Shimada histology, DNA ploidy, serum ferritin or lactate dehydrogenase levels, and *MYCN* amplification (1–7). Others, such as deletion or allelic loss of chromosome 1p (8, 9), allelic gain of 17q (10), *TrkA* expression (11–13), and CD44 expression (14), are also significant prognostic markers of NB. Although these factors have been known for some time, the molecular mechanism as to why these factors are predictive of NB outcome has remained elusive.

EPH (erythropoietin-producing hepatoma amplified sequence) family receptor tyrosine kinases and ephrin ligands are involved in fundamental developmental processes in the nervous system (15–19). Their participation in angiogenesis during cardiovascular development also has been demonstrated (20–23). In addition, their involvement in human cancers through autocrine and/or juxtacrine activation has been suggested (24–27). Based on the sequence relationships and structures, ephrin ligands are divided into two subgroups: ephrin-A and ephrin-B, which are encoded by *EFNA* and *EFNB* genes, respectively. EPH family receptors also are divided into subgroups based on the relatedness of their extracellular domain sequences and on their ability to bind to the two subgroups of ephrins. The EPHA subgroup interacts preferentially with ephrin-A ligands, whereas the EPHB subgroup interacts preferentially with ephrin-B ligands (28).

We recently demonstrated that transcripts encoding EPHB receptors and ephrin-B ligands were coexpressed in NB, suggesting that these molecules modulate biological and clinical behaviors of NB. Furthermore, high-level expression of *EPHB6*, *EFNB2*, and *EFNB3* was associated with low tumor stage and with high *TrkA* expression but inversely correlated with *MYCN* amplification in NB (26). This study was undertaken to determine whether the expressions of *EPHB6*, *EFNB2*, and *EFNB3* have any effects on the prognosis of NB, and if so, to elucidate the underlying mechanisms by which these effects are mediated. We show here that high-level expression of *EPHB6*, *EFNB2*, and *EFNB3* is a significant prognostic indicator of favorable NB, and that expression levels of genes associated with favorable NB disease outcome such as *EPHB6* can in fact determine benign or malignant phenotype of the tumor.

Materials and Methods

Primary NB Tumor Samples. Fifty NB tumor specimens were obtained from the Tumor Bank of The Children's Hospital of Philadelphia, the Tumor Bank of The Pediatric Oncology Group, and Memorial Sloan-Kettering Cancer Center. These included 10 tumors of stage 1, eight of stage 2, five of stage 4S, 12 of stage 3, and 15 of stage 4. Two stage 3 tumors and seven stage 4 tumors had *MYCN* amplification. The Shimada histology was absent from about a half of the cases and thus was not included in our analysis. The overall survival of this cohort was 71.43%, which was slightly higher than that expected from the general NB population (≈65%). This is because of a lower representation of *MYCN*-amplified cases in our cohort (18%) than that in the general NB population (≈25%) and because of lower representation of stage 4 tumors (≈30% in our cohort vs. ≈50% in the general NB population). Although the expression study was performed on 50 NB samples, the survival data were available for 49 NB.

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Abbreviations: NB, neuroblastoma; EPH, erythropoietin-producing hepatoma amplified sequence.

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Table 1. Prognostic significance of four established prognostic markers in the study cohort

Variable	Hazard ratio	95% CI	*P*
Age (>1 vs. <1)	8.01	1.05–61.24	0.045
Stage (3, 4 vs. 1, 2, 4S)	14.49	1.89–111.22	0.010
MYCN (amplified vs. normal)	7.91	2.59–24.14	<0.0005
*TrkA**	0.241	0.113–0.518	<0.0005

Univariate Cox regression analysis was used to assess prognostic significance of variables indicated. CI, Confidence interval.
*Continuous variable.

Quantitative Reverse Transcription–PCR (RT-PCR). Experimental procedures for quantitative RT-PCR were described elsewhere (26, 27). Results of this semiquantitative RT-PCR were shown to be consistent with those obtained by Northern blot analysis (27).

Statistical Analysis. Survival probabilities in various subgroups were estimated according to the method of Kaplan and Meier (29). Survival distributions were compared by using log-rank tests (30). The Cox regression models were used to determine the relationship among the prognostic markers (31). Both *EFNB2* and *EFNB3* expressions had skewed distributions. Thus, the Wilcoxon rank-sum test was used to assess differential expression of *EPHB6*, *EFNB2*, *EFNB3*, and *TrkA* by age (< 1 vs. >1 year), and by stage (1, 2, and 4S vs. 3 and 4). *P* value <0.05 is considered statistically significant.

Stable Transfection of NB Cells with *EPHB6*. Human *EPHB6* cDNA clones were obtained by PCR from fetal brain cDNA (CLONTECH), and the nucleotide sequences were confirmed by DNA sequencing and the BLAST homology search. One and a half million SY5Y or IMR5 cells were transfected by electroporation with either pCEP4 eukaryotic expression vector (Invitrogen) alone or the vector containing a human *EPHB6* cDNA. The resulting transfectants were plated into three wells of a 6-well plate and selected for 14 days with 250 μg/ml hygromycin. After the selection, the cells were treated with 0.5 μg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide for 4 h to stain viable cells and to examine the effect of *EPHB6* on clonogenicity of NB cells.

Mouse Xenograft Studies. A total of 3×10^7 SY5Y cells were transiently transfected with 300 μg linearlized DNA [pcDNA3.1/Hygro(+) vector (Invitrogen) or the vector containing an *EPHB6* cDNA], using FuGENE6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. Twenty-four hours after the transfection, the cells were harvested and injected s.c. in the flank of nude mice (5×10^6 cells in 0.2 ml Matrigel per mouse). The difference in tumor size between the vector control group and the *EPHB6* group was assessed on day 40 by the Wilcoxon rank-sum test by using the median value of each group.

Results

The NB Cohort. We first examined the prognostic values of four well-established prognostic markers of NB to evaluate our study cohort. The markers included patient age at diagnosis (1, 32), tumor stage (1, 32), *MYCN* amplification (1–7), and *TrkA* expression (11–13). As shown in Table 1, the univariate Cox regression survival analysis demonstrated that all of the variables tested predicted the disease outcome of NB as expected. We also confirmed that age, stage, *MYCN* amplification (data not shown), and *TrkA* expression (Fig. 1*A*) predicted disease outcome of the NB cohort by the Kaplan-Meier method. Although our NB cohort had some differences in its composition from the

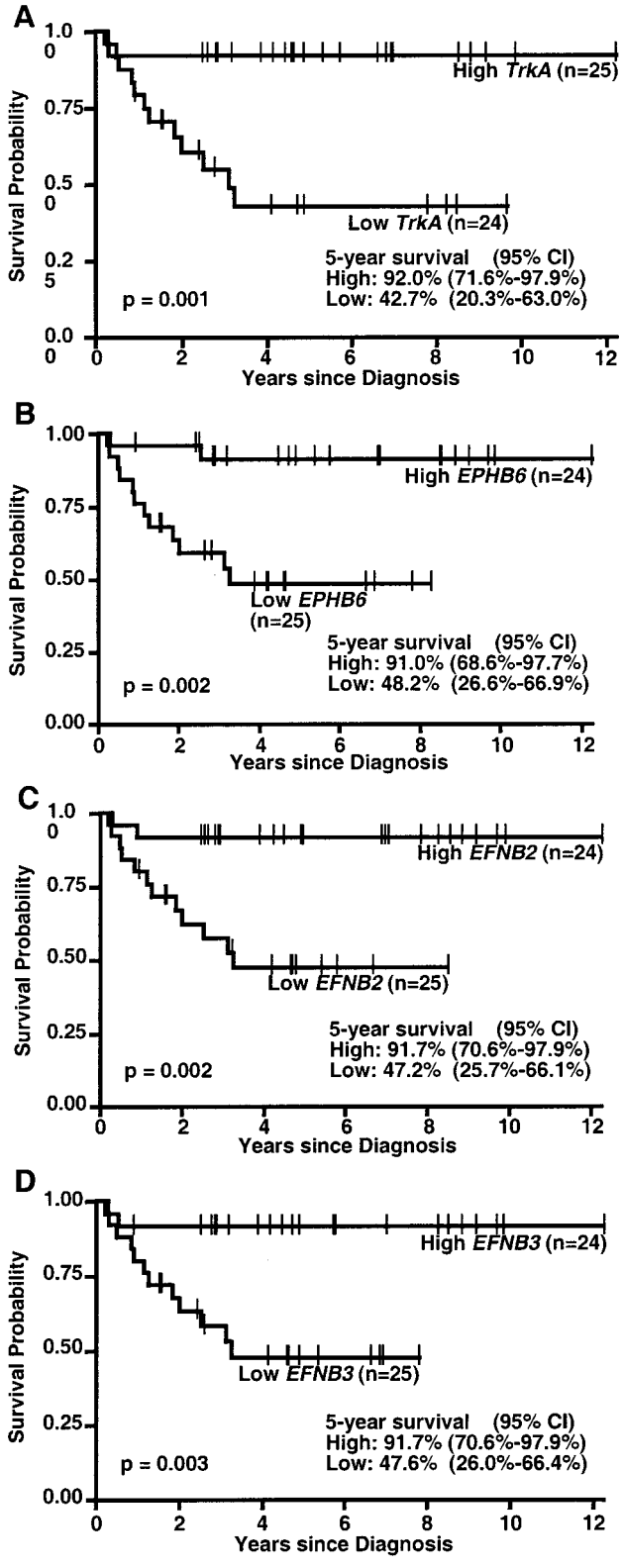

Fig. 1. The expression of *EPHB6*, *EFNB2*, and *EFNB3* predicts disease outcome of NB. Survival probabilities of groups of NB with low- or high-level expression of *TrkA* (*A*), *EPHB6* (*B*), *EFNB2* (*C*), or *EFNB3* (*D*) were estimated by the method of Kaplan-Meier. The median value of each variable was used as a cut-off to define high- and low-expression subgroups. Five-year survival and 95% confidence interval (CI) also were calculated for each subgroup. The log-rank test was used to compare survival probabilities of the two groups.

Table 2. Cox models with age, stage, *MYCN* amplification, and the expression of *EPHB6*, *EFNB2*, and *EFNB3*

Model	Variable	HR (95% CI)	*P*	Variable	HR (95% CI)	*P*
A1	Age*	5.31 (0.66–42.64)	0.116	*EPHB6*§	0.09 (0.02–0.54)	0.009
A2	Stage†	8.58 (1.06–69.40)	0.044	*EPHB6*	0.11 (0.01–0.84)	0.034
A3	*MYCN*‡	4.03 (1.20–13.50)	0.024	*EPHB6*	0.10 (0.01–0.80)	0.030
B1	Age	3.14 (0.35–28.07)	0.306	*EFNB2*§	0.20 (0.07–0.60)	0.004
B2	Stage	6.7 (0.79–56.50)	0.080	*EFNB2*	0.24 (0.09–0.69)	0.008
B3	*MYCN*	1.56 (0.28–8.76)	0.615	*EFNB2*	0.20 (0.04–0.90)	0.036
C1	Age	5.33 (0.67–42.38)	0.113	*EFNB3*§	0.11 (0.02–0.57)	0.009
C2	Stage	9.88 (1.26–77.27)	0.029	*EFNB3*	0.10 (0.02–0.60)	0.012
C3	*MYCN*	3.75 (1.17–12.01)	0.026	*EFNB3*	0.11 (0.02–0.74)	0.024

Cox regression models were used to assess prognostic significance of variables indicated. HR, Hazard ratio. CI, Confidence interval.
*>1 year vs. <1 year.
†Stage 3, 4 vs. 1, 2, 4S.
‡Amplified vs. normal.
§Continuous variable.

general NB population, these data collectively demonstrated that the study cohort would allow us to evaluate the clinical and biological significance of *EPHB6*, *EFNB2*, and *EFNB3* expressions in NB.

Expression of *EPHB6*, *EFNB2*, and *EFNB3* Predicts Disease Outcome of NB. We next investigated whether *EPHB6*, *EFNB2*, and *EFNB3* expressions were predictive of NB outcome by using the Kaplan-Meier method. Survival probabilities of high- and low-expression subgroups for each transcript were estimated, and survival of groups was compared by log-rank tests. As shown in Fig. 1 *B–D*, high-level expression of *EPHB6*, *EFNB2*, and *EFNB3* predicted favorable outcome of NB ($P = 0.002$, $P = 0.002$, and $P = 0.003$, respectively). NB with high-level expression of *EPHB6*, *EFNB2*, and *EFNB3* had better 5-year survival probabilities (91.0%, 91.7%, and 91.7%, respectively) than those with low-level expression of these transcripts (48.2%, 47.2%, and 47.6%, respectively). The univariate Cox regression analysis confirmed that high-level expression of *EPHB6*, *EFNB2*, and *EFNB3* was a strong predictor of favorable NB outcome ($P = 0.005$, $P < 0.0005$, and $P = 0.002$, respectively) (data not shown). In addition, we examined the effect of age, stage, or *MYCN* amplification on prognostic significance of *EPHB6*, *EFNB2*, and *EFNB3* expressions. Cox regression analysis showed that after including age, stage, or *MYCN* amplification separately in the models, *EPHB6*, *EFNB2*, and *EFNB3* expressions remained prognostic (Table 2). However, further studies on a larger cohort of NB will be needed to confirm these findings by using single Cox models including these variables.

***EPHB6* and *EFNB3* Expressions Are Associated with Stage Whereas *EFNB2* and *TrkA* Expressions Are Associated with Both Stage and Age.** Previously, we showed that high-level expressions of *EPHB6*, *EFNB2*, and *EFNB3* were associated with low tumor stage and correlated with high *TrkA* expression in NB (26). Moreover, high-level expression of *EPHB6*, *EFNB2*, *EFNB3*, and *TrkA* was predictive of favorable NB outcome. These observations raised the question of whether these genes had the same expression pattern in NB. Because *TrkA* expression is known to be associated with both stage and age (13, 33), we analyzed expression patterns of these four transcripts in our cohort of NB based on age (<1 vs. >1 year). As shown in Table 3, *EFNB2* and *TrkA* expressions were associated with age and stage. In contrast, expressions of *EPHB6* and *EFNB3* were associated only with stage. These results thus suggest that although these four genes shared similar prognostic characteristics in NB, their expression patterns were different.

***EPHB6*, *EFNB2*, or *EFNB3* Expression Predicts NB Disease Outcome Independent of *TrkA* Expression.** The Kaplan-Meier analysis was used to further explore the prognostic relationship between *TrkA* expression and the expression of *EPHB6*, *EFNB2*, or *EFNB3* in NB. The study cohort was divided into groups based on expression levels of the following gene combinations: *EPHB6* and *TrkA*; *EFNB2* and *TrkA*; *EFNB3* and *TrkA*. For example, there were four groups for the *EPHB6* and *TrkA* combination: low *TrkA*/low *EPHB6*; low *TrkA*/high *EPHB6*; high *TrkA*/low *EPHB6*; and high *TrkA*/high *EPHB6*. The patterns of patient distribution and survival probabilities of each of the four groups then were examined.

Table 3. Differential expression of *EPHB6*, *EFNB2*, *EFNB3*, and *TrkA* in NB subsets defined by age at diagnosis or tumor stage

	Age at diagnosis			Tumor stage		
Variable	<1 year (*n* = 17) Median	>1 year (*n* = 33) Median	*P*	1, 2, 4S (*n* = 23) Median	3, 4 (*n* = 27) Median	*P*
EPHB6	0.81	0.69	0.1546	0.90	0.52	0.0004
EFNB2	2.01	1.38	0.0022	2.01	1.31	0.0003
EFNB3	0.65	0.46	0.3621	0.77	0.43	0.0216
TrkA	2.18	1.09	0.0001	1.79	0.91	0.0005

The Wilcoxon rank-sum test was used to assess differential expression of *EPHB6*, *EFNB2*, *EFNB3*, and *TrkA* in subsets of NB indicated.

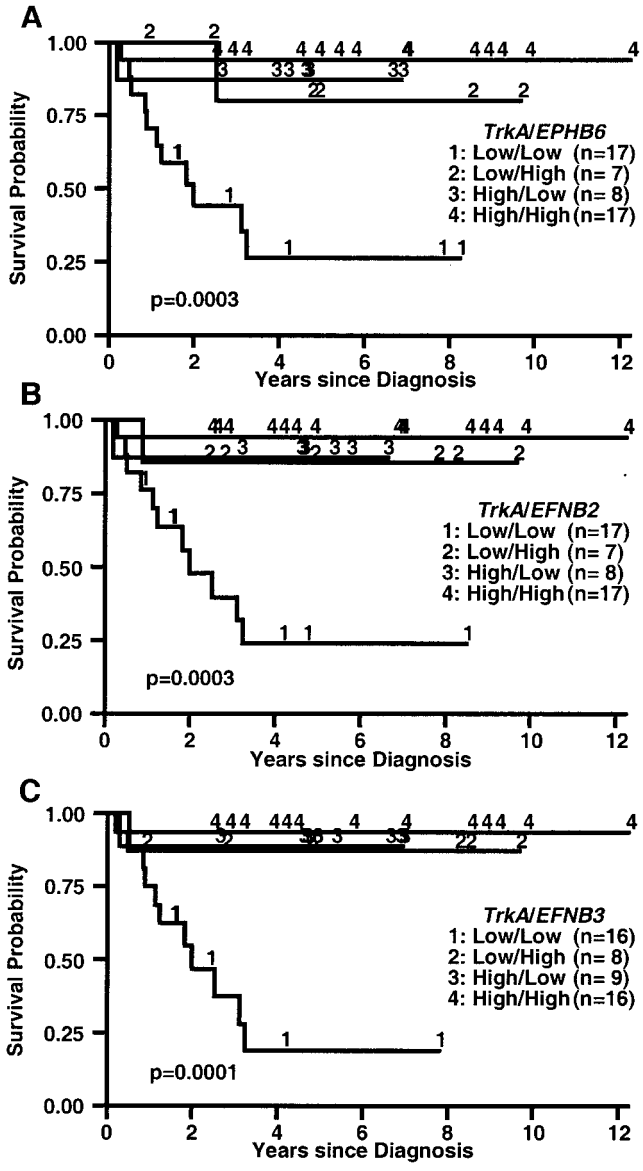

Fig. 2. High-level expression of *EPHB6*, *EFNB2*, and *EFNB3* predicts favorable outcome of NB independent of *TrkA* expression. Survival probabilities of four groups of NB defined by expression levels (1: low/low; 2: low/high; 3: high/low; 4: high/high) of each gene combination (*A*: *TrkA* and *EPHB6*; *B*: *TrkA* and *EFNB2*; *C*: *TrkA* and *EFNB3*) were estimated by the method of Kaplan-Meier. Five-year survival and 95% confidence intervals also were calculated for each subgroup. The log-rank test was used to compare survival probabilities of the two groups.

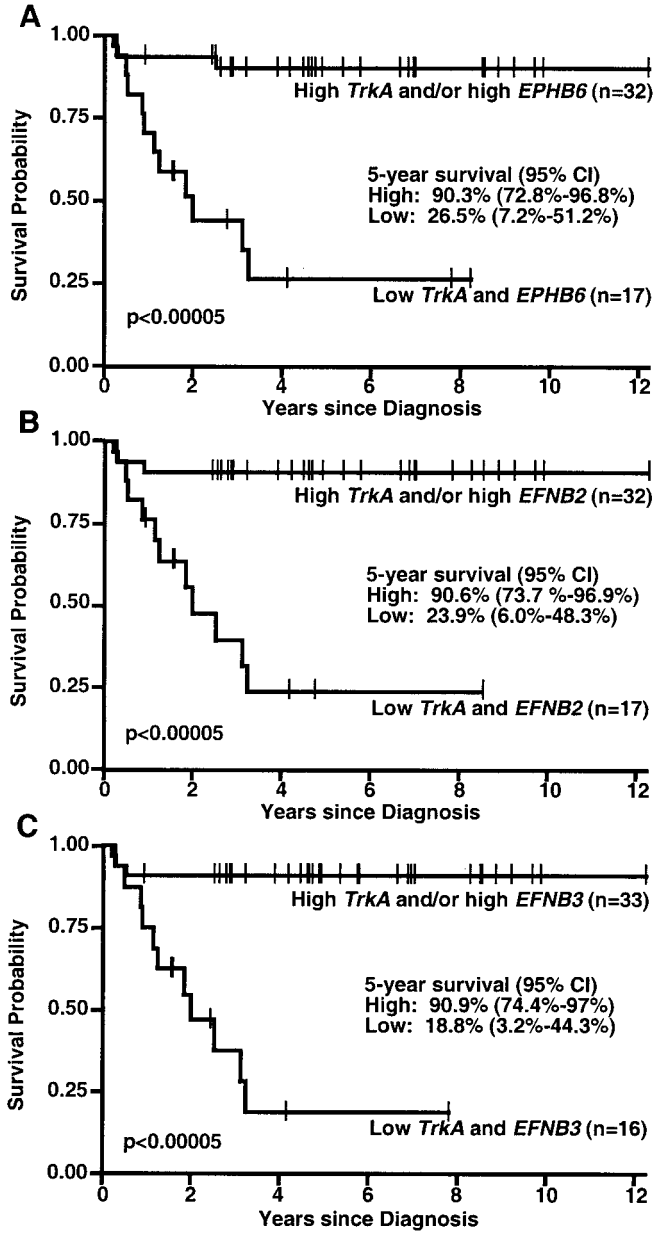

Fig. 3. Dichotomized combination variables of *EPHB6*/*TrkA*, *EFNB2*/*TrkA*, and *EFNB3*/*TrkA* strongly predict outcome of NB. Dichotomized variables were generated based on *TrkA* expression and *EPHB6*, *EFNB2*, or *EFNB3* expression. One category included NB expressing low levels of both *TrkA* and *EPHB6*, *EFNB2* or *EPNB3*, (group 1 in Fig. 2), whereas the other included NB expressing high levels of either one or both (combination of groups 2, 3, and 4 in Fig. 2). Survival probabilities of each two groups of NB defined by the dichotomized combination variables, *EPHB6*/*TrkA* (*A*), *EFNB2*/*TrkA* (*B*), and *EFNB3*/*TrkA* (*C*), were estimated by the method of Kaplan-Meier. The log-rank test was used to compare survival probabilities of the groups. CI, Confidence interval.

As shown in Fig. 2*A*, one-third of the NB expressed both low *TrkA* and low *EPHB6* (group 1). The second one-third expressed both high *TrkA* and high *EPHB6* (group 4). The last one-third included NB expressing high *EPHB6* but low *TrkA* (group 2) and those expressing high *TrkA* but low *EPHB6* (group 3). The existence of groups 2 and 3 indicated that favorable and unfavorable groups of NB identified by *EPHB6* expression were different from those by *TrkA* expression or vice versa. Therefore, *EPHB6* expression was predictive of NB disease outcome independent of *TrkA* expression. Similar results were obtained for *TrkA*/*EFNB2* and *TrkA*/*EFNB3* combinations (Fig. 2 *B* and *C*), although individual NB in the corresponding groups identified by these three variable combinations were different. Collectively, these data demonstrated that *EPHB6*, *EFNB2*, and *EFNB3* expression predicted disease outcome of NB independent of *TrkA* expression.

Expression of *EPHB6*, *EFNB2*, or *EFNB3* in Combination with *TrkA* Expression Predicts NB Disease Outcome More Accurately than Each Variable Alone. Because the survival curves of groups 2, 3, and 4 of each variable were statistically indistinguishable (Fig. 2), we

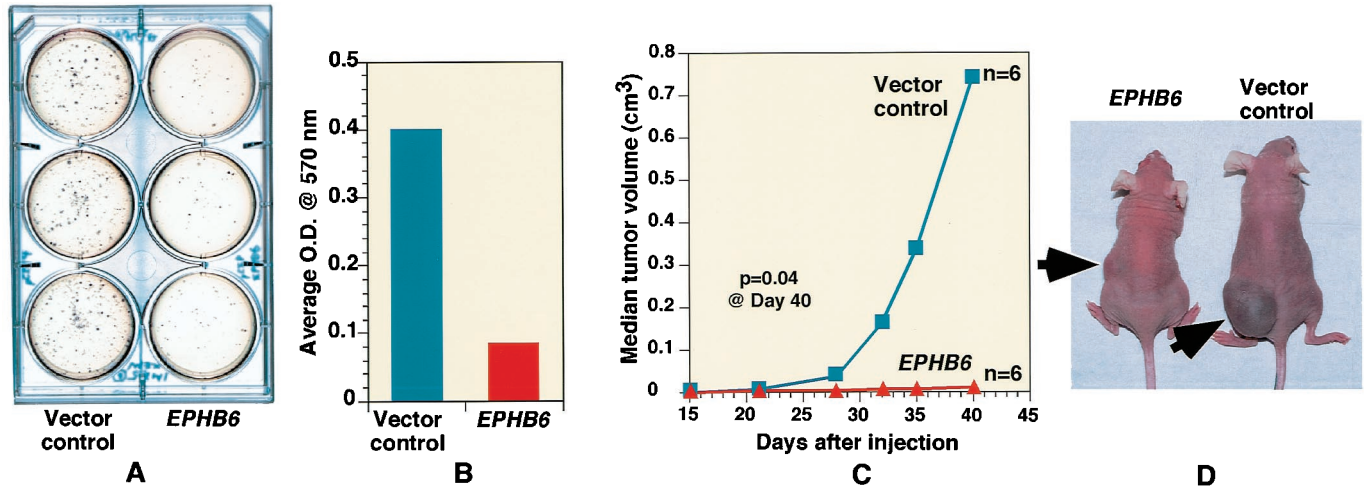

Fig. 4. EPHB6 suppresses NB cell growth *in vitro* and *in vivo*. (*A*) IMR5 cells were transfected by electroporation with either pCEP4 eukaryotic expression vector alone or the vector containing a human *EPHB6* cDNA. The resulting transfectants were plated into three wells of a 6-well plate and selected for 14 days with hygromycin. After the selection, the cells were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to stain viable cells. A digital scanner was used to capture the image of dark green MTT-stained cells. (*B*) To quantitatively analyze the amount of viable cells, the MTT-stained cells were solubilized by acidic isopropanol, and optical density was measured at 570 nm. (*C*) SY5Y cells were transiently transfected with linearlized DNA [pcDNA3.1/Hygro(+) vector control or the vector containing *EPHB6* cDNA]. Twenty-four hours after the transfection, the cells were harvested and injected s.c. in the flank of nude mice. Median tumor sizes of control and *EPHB6* groups at given time points are shown. At day 40, the difference in tumor size between the two groups reached a statistical significance ($P = 0.04$). Median values for the control and *EPHB6* groups were 0.74 and 0.008, respectively. (*D*) A representative animal from each group is shown.

generated dichotomized combination variables based on *TrkA* expression and *EPHB6*, *EFNB2*, or *EFNB3* expression (i.e., *EPHB6*/*TrkA*, *EFNB2*/*TrkA*, and *EFNB3*/*TrkA*) and performed the Kaplan-Meier analysis. For example, one category included NB expressing low levels of both *TrkA* and *EPHB6* (group 1 in Fig. 2), whereas the other included NB expressing high levels of either one or both (i.e., combination of groups 2, 3, and 4 in Fig. 2). This approach allowed us to further explore the prognostic significance of *EPHB6*, *EFNB2*, *EFNB3*, and *TrkA* expressions in NB. As shown in Fig. 3, all of the dichotomized combination variables (*EPHB6*/*TrkA*, *EFNB2*/*TrkA*, and *EFNB3*/*TrkA*) were highly predictive of NB outcome ($P < 0.00005$), and they remained prognostic among NB without *MYCN* amplification ($P < 0.01$, data not shown). The data also indicated that the dichotomized combination variables further separated NB into favorable and unfavorable groups compared with each variable alone (*EPHB6*, *EFNB2*, *EFNB3*, or *TrkA*) (Fig. 1 vs. Fig. 3), because *EPHB6*, *EFNB2*, or *EFNB3* expression identified additional favorable NB among those tumors expressing low levels of *TrkA*. Collectively, these data indicate that expression of *EPHB6*, *EFNB2*, or *EFNB3* in combination with *TrkA* expression predicted disease outcome of NB more accurately than each variable alone.

Favorable NB Outcome Results from High-Level Expression of Any One of the Four Genes (*EPHB6, EFNB2, EFNB3,* or *TrkA*). Interestingly, the results presented in Figs. 2 and 3 showed that if any one of the four genes (*EPHB6*, *EFNB2*, *EFNB3*, or *TrkA*) was expressed at high levels, that alone predicted favorable outcome of NB. What one may infer from these data is that a good disease outcome of NB is a consequence of high-level expression of at least one of these favorable NB genes. If so, increased expression of a favorable NB gene (e.g., *EPHB6*, *EFNB2*, *EFNB3*, or *TrkA*) in an otherwise unfavorable NB may suppress their malignant phenotype.

***EPHB6* Suppresses Clonogenicity and Tumorigenicity of NB Cells *in Vitro* and *in Vivo*.** In an attempt to test this hypothesis, we examined the effect of *EPHB6* on NB cells *in vitro* and *in vivo*. Two human NB cell lines, IMR5 (*MYCN*-amplified) and SY5Y (normal *MYCN*), were chosen for this analysis because they were derived from unfavorable NB and expressed little or no endogenous *EPHB6*. In fact, low expression of *EPHB6* is a general feature of NB cell lines (unpublished observation). As shown in Fig. 4 *A* and *B*, transfection of IMR5 with *EPHB6* cDNA inhibited its clonogenicity *in vitro*. Additional transfection experiments were performed by using another eukaryotic expression vector, pcDNA3.1/Hygro(+), and the results of these experiments were consistent with those in which the pCEP4 vector was used. *EPHB6* also inhibited clonogenicity of SY5Y in culture (data not shown). We further examined the effect of *EPHB6* on the tumorigenicity of NB xenografts in athymic nude mice. As shown in Fig. 4 *C* and *D*, the introduction of *EPHB6* into SY5Y suppressed its tumorigenicity *in vivo*. At 40 days after the injection of SY5Y transfectants into nude mice, the difference in tumor size between the control group and the *EPHB6*-transfected group was statistically significant ($P = 0.04$) (Fig. 4*C*). These results thus demonstrated that *EPHB6* suppressed malignant phenotype of NB cell lines derived from unfavorable NB.

Discussion

Considerable efforts have been made in recent years to elucidate the biological functions of EPH family receptors and their ligand ephrins in neural and cardiovascular development. Nonetheless, little has been learned about their roles in cancer biology. Our previous study has shown that NB expresses transcripts encoding EPHB receptors (*EPHB*) as well as ephrin-B ligands (*EFNB*). Among the *EPHB* and *EFNB* transcripts expressed in NB, levels of *EPHB6*, *EFNB2*, and *EFNB3* expressions are significantly higher in low-stage NB than in advanced-stage disease.

In this study, we showed that high-level expression of *EPHB6*, *EFNB2*, and *EFNB3* is predictive of favorable NB disease outcome. We also have found that the expression pattern of *TrkA* and *EFNB2* is distinct from that of *EPHB6* and *EFNB3*. Namely, *EPHB6* and *EFNB3* expressions are associated with stage, whereas *EFNB2* and *TrkA* expressions are associated with both age and stage. Interestingly, CD44, another prognostic factor of favorable NB, shows the same expression pattern as *EFNB2* and

TrkA in NB (14). Thus, *EPHB6* and *EFNB3* expressions are the only known favorable prognostic factors solely associated with tumor stage. Collectively, these data suggest that stage-associated expression of *EPHB6* and *EFNB3* and age/stage-associated expression of *EFNB2*, *TrkA*, and CD44 may account for molecular mechanisms underlying why tumor stage and patient age at diagnosis are independent prognostic indicators of NB (1).

Because *TrkA* expression is among the most informative prognostic markers of NB (11–13), the prognostic relationship between *TrkA* expression and *EPHB6*, *EFNB2*, or *EFNB3* expression is of particular interest. Our results show that *EPHB6*, *EFNB2*, and *EFNB3* expressions are predictive of NB disease outcome independent of *TrkA* expression and combination of *TrkA* expression and *EPHB6*, *EFNB2*, or *EFNB3* expression predicts NB disease outcome more accurately than each variable alone. The survival analysis using the combination of *TrkA*/*EPHB6*, *TrkA*/*EFNB2*, and *TrkA*/*EFNB3* expression also reveals an intriguing phenomenon. If one of the genes (*EPHB6*, *EFNB2*, *EFNB3*, or *TrkA)* is expressed at high levels in NB, that alone predicts favorable NB outcome. In contrast, low expression of *EPHB6*/*TrkA*, *EFNB2*/*TrkA*, or *EFNB3*/*TrkA* defines NB with the worst outcome, including NB with *MYCN* amplification as well as those with normal *MYCN*. These observations thus suggest that expression levels of genes associated with favorable NB outcome or favorable NB genes determine survival of NB patients. In other words, unfavorable phenotype of NB may result from a lack of or diminished expression of the favorable NB genes, such as *EPHB6*, *EFNB2*, *EFNB3*, and *TrkA.* To address this question, we examined the effect of *EPHB6* on the biological behavior of NB cell lines, which originally were derived from unfavorable advanced NB. We demonstrate that the introduction of *EPHB6* can in fact suppress malignant phenotype of NB cell lines, including those with *MYCN* amplification. *EPHB6* thus can be considered a tumor suppressor of NB.

There is a great biological interest underlying the fact that high-level expression of transcripts encoding EPHB6, ephrin-B2, and ephrin-B3 predicts favorable outcome of NB. First, EPHB6 lacks its kinase activity because of a mutation at the ATP acceptor site (34, 35). Second, ephrin-B ligands are bifunctional molecules in that their extracellular domains promote angiogenesis (22) and their cytoplasmic domains suppress the growth-promoting activity of activated protein-tyrosine kinases (36). Thus, it is anticipated that EPHB6 restricts growth, angiogenesis, and metastasis of NB cells by acting as a dominant negative member among the EPHB receptor family and/or by sequestering ephrin-B ligands from participating in angiogenesis. Our study showing that *EPHB6* suppresses growth of NB cell lines *in vitro* and *in vivo* supports this hypothesis.

In conclusion, this study reveals the biological significance of *EPHB6*, *EFNB2*, and *EFNB3* expressions in NB and illustrates that expression levels of genes associated with favorable NB disease outcome can in fact directly influence benign or malignant phenotype of the tumor. An understanding of the function and regulation of the favorable NB genes, their products, and the biochemical pathways therefore may help develop an effective therapeutic strategy for children with unfavorable NB whose survival chances need much improvement.

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1. Breslow, N. & McCann, B. (1971) *Cancer Res.* **31,** 2098–2103.
2. Shimada, H., Chatten, J., Newton, W. A., Jr., Sachs, N., Hamoudi, A. B., Chiba, T., Marsden, H. B. & Misugi, K. (1984) *J. Natl. Cancer Inst.* **73,** 405–416.
3. Brodeur, G. M., Pritchard, J., Berthold, F., Carlsen, N. L., Castel, V., Castelberry, R. P., De Bernardi, B., Evans, A. E., Favrot, M., Hedborg, F., *et al*. (1993) *J. Clin. Oncol.* **11,** 1466–1477.
4. Look, A. T., Hayes, F. A., Shuster, J. J., Douglass, E. C., Castleberry, R. P., Bowman, L. C., Smith, E. I. & Brodeur, G. M. (1991) *J. Clin. Oncol.* **9,** 581–591.
5. Hann, H. W., Evans, A. E., Siegel, S. E., Wong, K. Y., Sather, H., Dalton, A., Hammond, D. & Seeger, R. C. (1985) *Cancer Res.* **45,** 2843–2848.
6. Seeger, R. C., Brodeur, G. M., Sather, H., Dalton, A., Siegel, S. E., Wong, K. Y. & Hammond, D. (1985) *N. Engl. J. Med.* **313,** 1111–1116.
7. Shuster, J. J., McWilliams, N. B., Castleberry, R., Nitschke, R., Smith, E. I., Altshuler, G., Kun, L., Brodeur, G., Joshi, V., Vietti, T., *et al*. (1992) *Am. J. Clin. Oncol.* **15,** 295–303.
8. Maris, J. M., White, P. S., Beltinger, C. P., Sulman, E. P., Castleberry, R. P., Shuster, J. J., Look, A. T. & Brodeur, G. M. (1995) *Cancer Res.* **55,** 4664–4669.
9. Caron, H., van Sluis, P., de Kraker, J., Bokkerink, J., Egeler, M., Laureys, G., Slater, R., Westerveld, A., Voute, P. A. & Versteeg, R. (1996) *N. Engl. J. Med.* **334,** 225–230.
10. Bown, N., Cotterill, S., Lastowska, M., O'Neill, S., Pearson, A. D., Plantaz, D., Meddeb, M., Danglot, G., Brinkschmidt, C., Christiansen, H., *et al*. (1999) *N. Engl. J. Med.* **340,** 1954–1961.
11. Kogner, P., Barbany, G., Dominici, C., Castello, M. A., Raschella, G. & Persson, H. (1993) *Cancer Res.* **53,** 2044–2050.
12. Suzuki, T., Bogenmann, E., Shimada, H., Stram, D. & Seeger, R. C. (1993) *J. Natl. Cancer Inst.* **85,** 377–384.
13. Nakagawara, A., Arima-Nakagawara, M., Scavarda, N. J., Azar, C. G., Cantor, A. B. & Brodeur, G. M. (1993) *N. Engl. J. Med.* **328,** 847–854.
14. Combaret, V., Gross, N., Lasset, C., Frappaz, D., Peruisseau, G., Philip, T., Beck, D. & Favrot, M. C. (1996) *J. Clin. Oncol.* **14,** 25–34.
15. Tessier-Lavigne, M. (1995) *Cell* **82,** 345–348.
16. Winslow, J. W., Moran, P., Valverde, J., Shih, A., Yuan, J. Q., Wong, S. C., Tsai, S. P., Goddard, A., Henzel, W. J., Hefti, F., *et al*. (1995) *Neuron* **14,** 973–981.
17. Wang, H. U. & Anderson, D. J. (1997) *Neuron* **18,** 383–396.
18. Magal, E., Holash, J. A., Toso, R. J., Chang, D., Lindberg, R. A. & Pasquale, E. B. (1996) *J. Neurosci. Res.* **43,** 735–744.
19. Lumsden, A. & Krumlauf, R. (1996) *Science* **274,** 1109–1115.
20. Pandey, A., Shao, H., Marks, R. M., Polverini, P. J. & Dixit, V. M. (1995) *Science* **268,** 567–569.
21. Daniel, T. O., Stein, E., Cerretti, D. P., St. John, P. L., Robert, B. & Abrahamson, D. R. (1996) *Kidney Intl.*, Suppl., **57,** S73–S81.
22. Adams, R. H., Wilkinson, G. A., Weiss, C., Diella, F., Gale, N. W., Deutsch, U., Risau, W. & Klein, R. (1999) *Genes Dev.* **13,** 295–306.
23. Wang, H. U., Chen, Z. F. & Anderson, D. J. (1998) *Cell* **93,** 741–753.
24. Easty, D. J., Guthrie, B. A., Maung, K., Farr, C. J., Lindberg, R. A., Toso, R. J., Herlyn, M. & Bennett, D. C. (1995) *Cancer Res.* **55,** 2528–2532.
25. Vogt, T., Stolz, W., Welsh, J., Jung, B., Kerbel, R. S., Kobayashi, H., Landthaler, M. & McClelland, M. (1998) *Clin. Cancer Res.* **4,** 791–797.
26. Tang, X. X., Evans, A. E., Zhao, H., Cnaan, A., London, W., Cohn, S. L., Brodeur, G. M. & Ikegaki, N. (1999) *Clin. Cancer Res.* **5,** 1491–1496.
27. Tang, X. X., Brodeur, G. M., Campling, B. G. & Ikegaki, N. (1999) *Clin. Cancer Res.* **5,** 455–460.
28. Gale, N. W., Holland, S. J., Valenzuela, D. M., Flenniken, A., Pan, L., Ryan, T. E., Henkemeyer, M., Strebhardt, K., Hirai, H., Wilkinson, D. G., *et al*. (1996) *Neuron* **17,** 9–19.
29. Kaplan, E. L. & Meier, P. (1958) *J. Am. Stat. Assoc.* **53,** 457–481.
30. Mantel, N. (1966) *Cancer Chemother. Rep.* **50,** 163–170.
31. Cox, D. R. (1972) *J. R. Stat. Soc. B* **74,** 187–220.
32. Silber, J. H., Evans, A. E. & Fridman, M. (1991) *Cancer Res.* **51,** 1426–1433.
33. Combaret, V., Gross, N., Lasset, C., Balmas, K., Bouvier, R., Frappaz, D., Beretta-Brognara, C., Philip, T., Favrot, M. C. & Coll, J. L. (1997) *Br. J. Cancer* **75,** 1151–1155.
34. Matsuoka, H., Iwata, N., Ito, M., Shimoyama, M., Nagata, A., Chihara, K., Takai, S. & Matsui, T. (1997) *Biochem. Biophys. Res. Commun.* **235,** 487–492.
35. Gurniak, C. B. & Berg, L. J. (1996) *Oncogene* **13,** 777–786.
36. Bruckner, K., Pasquale, E. B. & Klein, R. (1997) *Science* **275,** 1640–1643.