High-Level Expression of EPHB6, EFNB2, and EFNB3 Is Associated with Low Tumor Stage and High TrkA Expression in Human Neuroblastomas

Xao X. Tang, Audrey E. Evans, Huaqing Zhao, Avital Cnaan, Wendy London, Susan L. Cohn, Garrett M. Brodeur, and Naohiko Ikegaki


ABSTRACT

Neuroblastoma (NB) is a common pediatric tumor of neural crest origin that is biologically and clinically heterogeneous. EPH family receptor tyrosine kinases and ephrin ligands play fundamental roles in neurodevelopmental processes. Recently, we found that NB cell lines expressed several EPHB and EFNB transcripts, which encode EPHB subgroup receptors and ephrin-B subgroup ligands, respectively. To explore the role of EPHB receptors and ephrin-B ligands in the biology of NB, we examined the expression of EPHB and EFNB transcripts in 47 primary NB specimens. Multiple EPHB and EFNB transcripts were expressed in all of the NB tumors examined, suggesting the involvement of these transcripts in modulating the biological behavior of NB. Higher levels of EPHB6, EFNB2, and EFNB3 expression were found in low-stage tumors (stage 1, 2, and 4S) than in advanced-stage tumors (stage 3 and 4; P = 0.0013, P = 0.0048, and P = 0.027, respectively). Expression of TrkA, a well-established prognostic marker of favorable NB, was positively correlated with EPHB6, EFNB2, and EFNB3 expression (P < 0.0001, P = 0.0019, and P = 0.0001, respectively). MYCN-amplified tumors expressed lower levels of EPHB6, EFNB2, EFNB3, and TrkA transcripts compared to nonamplified tumors (P = 0.0006, P = 0.0023, P = 0.0048, and P = 0.0001, respectively). These data suggest that high-level expression of EPHB6, EFNB2, and EFNB3 is associated with favorable NB and that low-level expression of EPHB6, EFNB2, and EFNB3 correlates with aggressive MYCN-amplified NB. Thus, EPHB6, EFNB2, and EFNB3 may have biological relevance in NB. Further investigation on the biology of these genes may help provide insight into the treatment of NB.

INTRODUCTION

NB is a common pediatric tumor of neural crest origin. The tumor occurs frequently in infants and young children, with the primary lesion in the adrenals or the sympathetic chain. It is known that NB exhibits a wide range of clinical, genetic, and biological heterogeneity. Previous studies suggest that there are three distinct subsets of NB (1–4). Of those, two subsets show apparently opposite outcomes; low-risk NB is curable with minimal or no treatment, whereas high-risk NB is usually lethal despite the most aggressive treatments, including bone marrow transplantation. Patients with low-risk NB are likely to be infants having low-stage disease (stage 1, 2, or 4S). These NBs are near triploid in karyotype, rarely show chromosomal abnormalities, and have a high TrkA expression. In contrast, patients with high-risk NB are more likely to be >1 year of age and have advanced-stage disease (stage 3 or 4). High-risk NBs are near diploid in karyotype and have numerous structural chromosomal abnormalities, including MYCN amplification and deletion or allelic loss of chromosome 1p. TrkA expression is low or absent in the high-risk NB, but those tumors with MYCN amplification often express TrkB (5).

Between these two NB subsets is the intermediate-risk NB subset, which accounts for about 25–30% of total NB cases. Intermediate-risk NBs are characterized by patients with advanced-stage disease (stage 3 and 4), a near diploid karyotype, and no MYCN amplification. About 25–50% of them have deletion or allelic loss of 1p or other chromosomes. TrkA expression is generally low in this group. The 3-year survival for intermediate-risk NB is between 25–50%, suggesting that this subset of NB may be heterogeneous.

EPH family receptor kinases and their ephrin ligands are involved in fundamental developmental processes in the nervous system, including axon guidance (6), axon fasciculation (7), neural crest cell migration (8), acquisition of brain subregional identity (9), and neuronal cell survival (10). Moreover, evidence suggests that some members of the EPH family and their ligands are involved in angiogenesis and oncogenesis (11–14). The EPH family is the largest subfamily of receptor protein tyrosine kinases, consisting of 14 known members (15). Similarly, ephrin
ligands constitute a large family, which includes eight members (15).

Ephrins are cell surface-bound ligands and are divided into two subgroups (ephrin-A and ephrin-B), depending on how they are anchored to the cell membrane (16). The ephrin-A ligands are glycosylphosphatidylinositol-linked proteins and are encoded by the EFNA genes. In contrast, ephrin-B ligands are transmembrane proteins and are encoded by the EFNB genes. The EPH family receptors can also be divided into two 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 (16).

We reported previously that EPHB2 transcripts were highly expressed in NB cell lines (17). Subsequently, we found that transcripts encoding several EPHB receptors and their ephrin-B ligands were coexpressed in these NB cell lines.4 In this study, we examined the expression of transcripts encoding five members of the EPHB subgroup and three members of the ephrin-B subgroup in 47 NB tumor specimens representing all clinical stages. We found that transcripts encoding multiple EPHB receptors and ephrin-B ligands were expressed at various levels in all of the NB tumors examined. High levels of EPHB6, EFNB2, and EFNB3 expression were associated with low-stage NB, and their expression was positively correlated with TrkA expression. In contrast, MYCN-amplified tumors expressed lower levels of EPHB6, EFNB2, EFNB3, and TrkA transcripts compared to nonamplified tumors. The implications of these observations in the biology of NB are discussed.

MATERIALS AND METHODS

NB Tumor Samples. Forty-seven NB tumor specimens were obtained from the Tumor Bank of The Children’s Hospital of Philadelphia and from the Tumor Bank of The Pediatric Oncology Group. These include 10 stage 1 tumors, 8 stage 2 tumors, 5 stage 4S tumors, 12 stage 3 tumors, and 12 stage 4 tumors. Among these tumors, two stage 3 tumors and four stage 4 tumors had MYCN amplification.

RNA Extraction and RT. Total cellular RNA was prepared by the Totally RNA kit (Ambion) or by the method described by Auffray and Rougeon (18). Total RNA (1 μg) extracted from primary NB specimens was mixed with 35 ng of random hexamers and 250 ng of oligothymidylic acid (15-mer) in a volume of 10 μl. The resultant mixture was heated at 70°C for 10 min and chilled on ice. Each RT reaction was carried out in a volume of 10 μl containing the RNA-primer mixture, 10 mM DTT, 500 μM deoxynucleotide triphosphates, 2 mM of the RT reaction, 50 mM KCl, 2 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), and 1 unit of AmpliTaq Gold (PE Applied Biosystems). RT reactions were followed by 20 cycles of 95°C for 12 min followed by 20 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 5 min, followed by 72°C for 10 min. Nucleotide sequences of EPHB1, EPHB2, EPHB3, EPHB4, EPHB6, EFNB1, EFNB2, EFNB3, and GAPD were amplified and were reported elsewhere (21). TrkA PCR primers have the following nucleotide sequences: sense primer, 5′-TGCCTGCTCCTTCTCTTTCTA-3′; antisense primer, 5′-GTGGTGACACAGGCATCAC-3′.

PCR products (10 μl) of a total of 20 μl of PCR products were subjected to 6% PAGE. DNA bands were electrotransferred onto nylon membrane (Hybond N⁺; Amersham) and immobilized to the membrane by baking the filter for 30 min at 80°C, followed by 1 min of UV irradiation. The biotinylated PCR products were then detected using the Southern Light chemiluminescence detection procedure (Tropix, Inc.). Quantification of RNA transcript expression was performed by densitometric analysis on X-ray films. The

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expression of a given transcript was then normalized by taking the ratio between the densitometric unit of the transcript and that of the internal control, GAPD.

This semiquantitative RT-PCR analysis requires much less RNA and allowed us to examine the expression of 15 or more transcripts from a few micrograms of RNA sample. This was critical because in some cases, we could only obtain a very limited amount of tumor RNA. Moreover, the semiquantitative RT-PCR analysis provided results consistent with those obtained by Northern blot analysis, indicating the validity of this method for a quantitative analysis of transcript expression (21).

** Statistical Analysis. ** A t test was used to examine possible associations between clinical stages and the expression of genes of interest as well as differential expression of EPHB and EFNB transcripts in MYCN-amplified and nonamplified NBs. The Pearson correlation coefficient (r) and P for each gene pair examined were calculated. Analysis was performed using Stata version 5.0 (State Corp., College Station, TX).

**RESULTS**

We found previously that EPHB2 and EFNB transcripts were expressed in NB cell lines (17). These observations prompted us to examine the expression of EPHB2 transcripts as well as EFNB1, EFNB2, and EFNB3 transcripts encoding ephrin-B1, ephrin-B2, and ephrin-B3, respectively, in primary NB. Because all three members of the ephrin-B subgroup can interact with all members of the EPHB receptor subgroup, the expression of EPHB1, EPHB3, EPHB4, and EPHB6 was also examined. The expression of EPHB5 was not examined because a human EPHB5 gene has not been identified.

Semi quantitative RT-PCR analysis was used to examine EPHB and EFNB expression in NB tumors (see “Materials and Methods”). Human fetal brain RNA was included in the RT-PCR analysis as a positive control because transcripts encoding EPH family receptors and ephrin ligands are known to be highly expressed in the developing nervous system (17, 22, 23). As described below, multiple EPHB transcripts (EPHB2, EPHB3, EPHB4, and EPHB6) were expressed at various levels in all NBs examined. EPHB1 expression was found to be either absent or very low (data not shown).

**Expression of EPHB2, EPHB3, and EPHB4 Transcripts in NB.** As shown in Fig. 1, all NBs examined expressed EPHB2, EPHB3, and EPHB4 transcripts at various levels. There was no association between the expression levels of these transcripts and the clinical stage of NB. In addition, there was no differential expression of EPHB2 or EPHB4 in MYCN-amplified and nonamplified tumors. However, there was a trend in which MYCN-amplified tumors expressed lower levels of EPHB3 than nonamplified tumors (P = 0.0391; Fig. 1).

**Association between High-Level EPHB6 Expression and Low-Stage NB, and Low-Level Expression of EPHB6 in MYCN-amplified Tumors.** Like EPHB2, EPHB3, and EPHB4, EPHB6 transcripts were also expressed at various levels in all 47 NBs examined (Fig. 1). However, EPHB6 was found to be expressed at higher levels in low-stage tumors (stage 1, 2, and 4S) than in advanced-stage tumors (stage 3 and 4) (P = 0.0013; Fig. 1).
Expression of EFNB Transcripts in NB and Association of High-Level EFNB2 and EFNB3 Expression with Low-Stage NB. EFNB1, EFNB2, and EFNB3 transcripts were also detected at various levels in the same set of NB samples (Fig. 2). There was no association of EFNB1 expression with NB stage, and there was no differential expression of EFNB1 expression in MYCN-amplified and nonamplified tumors. In contrast, higher levels of EFNB2 expression were found to be associated with low-stage NB ($P = 0.0048$; Fig. 2). Moreover, MYCN-amplified tumors expressed lower levels of EFNB2 than nonamplified tumors, and this differential expression of EFNB2 was statistically significant ($P = 0.0023$; Fig. 2). Similarly, high levels of EFNB3 expression were associated with low-stage NB ($P = 0.027$; Fig. 2). As found in EPHB6 and EFNB2, MYCN-amplified tumors expressed lower levels of EFNB3 than nonamplified tumors. This differential expression of EFNB3 was statistically significant as well ($P = 0.0048$; Fig. 2).

Correlation of EPHB6, EFNB2, and EFNB3 Expression with TrkA Expression. Because the expression of EPHB6, EFNB2, and EFNB3 was found to be associated with low-stage NB tumors, we examined whether their expression was correlated with TrkA expression, a well-established prognostic marker of favorable NB (24–26). This analysis was designed to evaluate the potential contribution of EPHB6, EFNB2, and EFNB3 expression to a favorable outcome of NB. The examination of TrkA in our study cohort of NB also served as a control for sampling bias (see “Discussion”). As shown in Fig. 3, TrkA expression was positively correlated with the expression of EPHB6, EFNB2, and EFNB3 ($P < 0.0001$, $P = 0.0019$, and $P = 0.0001$, respectively).

DISCUSSION

A number of growth/differentiation factor receptors are known to influence the biological behavior of various tumors through autocrine and/or paracrine activation by interacting with their cognate ligands. Trk family receptor protein tyrosine kinases and their ligands, neurotrophins, have been suggested to play important roles in the biological behavior of NB. High TrkA expression without concomitant expression of its ligand, nerve growth factor, is associated with low-stage, favorable-prognosis NB (3). In contrast, the coexpression of TrkB and its ligand, brain-derived neurotrophic factor, is associated with advanced-stage, unfavorable-prognosis NB, especially in NBs with MYCN amplification (5).

In this study, we found that transcripts encoding multiple members of the EPHB receptor kinases and the ephrin-B ligands were expressed together in primary NB. It should be mentioned that coexpression of multiple EPHB and EFNB transcripts was also detected in NB cell lines (data not shown), indicating that NB cells expressed these transcripts. The coexpression of EPHB and EFNB transcripts suggests that there are multiple autostimulation loops of EPHB receptors mediated by their ephrin-B ligands in NB. These autostimulation loops may, in turn, modulate biological behaviors of NB cells.

To further explore the biological significance of EPHB receptors and their ephrin-B ligands in NB, we examined whether their expression was associated with the clinical stage of NB and with well-established favorable and unfavorable prognostic markers of NB, namely TrkA expression and MYCN amplification, respectively. The examination of TrkA expression also served as a control for sampling bias because TrkA expres-
EFNB2, EPHB6, and EFN3 in the general NB population. The expression of these genes has been investigated in large study cohorts, and its expression pattern in NB has been well established (24–26). In fact, our data on TrkA expression in NB in this study were consistent with the previous finding: high TrkA expression was associated with low-stage, favorable NB (P = 0.0011; data not shown). These data suggest that there is no apparent sampling bias in our study cohort; thus, the expression pattern of EPHB6, EFN2, and EFN3 in primary NBs were plotted against those of TrkA in the corresponding tumors. The Pearson correlation coefficient (r) and P for each pair were calculated using Stata version 5.0 software.

As described earlier, we found that higher levels of EPHB6, EFN2, and EFN3 expression were associated with low-stage NB, and this association was statistically significant. In addition, the expression of EPHB6, EFN2, and EFN3 was positively correlated with TrkA expression. Collectively, these data suggest that high-level expression of EPHB6, EFN2, and EFN3 is associated with a favorable outcome of NB. Although it is not clear if this association indicates a causal relationship, it is worth examining whether high-level expression of EPHB6, EFN2, and EFN3 could suppress aggressive behaviors of NB in experimental model systems.

It should also be mentioned that whereas the association between high-level expression of EPHB6, EFN2, EFN3, and TrkA and low-stage NB was statistically significant, there was an overlap in the expression of these genes between low- and advanced-stage NB. This reflects the fact that the NB staging system is not a perfect prognostic parameter for risk assessment of NB. In fact, one of our goals is to identify such prognostic markers that clearly distinguish high-risk groups from low-risk groups in the general NB population. This raised the question of whether the expression of EPHB6, EFN2, and EFN3 could predict the outcome of NB, especially of intermediate-risk NB. In our study cohort, there were only 12 patients in this category. Nonetheless, there was a trend in which high expression of EPHB6, EFN2, and EFN3 was associated with a favorable outcome and low expression was associated with a poor outcome in the intermediate-risk NB group. Future studies in larger study cohorts will be required to determine whether the expression of EPHB6, EFN2, and EFN3 is predictive of NB outcome.

In contrast to the positive correlation between EPHB6, EFN2, and EFN3 expression and TrkA expression, we found an inverse relationship between MYCN amplification and the expression of these genes. MYCN-amplified tumors expressed significantly lower levels of EPHB6, EFN2, and EFN3 than nonamplified tumors. TrkA expression in these MYCN-amplified tumors was also very low (P = 0.0001; data not shown). As expected, MYCN-amplified tumors expressed substantially higher levels of MYCN than nonamplified tumors (data not shown). A similar finding has also been made for cell adhesion molecule CD44. High levels of CD44 expression are associated with low tumor stage and favorable outcome of NB, and MYCN-amplified tumors express lower levels of CD44 than nonamplified tumors (27, 28).

The inverse relationship between MYCN amplification and the expression of genes associated with favorable NB, including TrkA, CD44, EPHB6, EFN2, and EFN3, is an intriguing observation. However, mechanisms that account for this phenomenon remain elusive. Nonetheless, there are at least two possible explanations. High levels of MYCN expression in MYCN-amplified tumors may negatively regulate the expression of these genes that would provide NB with the favorable phenotype. Alternatively, the process leading to MYCN amplification may cause repression of these genes. Whichever the case, suppression of genes that are associated with the favorable phenotype in MYCN-amplified NB may result in poor outcome. This feature, along with the other known effect of MYCN, namely, augmentation of NB cell growth (29), may ultimately cause the progression of MYCN-amplified NB.

The association of high EPHB6, EFN2, and EFN3 expression with low-risk NB and the low EPHB6, EFN2, and EFN3 expression in MYCN-amplified tumors raise some intriguing possibilities as to their roles in the biology of NB. EPHB6 lacks tyrosine kinase activity due to a nucleotide mutation at the region corresponding to the ATP acceptor site (30, 31), suggesting that EPHB6 acts as a dominant negative member of the EPHB subgroup. Thus, EPHB6 might negatively regulate the kinase activity of other EPHB members by reducing the density of unoccupied ephrin-B ligands on the cell surface, which are otherwise available to the other EPHB receptors. On the other hand, the cytoplasmic domain of ephrin-B1 has been shown to suppress the transformation activity of several activated protein
tyrosine kinases, including the TrkB/brain-derived neurotrophic factor combination, in NIH3T3 transformation assays (32). Because the cytoplasmic domains of ephrin-B2 and ephrin-B3 are highly similar in sequence to that of ephrin-B1 (23), ephrin-B2 and ephrin-B3 may also exhibit similar characteristics. It is thus possible that EPHB receptors and ephrin-B ligands could play several distinct biological roles in NB, such as modulating growth, survival, differentiation, and motility as well as suppressing tumorigenesis. Additional studies will be required to dissect these functions of EPHB receptors and ephrin-B ligands in the biology of NB.

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