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Inverse Relationship between trk Expression and N-myc Amplification in Human Neuroblastomas

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Abstract

We examined the expression of the trk protooncogene in a series of 82 neuroblastomas to determine its relationship to N-myc amplification and expression, disease stage, patient age, and survival. We found that virtually all stage I, II, and IV-S patients had moderate to high levels of trk expression, whereas most advanced stage neuroblastomas had low or absent levels. All but one tumor with N-myc amplification had low or absent trk expression, and the one exception was regressing at the time it was resected. Conversely, all neuroblastomas identified by mass screening had moderate to high expression of trk, and all these patients are surviving. Thus, trk expression was associated with an absence of N-myc amplification, lower disease stage, lower patient age, and favorable outcome. Tumors with high trk expression may be more likely to differentiate, regress spontaneously, or respond well to therapy.

Introduction

Developing sympathetic neuroblasts require NGF for survival, and they respond to NGF by undergoing neuronal differentiation. This response is mediated by the NGFR. Neuroblastomas are derived from immature sympathetic neuroblasts, but most neuroblastoma cell lines either do not express the NGFR or they fail to undergo morphological differentiation in response to NGF (1-4). Current evidence suggests that the biologically active NGFR is formed by two genes, LNGFR and trk, which encode two transmembrane proteins, called p75LNGFR and p140pro-trk (5, 6). The trk protooncogene encodes a transmembrane tyrosine kinase that is required for high-affinity NGF binding and for biological activity (7). We have determined previously (4) that neuroblastoma cell lines have a variety of abnormalities in the expression or function of the p75LNGFR component of the NGFR. For the present study, we have analyzed the level of trk mRNA expression by Northern analysis in 82 primary neuroblastomas and compared its expression to a variety of clinical and biological variables in order to determine the significance of trk expression in these tumors. Our findings demonstrate an inverse relationship between trk expression and MYCN (N-myc) amplification, a marker of aggressive neuroblastomas (8), and they suggest that tumors expressing trk represent a group of tumors with a favorable outcome.

Materials and Methods

Sixty-six of the 82 neuroblastomas were obtained from patients identified at Kyushu University in Fukuoka or other Kyushu Neuroblastoma Study Group Institutions and cooperative Hospitals in Japan. Twenty-seven of these patients were identified by a mass screening program that began in 1985 (9, 10). Fifteen patients were seen at Washington University in St. Louis or other Pediatric Oncology Group Institutions, and one was seen elsewhere. Neuroblastoma tissue was obtained from the primary tumors of untreated patients in almost all cases. A subset of the patients with advanced stages of disease seen in Japan received one course of chemotherapy prior to resection of their tumors. One ganglioneuroma was obtained at the time of recurrence 7 years after initial diagnosis, but the patient had received no chemotherapy or radiation. Two tumor samples were obtained from a metastatic tumor (lymph node, liver nodule), but these samples were replaced with tumor. Because the majority of the patients were diagnosed and staged in Japan, all patients were staged according to the Evans staging system (11). The median follow-up of the patients from diagnosis was 36 months (range, 8 to 116 months). There were 60 neuroblastomas, 17 ganglioneuroblastomas, and 5 ganglioneuromas. The 77 neuroblastomas and ganglioneuroblastomas were combined and analyzed as neuroblastomas. Unless stated otherwise, the 5 ganglioneuromas were examined separately from the neuroblastomas.

The trk probe was a generous gift from Dr. Luis Parada, at the NCI-Frederick Cancer Center (6), and the N-myc probe was provided generously by Dr. J. Michael Bishop at the University of California, San Francisco (12). The N-myc copy number was determined by Southern analysis, serial dilution, and densitometry (see below). RNA was prepared and the RNA expression was determined by Northern analysis, as described previously (4). All bands of N-myc DNA and RNA, as well as trk and β-actin RNA, were analyzed by scanning the autoradiographs on an Apple Scanner. We used a computer-based program to analyze captured images from autoradiograms (Densitometer-on-a-Disk, or DoaD; compliments of IMAGENETICS and AMOCO Technology Co., Naperville, IL). N-myc DNA copy number was analyzed by serial dilution compared to single-copy and amplified controls, as described previously (12, 13). RNA expression of the trk and N-myc genes was normalized to β-actin, expressed as arbitrary density units (d.u.), and then converted to the following scale: 0 (undetectable); 1+ (1-99 d.u.; median, 19 d.u.; range, 6-87); 2+ (100-499 d.u.; median, 334; range, 136-490); 3+ (500-999 d.u.; median, 751; range, 511-990); and 4+ (>1,000 d.u.; median, 2228; range, 1000-4864). This gave good discrimination between absent to low (0-1+) and moderate to high (2+-4+) levels of expression. Comparisons between two clinical or biological variables was done by χ² analysis, and comparison of individual variables to survival was done by Kaplan-Meier life table analysis.

Results

trk Expression. Moderate to high trk expression (2+-4+) was seen in the majority of the patients. This included 20 of 20 with stage I, 14 of 14 with stage II, and 11 of 12 with stage IV-S. Interestingly, all 27 patients identified by mass screening had substantial trk expression (20 with 4+, 6 with 3+, 1 with 2+). In contrast, only 18 of 31 patients with stages III and IV had this level of trk expression (χ² = 17.10; P < 0.001). Five
ganglioneuromas were included in this study, and trk expression in these tumors was quite variable. Representative autoradiograms for the expression of trk in the neuroblastomas are shown in Fig. 1 (first row), and a comparison of the level of trk expression with clinical stage is shown in Fig. 2.

N-myc Expression. We analyzed the N-myc copy number of the 77 neuroblastomas, and 11 had N-myc amplification, ranging from 10 to 200 copies/haploid genome (data not shown). As expected, none of the five ganglioneuromas had N-myc amplification. All patients with N-myc amplification had stage III, IV, or IV-S. In addition, we analyzed N-myc expression in these tumors (Fig. 1, second row), and there was a general trend towards higher N-myc expression in more advanced stage (Fig. 3), but this was not statistically significant ($\chi^2 = 0.16; P > 0.1$).

Indeed, most of this could be accounted for by the cases with N-myc amplification, which was associated with advanced stages of disease ($\chi^2 = 7.31; P < 0.01$). Ten of the 11 cases with N-myc amplification had 3+–4+ expression, as determined by our quantitative assessment of Northern blots, whereas only 2 of the 66 single-copy neuroblastomas had this level of expression. None of the ganglioneuroblastomas had substantial N-myc expression.

Comparison of trk and N-myc. We compared the expression of trk and N-myc in the 82 tumors to determine if there was a relationship between the expression of these genes. We considered those with 2+-4+ expression to be positive, those with 0–1+ to be negative. Tumors were classified according to the four possible combinations of these variables (Fig. 4). Tumors with high trk and low or absent N-myc represented the largest group, which included the vast majority of the low stage tumors (stages I and II) as well as the majority of the tumors detected by mass screening (22 of 27). It also included a substantial number of tumors from patients with more advanced stages of disease (III, IV, IV-S). The second largest group was composed of tumors expressing moderate to high levels of both trk and N-myc. This group represented the spectrum of stages from I to IV, but interestingly, almost all of these tumors were derived from
patients less than 1 year of age (17 of 19). The third group consisted of tumors with high N-myc expression and low or absent trk expression, and this group of ten tumors was composed exclusively of cases with N-myc amplification. The only other tumor with N-myc amplification had high expression of both genes, but this tumor was regressing clinically at the time of resection (14) (see below). The last group of tumors had low or absent expression of both genes: two were ganglioneuromas, two were stage III tumors, and two were stage IV tumors.

High trk expression was significantly correlated with low or absent N-myc expression, and vice versa ($\chi^2 = 6.64; P < 0.01$). However, this inverse relationship was even more apparent in the cases with N-myc amplification; almost all of which had high expression of N-myc and low or absent expression of trk ($\chi^2 = 40.10; P < 0.001$).

Survival Analysis. Estimated survival at 36+ months by Kaplan-Meier analysis was 100% for patients with ganglioneuroma, 100% for stage I and stage II, 82% for stage IV-S, 64% for stage III, and 26% for stage IV. These results are somewhat higher than expected for neuroblastoma, probably due to the substantial number of patients in this study who were identified by mass screening. All of the mass screening patients are alive, whereas all 11 patients with N-myc amplification died of progressive disease. The last group had N-myc amplification, which may account for their poor outcome.

Only 2 of 12 patients (17%) with 3+–4+ N-myc expression were alive, and the 2 survivors were the only ones without N-myc amplification. The survival rates for patients with 0, 1+, and 2+ expression were comparable (91, 84, and 88%, respectively). Thus, the analysis of survival relative to the two variables together correlated more with trk expression than with N-myc expression, after accounting for N-myc amplification. The survival of those expressing at least moderate levels of trk was not affected by the presence or absence of N-myc expression (89% versus 91%), whereas none of those expressing N-myc and not trk were alive.

Discussion

Our results indicate that the majority of primary neuroblastomas express substantial levels of trk, including all tumors from patients with lower stages of disease ($\chi^2 = 17.10; P < 0.001$) and all tumors from patients identified by mass screening. trk expression is required to form the biologically active, high-affinity NGFR, which in turn renders neuronal cells capable of responding to NGF and undergoing neuronal differentiation (6, 7). However, the fact that trk is expressed does not mean necessarily that it functions normally. We have described previously (4) that some neuroblastoma cell lines express p75NGFR, the other component of the NGFR, in conjunction with high-affinity NGF binding, but they do not differentiate in response to NGF. All of these cell lines were derived from advanced-stage tumors that had been in culture for years. Thus, expression of the components of the NGFR are necessary for normal responsiveness to NGF, but expression alone does not indicate whether or not the receptor is functional or if the signal transduction pathway is intact. Nevertheless, based on the data presented here, trk-positive tumors generally have a favorable prognosis.

Roughly one-third of the tumors in this study were derived from patients who had been identified by mass screening for neuroblastoma in Japan (9). This biases our sample in favor of improved survival overall, but it was our intention to gain insight into the biology of this favorable group of tumors, as well as the tumors from infants with favorable disease who are diagnosed clinically. It has been suggested that a substantial number of neuroblastomas identified by mass screening might regress spontaneously, because the prevalence of neuroblastoma in screened populations is increased, especially in the first year of life (10, 15, 16). All of the tumors had high trk expression, none had N-myc amplification, and all are alive, similar to previous studies (9, 10, 15, 16). Although this does not prove that there is a causal relationship between the two, trk expression clearly is important in the formation of high-affinity NGFR. Exposure of NGFR-expressing neuroblasts to NGF should result in differentiation (17), whereas trophic factor deprivation in susceptible cells may result in programmed cell death (18). Thus, there is a plausible functional relationship between trk expression and the favorable outcome of these tumors. On the other hand, all but one of the patients with N-myc amplification had low or absent trk expression, and all died of progressive disease.

High N-myc expression was seen in all the tumors with N-myc amplification, but there were two tumors with comparable expression without amplification. These patients had a favorable outcome, so that N-myc expression, in the absence of...
amplification, did not correlate necessarily with a poor outcome. This conclusion is similar to several previous studies (19, 20, 21). One possible reason for this finding is that some other gene in the amplified domain is responsible for the aggressive phenotype associated with N-myc amplification and that N-myc is just a marker for the amplified domain. Alternatively, there may be a threshold of N-myc expression, above which the effects are deleterious. High N-myc expression associated with amplification may alter cell behavior by saturating possible DNA or protein interactions that are characteristic for this protein, or the overabundance of expression may have deleterious effects by causing other genes or pathways that seldom are affected by lower levels of expression to interact.

Our studies have shown some overlap between the higher-expressing, nonamplified tumors and the lower-expressing, amplified tumors, as seen in earlier studies that examined N-myc expression at the RNA level (19–21). However, N-myc expression must be examined quantitatively at the protein level. There is heterogeneity in the level of expression of N-myc protein in tumors with N-myc amplification, which probably is due to unequal distribution of the double minutes which carry the amplified genes (12, 19, 22). Thus, even if the average level of expression is similar, there may be a substantial number of cells with higher levels of expression. In addition, the efficiency of translation, the stability of the protein (23), alterations in the protein sequence, or other factors could have a substantial effect on the net amount of steady-state, functional protein in the cell.

The subset of tumors with high N-myc and low trk was composed exclusively of amplified tumors, and all but one of the tumors with N-myc amplification had low or absent trk expression (χ² = 40.10; P < 0.001). The one exception was a tumor with N-myc amplification and high expression of both N-myc and trk. Interestingly, this tumor was regressing at the time that the tumor was removed (14). Although the tumor eventually recurred and killed the patient, the regressing behavior of the tumor in this patient was associated with high trk expression, even in the presence of high N-myc expression. Unfortunately, a tumor sample was not available at the time of tumor recurrence, so we cannot determine if the trk expression was low at this time, but our experience with the pattern of expression in the other tumors with N-myc amplification would suggest that trk expression probably was low.

A number of other genes the expression of which is associated with a more differentiated phenotype are correlated inversely with N-myc amplification (24–29). Interestingly, one of these studies showed an inverse relationship with LNGFR, the other component of the NGFR (28). The relationship between LNGFR and trk expression remains to be determined. Thus, it is difficult to say at the present time if there is a direct relationship between the regulation of expression of N-myc compared to trk and LNGFR in neuroblastomas. Nevertheless, our results suggest that trk may play a role in the favorable outcome of patients whose tumors have at least moderate expression of this gene. This gene, together with LNGFR, may permit neuroblastoma cells to respond to NGF by undergoing differentiation, or alternatively to regress in the absence of this neurotrophic factor.

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