TrkA Expression in Peripheral Neuroblastic Tumors

Prognostic Significance and Biological Relevance

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Supported in part by grants CA 13539, CA60104, and CA22794 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, and by The T. J. Martell Foundation for Leukemia, Cancer, and AIDS Research.

The authors thank Dr. R. Sposto for his generous support in the statistics portion of this study.

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Received May 24, 2004; revision received June 29, 2004; accepted July 1, 2004.

BACKGROUND. This study was conducted to investigate the prognostic significance and biologic relevance of trkA expression levels in peripheral neuroblastic tumors (pNTs) (i.e., neuroblastoma, ganglioneuroblastoma, and ganglioneuroma).

METHODS. Levels of trkA expression from a total of 265 pNTs were determined by quantitative polymerase chain reaction analysis with Genescan software. The results were analyzed according to histopathology (favorable histology [FH] vs. unfavorable histology [UH] according to the International Neuroblastoma Pathology Classification) and MYCN tumor status (amplified vs. nonamplified) along with clinical stage and outcomes of the patients.

RESULTS. The levels of trkA expression differed significantly between the group of patients who were alive and well (n = 170 patients) and the group that had progressed or died (n = 95 patients) and between the group that was alive (n = 188 patients) and the group that died (n = 77 patients). However, the trkA expression levels were not independent predictors of clinical outcome when the proportional hazards model contained the known prognostic variables of clinical stage, histopathology, and MYCN status (all tests were done in 196 patients). In the neuroblastoma category (n = 173 tumors), tumors in the FH/nonamplified MYCN subset (n = 112 tumors) expressed higher levels of trkA and showed an age-dependent neuroblastic differentiation: They were classified into either a poorly differentiated subtype (n = 91 tumors; all patients age < 1.5 years at diagnosis) or a differentiating subtype (n = 21 tumors; 57% of patients ages 1.5–5.0 years). Tumors in the UH/amplified MYCN subset (n = 30 tumors) expressed significantly lower levels of trkA and showed very limited neuroblastic differentiation. Tumors in the FH/amplified MYCN subset were very rare (n = 3 tumors) and expressed higher levels of trkA. Tumors in the UH/nonamplified MYCN subset (n = 28 tumors) had trkA levels in a wide range and showed limited neuroblastic differentiation.

CONCLUSIONS. For patients with pNTs, levels of trkA expression did not add significant information to prognostic grouping, as defined by the combination of clinical stage, histopathology, and MYCN status. There was a biologically relevant correlation between molecular properties (trkA expression and MYCN status) and histopathologic features of the tumors in the neuroblastoma category. Cancer 2004;101:1873–81. © 2004 American Cancer Society.

KEYWORDS: peripheral neuroblastic tumors, trkA, quantitative polymerase chain reaction, MYCN, histopathology, clinical stage, International Neuroblastoma Pathology Classification, prognosis.

Peripheral neuroblastic tumors (pNTs), a group of tumors that includes neuroblastoma, ganglioneuroblastoma, and ganglioneuroma, constitute one of the most common solid tumors in children.1 Because of the recent advances in research, pNTs are now considered biologically heterogeneous; and their unique clinical behaviors, such as involution/spontaneous regression, maturation, and aggressive
progression, are related closely to their individual genetic/molecular properties. The Children’s Oncology Group (COG) neuroblastoma studies currently are using various factors for defining risk groups for patient stratification and protocol assignment. Those factors that predict the prognosis of patients with this disease include clinical stage, age at diagnosis, histopathology, MYCN status, and DNA index. Prognostic effects of other genetic/molecular markers, such as trkA (high-affinity nerve growth factor receptor) expression, 1p deletion, 17q gain, and telomerase activity, etc., also have been investigated in this group of tumors. In particular, the prognostic significance of trkA expression in pNTs has been reported and discussed by many investigators: trkA is expressed highly by clinically favorable tumors, with a propensity to either differentiate or regress.

In our previous reports, prognostically as well as biologically distinct subsets were defined by combination of histopathology (favorable histology [FH] vs. unfavorable histology [UH]) and MYCN status (amplified vs. nonamplified) in pNTs: They are 1) the FH/nonamplified MYCN subset (patients who have an excellent prognosis); 2) the FH/amplified MYCN subset, which is extremely rare; 3) the UH/nonamplified MYCN subset (patients who have a poor prognosis), and 4) the UH/amplified MYCN subset (patients who have a very poor prognosis). In the current study, trkA expression was determined quantitatively in pNTs, and the results were analyzed along with clinical stage and patient outcomes. Prognostic significance and biologic relevance of trkA expression also were examined by stratifying the data according to tumor histopathology and MYCN status.

MATERIALS AND METHODS
Study Cases
Frozen tumor specimens (from 265 patients) that had been filed at the Neuroblastoma Reference Laboratory, Childrens Hospital Los Angeles, including specimens from patients who participated in the Children’s Cancer Group (CCG) studies (109 specimens from CCG-3881, 59 specimens from CCG-3891, and 25 specimens from CCG-321P) and from non-CCG patients (72 specimens), were tested for trkA expression in this study. Those patients were evaluated clinically and diagnosed with Stage I disease (27 patients), Stage II disease (53 patients), Stage III disease (47 patients), Stage IV disease (112 patients), and Stage IVS disease (26 patients), and they were treated mainly on or following the CCG protocols. Briefly, patients with Stage I and II disease underwent surgery alone; patients with Stage IVS disease received supportive care or modest chemotherapy; patients with Stage III and IV disease in the intermediate-risk group received chemotherapy; and patients with Stage III and IV disease in the high-risk group received aggressive treatment with or without bone marrow transplantation.

All specimens were obtained prior to chemotherapy and/or irradiation therapy. Appropriate informed-consent procedures were followed, and consent was obtained from parents or guardians. Follow-up information (follow-up range, 0–140 months; median follow-up, 46 months after diagnosis) from all patients was collected and recorded as alive and well, progressed (alive but with progressed disease), or died (deceased). It was noted that there were only 4 patients who had 0 months of follow-up in this series; they had Stage I disease (1 patient), Stage II disease (2 patients), and Stage III disease (3 patients).

Of the 265 tumors, histopathology review and MYCN test were performed in 196 tumors (from 27 patients with Stage I disease, 43 patients with Stage II disease, 29 patients with Stage III, 75 patients with Stage IV disease, and 22 patients with Stage IVS disease). Histopathology evaluation was done by using the International Neuroblastoma Pathology Classification (Shimada system), and the tumors were classified into either a favorable histology (FH) group or an unfavorable histology (UH) group. MYCN status (amplified or nonamplified) was determined either by Southern blot analysis of gene copy number or by the pattern of MYCN protein expression in immunostaining combined with a semiquantitative polymerase chain reaction technique for MYCN gene copy number. Tumors with >10 copies of MYCN were classified into the amplified group. Follow-up of the 196 patients ranged from 0 months to 133 months (median, 53 months) after diagnosis. Among these patients, only 2 had 0 months of follow-up clinically: One patient had Stage I disease, the other patient had Stage II disease, and both patients had FH/nonamplified MYCN amplified tumors.

Determination of trkA Expression
Synthesis of cDNA.
Using the TRIzol reagent modification of the method of Chomczynski and Sacchi, total RNA was extracted from frozen tumor specimens and was treated with RNase-free DNase I (3 U/reaction). The synthesis of cDNA from 200 ng of total RNA by Superscript II Rnase H(−) reverse transcriptase was carried out under standard conditions, except that random nanamer (100 pmole/reaction) was used as the primer. This modification increased the yield of transcript-specific signal by threefold to fourfold.
Quantitation of trkA expression.
Competitive reverse transcriptase-polymerase chain reaction analysis was used to quantitate the total number of TrkA-specific transcripts, the sum of TrkAI and TrkAII splice variants, which differ by an 18-nucleotide exon. The TrkA primers (sense: 5′/H11032 GCTG-GCTCTTCAATGGCTCCG3′, amino acids 1025–1045; antisense: 5′/H11032 GTTTTCGTCCTTCTTCTCCACC3′, amino acids 1287–1307) bracketing the insert yielded a 284-base pair (bp) product from TrkAI and a 302-bp product from TrkAII. A deletion product with primer sites identical to the normal product was constructed using the fusion primer method of Celi et al. The fusion primer (5′/H11032 GTTTTCGTCCTTCTTCTCCACCGTGATG-3′), linking the 3′-end of the antisense primer to the 5′-end of an upstream sequence, was used along with the normal sense primer to generate a 162-bp deletion product. The deletion product was added to the cDNA and was coamplified at the same efficiency as the normal products.

For fluorescent quantitation, the sense primer was 5′-end labeled with hexachloro-6-carboxy-fluorescein phosphoramidite (Applied Biosystems, Foster City, CA). An ABI 373 DNA sequencer with Genescan software was used for measurement of the peak areas corresponding to the fluorescently labeled TrkAI and TrkAII and the deletion products. The level of total TrkA expression was calculated as \((TrkAI + TrkAII / deletion\) product) \(\times\) input copy number of deletion product. It was found that this measure was independent of cycle number (20–40 cycles) and input deletion product copy number (range, \(10^5\)–\(10^6\) copies). In summary, the level of trkA expression of the given tumor was determined quantitatively by its copy number and was recorded by using the term “trkA copy number” in this report.

Statistical Analysis
The data were analyzed using SAS statistical software (version 8.02; SAS Institute, Cary NC). Because the distribution of trkA level was skewed highly, all analyses were carried out on log-transformed (base 10) data. First, the expression levels of trkA were compared between patients who were alive and well and patients who had progressive disease or who had died of disease. Then, the trkA levels were stratified by the known prognostic variables of clinical stage (Stages I, II, III, IV, and IVS), histopathology (FH vs. UH), and MYCN status (amplified vs. nonamplified) using the Wilcoxon two-sample test with continuity correction. Because patients who were observed to be alive and well would fail in the future, this type of analysis may have a serious bias. Because the primary objective of this study was to ascertain the effect that the trkA level had on predicting survival, a Cox proportional hazards model was used to investigate the effects that the trkA level and the known prognostic variables had on survival (SAS procedure PHREG). A crude model (Model 1) with only the trkA level was constructed to study the effects of trkA on survival, and a proportional hazards model with the covariates (clinical stage, histopathology, and MYCN) was constructed (Model 2). Then, a proportional hazards model that contained the trkA level and the covariates (Model 3) was run to assess whether the trkA level predicted outcome with the known confounding variables included in the model.

RESULTS
The Prognostic Significance of trkA Expression
Among 265 patients (see Fig. 1), trkA expression was significantly higher in tumors from patients who were...
alive and well (\(n = 170\) patients) compared with tumors from patients who progressed or died (\(n = 95\) patients; \(P < 0.001\)). A significant difference in \(trkA\) expression also was seen between tumors from patients who were alive (including those who were alive and well and those who progressed; \(n = 188\) patients) and tumors from patients who died (\(n = 77\) patients; \(P < 0.001\)). Figure 2 shows the median \(trkA\) copy numbers for tumors by clinical stage. Among patients with Stage III, Stage IV, and Stage IVS disease, the levels of \(trkA\) expression were significantly lower in patients who died or had who had disease progression compared with patients who were alive with or without disease (\(P = 0.001\)).

The Prognostic Significance of \(trkA\) Expression Stratified by Histopathology, \(MYCN\) Status, and Clinical Stage

Among the 196 patients who had all 3 test results available (\(trkA\) level, histopathology, and \(MYCN\) status), again, \(trkA\) copy numbers were significantly greater in tumors from patients who were alive and well (\(n = 134\) patients; median \(trkA\) copy number, 5.42) compared with patients who had disease progression or died (\(n = 62\) patients; median \(trkA\) copy number, 4.58). Significant differences in \(trkA\) expression also were found between tumors from patients who were alive (\(n = 149\) patients; median \(trkA\) copy number, 5.36) and tumors from patients who died (\(n = 47\); median \(trkA\) copy number, 4.56; \(P < 0.001\)). With respect to histopathology, \(trkA\) levels were significantly greater in FH tumors compared with UH tumors (Fig. 3, left) (\(P < 0.001\)). Tumors with amplified \(MYCN\) status expressed significantly lower \(trkA\) compared with tumors with nonamplified \(MYCN\) status (Fig. 3, right) (\(P < 0.001\)).

The results of proportional hazards models (Models 1–3) are listed in Table 1. The crude proportional hazards model (Model 1) that contained the log \(trkA\) levels resulted in a significant hazard ratio (0.52; \(P < 0.001\)), indicating that lower levels of \(trkA\) were associated with worse survival. The proportional hazards model that included clinical stage, histopathology, and \(MYCN\) status (Model 2) is shown in the second column of Table 1. In the third column (Model 3) of Table 1, adding the log \(trkA\) levels to this model (Model 2) revealed that \(trkA\) was no longer statistically significant, and it also had minimal effect on the hazard ratios of the clinical prognostic variables.

FIGURE 2. \(trkA\) expression by clinical stage: Note the significant difference in median \(trkA\) copy numbers between prognostic groups among patients with Stage III (single asterisk), Stage IV (double asterisks), and Stage IVS (triple asterisks) disease (\(P < 0.001\)). Left: There were 170 patients who were alive and well with Stage I disease (\(n = 25\) patients; \(trkA\) median copy number, 5.49), Stage II disease (\(n = 47\) patients; median \(trkA\) copy number, 5.43), Stage III disease (\(n = 38\) patients; median \(trkA\) copy number, 5.35), Stage IV disease (\(n = 45\) patients; median \(trkA\) copy number, 5.57), and Stage IVS disease (\(n = 15\) patients; median \(trkA\) copy number, 5.57); and there were 95 patients who progressed or died with Stage I disease (\(n = 2\) patients; median \(trkA\) copy number, 6.11), Stage II disease (\(n = 6\) patients; median \(trkA\) copy number, 4.86), Stage III disease (\(n = 9\) patients; median \(trkA\) copy number, 3.92), Stage IV disease (\(n = 67\) patients; median \(trkA\) copy number, 4.56), and Stage IVS disease (\(n = 11\) patients; median \(trkA\) copy number, 4.28). Open bars: alive and well; solid bars: progressed/died. Right: One hundred eighty-eight patients who were alive had Stage I disease (\(n = 27\) patients; median \(trkA\) copy number, 5.63), Stage II disease (\(n = 51\) patients; median \(trkA\) copy number, 5.34), Stage III disease (\(n = 38\) patients; median \(trkA\) copy number, 5.35), Stage IV disease (\(n = 54\) patients; median \(trkA\) copy number, 5.42), and Stage IVS disease (\(n = 18\) patients; median \(trkA\) copy number, 5.47); and 77 patients died with Stage II disease (\(n = 2\) patients; median \(trkA\) copy number, 4.71), Stage III disease (\(n = 9\) patients; median \(trkA\) copy number, 3.92), Stage IV disease (\(n = 58\) patients; median \(trkA\) copy number, 4.56), and Stage IVS disease (\(n = 8\) patients; median \(trkA\) copy number, 4.15). Open bars: alive; solid bars: died.
**Biologic Relevance of trkA Expression**

The analyses to determine the biologic relevance of trkA expression levels were done by using tumors in the neuroblastoma (Schwannian stroma-poor) category (n = 173 tumors; 140 MYCN nonamplified tumors and 33 MYCN amplified tumors). There were 23 tumors that were excluded from this analysis: 6 ganglioneuroblastoma, intermixed (GNBi); 4 ganglioneuroma, maturing subtype (GN); and 11 ganglioneuroblastoma, nodular (GNBn). Those tumors in the GNBi and GN categories were excluded from the analyses, because they were composed predominantly of Schwannian stroma cells rather than neuroblastic cells. Tumors in the GNBn category, which were composed of biologically different clones, were also excluded from the analysis because of difficulty in identifying tissue areas of sampling for molecular tests.

Histologic features were assigned to the 173 tumors according to the International Neuroblastoma Pathology Classification as follows: 1) grade of differentiation, including 2 undifferentiated tumors, 144 poorly differentiated tumors, and 27 differentiating tumors; and 2) low mitosis-karyorrhexis index (MKI) in 101 tumors, intermediate MKI in 42 tumors, and high MKI in 30 tumors. Figure 4 shows that there was no significant difference in trkA expression between tumors in the undifferentiated/poorly differentiated subtypes and tumors in the differentiating subtype (also see Fig. 5). Whereas tumors that had a high MKI expressed significantly lower trkA compared with tumors that had either a low MKI or an intermediate MKI, it was noted that the majority of tumors with a high MKI (24 of 30 tumors; 80.0%) had amplified MYCN status in this series.

Figure 6 shows scattergrams for trkA expression by patient age at the time of diagnosis for four subsets defined by histopathology and MYCN status in the neuroblastoma category. There were 112 FH/nonamplified MYCN tumors, 3 FH/amplified MYCN tumors, 28 UH/nonamplified MYCN tumors, and 30 UH/amplified MYCN tumors.

1) FH/nonamplified MYCN tumors were classified into either the poorly differentiated subtype (91 tumors) or the differentiating subtype (21 tumors). Again, there was no difference in trkA expression level between tumors in these two subtypes. By definition, tumors of the poorly differentiated subtype were seen in patients age < 1.5 years at diagnosis. Also, by definition, tumors of the differentiating subtype were seen in patients age < 5 years at diagnosis, and > 50% of the patients (12 of 21 patients; 57%) were diagnosed after age 1.5 years.

2) FH/amplified MYCN tumors were rare (1.7%, 3 of 173 tumors) and were diagnosed in infants in this series. All three tumors in this subset expressed higher levels of trkA: Two tumors had features of the poorly differentiated subtype, and one tumor had the features of the differentiating subtype.

3) UH/nonamplified MYCN tumors showed a

**TABLE 1**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio (P value)</th>
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<tbody>
<tr>
<td></td>
<td>Model 1</td>
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<tr>
<td>Histopathology</td>
<td>4.58 (&lt;0.001)</td>
</tr>
<tr>
<td>Clinical stage</td>
<td>1.62 (&lt;0.01)</td>
</tr>
<tr>
<td>MYCN</td>
<td>1.74 (0.06)</td>
</tr>
<tr>
<td>Log trkA</td>
<td>0.52 (&lt;0.001)</td>
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**FIGURE 3.** trkA expression by histopathology and MYCN status in peripheral neuroblastic tumors. (A) Tumors that had favorable histology (n = 128 tumors; median trkA copy number, 5.42) had significantly higher trkA expression compared with tumors that had unfavorable histology (n = 68 tumors; median trkA copy number, 4.42; P < 0.001). MYCN-amplified tumors (n = 33 tumors; median trkA copy number, 3.71) had significantly lower trkA expression compared with MYCN-nonamplified tumors (n = 163 tumors; median trkA copy number, 5.41; P < 0.001).
wide range of trkA expression. There were 24 tumors of the poorly differentiated subtype and 4 tumors of the differentiating subtype. Those tumors infrequently demonstrated neuroblastic differentiation in any age group.

4) UH/amplified MYCN tumors generally expressed lower levels of trkA, and the large majority did not show neuroblastic differentiation regardless of the age of the patients. They were classified either as undifferentiated (2 tumors) or as poorly differentiated (27 tumors), and only 1 tumor (1 of 30 tumors; 3.3%) qualified as the differentiating subtype.

DISCUSSION

This is the first report presenting trkA expression by quantitative analysis in a large series of pNTs, and its prognostic significance and biologic relevance are discussed. First, as reported by other investigators, levels of trkA expression had significant prognostic effects for the patients: Higher trkA copy numbers were linked with a better prognosis. TrkA expression of the tumors also distinguished good and poor prognoses for patients with advanced-stage disease (Stages III and IV) and with Stage IVS disease in our series. The results of this study also clearly demonstrated that trkA levels differed significantly between FH and UH tumors and between MYCN nonamplified and MYCN amplified tumors. Furthermore, trkA levels were not independent predictors of patient outcome when the proportional hazards model contained the known prognostic variables of clinical stage, histopathology, and MYCN status. Accordingly, the determination of trkA expression levels in pNTs does not seem likely to serve as one of the front-end prognostic factors for...
patient stratification and treatment assignment, at least at the current stage of clinical trials.

It has been reported and discussed that activation of the neurotrophin receptor \textit{trkA} by its preferred ligand, nerve growth factor, initiates a cascade of signaling events that lead to neuronal differentiation in vitro\textsuperscript{35} and that may play an important role in the differentiation of biologically (clinically) favorable neuroblastoma in vivo.\textsuperscript{6} In our series, the FH/nonamplified \textit{MYCN} tumors had uniformly higher levels of \textit{trkA} expression. However, it was noted that those tumors showed different histologic grades of neuroblastic differentiation, i.e., poorly differentiated and differentiating. It also was noted that there was no difference in the level of \textit{trkA} expression between these two histologic subtypes in the FH/nonamplified \textit{MYCN} subset. According to the definition from the International Neuroblastoma Pathology Classification (the Shimada system), however, FH tumors of the poorly differentiated subtype were diagnosed in newborns and in patients age \(\leq 1.5\) years, and FH tumors of the differentiated subtype usually were diagnosed in older children age \(\leq 5\) years (see Fig. 6A). In other words, tumors that shared the same molecular properties (\textit{MYCN} nonamplified and high \textit{trkA} expression) had different grades of neuroblastic differentiation (see Fig. 5) based on the age of the patients within an age-linked framework of the Shimada system.\textsuperscript{26–28} The result suggests that, to demonstrate histologic evidence of neuroblastic differentiation, tumors with the same molecular properties required an in vivo latent period in individual patients.

In our previous studies, we documented a significant relation between \textit{MYCN} status and histologic features in pNTs and suggested that an excess amount of myc-max heterodimer formation by amplified \textit{MYCN} may be a powerful driving force for preventing neuroblastic differentiation and increasing mitotic and karyorrhectic activities in the tumors.\textsuperscript{18,19} It also has been reported that there is an inverse relation between \textit{MYCN} amplification and \textit{trkA} expression.\textsuperscript{36} In the current series of tumors, UH/amplified \textit{MYCN} tumors expressed significantly lower levels of \textit{trkA}, and 29 of 30 tumors did not show neuroblastic differentiation histologically (Fig. 6D).

There were only three FH/amplified \textit{MYCN} tumors: All three were diagnosed in infancy, and their \textit{trkA} levels were significantly higher compared with the UH/amplified \textit{MYCN} tumors (Fig. 6B,D). It has been suggested that \textit{trkA} overexpression may over-
come aggressiveness, even in tumors with MYCN amplification.37 Among the three patients with FH/amplified MYCN tumors, however, two patients (with Stage II and Stage IV disease) died of their disease, and one patient (with Stage IVS disease) is alive and well.

Finally, there was a subset of UH/nonamplified MYCN tumors in which the levels of trkA expression were distributed in a wide range, almost comparable to the trkA levels observed in the subset of FH/nonamplified MYCN tumors but significantly higher than the trkA levels in the subset of UH/amplified MYCN tumors. Figure 6C shows that tumors in this subset seemed to have no or limited potential for neuroblastic differentiation in any age group. It also was noted that considerable numbers of patients (9 of 28 patients; 32%) were diagnosed after age 5 years. Nonfunctional trkA and/or abnormalities of the downstream target may be responsible for at least some of the tumors in this subset.

In summary, trkA expression could distinguish prognostic groups among patients with pNTs. However, it did not seem to be practical, at least at this moment, to add prognostic information determined by the levels of trkA expression to the currently established risk grouping system for the COG neuroblastoma studies.4 At the same time, it should be stated here that trkA remains one of the important targets in pNT research for developing future management based on our continuous effort in understanding the biology of this disease.

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4. Weinstein JL, Katzenstein HM, Cohn SL. Advances in the expression to the currently established risk grouping system for the COG neuroblastoma studies.4 At the same time, it should be stated here that trkA remains one of the important targets in pNT research for developing future management based on our continuous effort in understanding the biology of this disease.

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