The MYCN Enigma: Significance of MYCN Expression in Neuroblastoma

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Abstract

MYCN amplification strongly predicts adverse outcome of neuroblastoma. However, the significance of MYCN expression in the clinical and biological behavior of neuroblastoma has been unclear. To address this question, we first examined the expression of MYCN in combination with TrkA (a favorable prognostic indicator of neuroblastoma) in 91 primary neuroblastoma by quantitative reverse transcription-PCR and investigated the relationship among patient survival, MYCN, and TrkA expressions. Three subsets of neuroblastoma were defined based on MYCN and TrkA expression. Neuroblastoma expressing the highest level of MYCN but little TrkA were MYCN-amplified cases, which had a 5-year survival of 9.3%. Interestingly, MYCN and TrkA expression showed a linear correlation ($r = 0.5664$, $P < 0.00005$) in neuroblastoma lacking MYCN amplification, and the 5-year survival of neuroblastoma patients with low MYCN and low TrkA expressions was 63.7%, whereas those with high expression of both had a 5-year survival of 88.1% ($P < 0.00005$). This nonlinear distribution of disease outcome relative to MYCN expression in neuroblastoma explains why MYCN expression is not predictive of neuroblastoma disease outcome by dichotomous division of the neuroblastoma cohort. However, high-level MYCN expression is associated with favorable outcome in neuroblastoma lacking MYCN amplification. Furthermore, forced expression of MYCN significantly suppresses growth of neuroblastoma cells lacking MYCN amplification by inducing apoptosis and enhancing favorable neuroblastoma gene expression. Collectively, these data suggest that high-level MYCN expression in neuroblastoma lacking MYCN amplification results in a benign phenotype. Thus, the high MYCN expression confers the opposite biological consequence in neuroblastoma, depending on whether or not MYCN is amplified. (Cancer Res 2006; 66(5): 2826-33)

Introduction

Neuroblastoma is a common pediatric solid tumor of neural crest origin. The tumor occurs in infants and young children with primary sites in adrenal glands or the sympathetic chain. Neuroblastoma is unique because of its clinical bipolarity. It comes in two very different forms: favorable or unfavorable (1). Several prognostic markers have been described to predict the outcome of neuroblastoma, including the age of the patient at diagnosis, tumor stage, Shimada histology, DNA ploidy, serum ferritin or lactate dehydrogenase levels, and MYCN amplification (2–7). There are additional genetic and biological markers of neuroblastoma that are predictive of disease outcome. These include deletion or allelic loss of chromosome 1p or 11q (8–11), allelic gain of 17q (12), and the expression of transcripts encoding receptor tyrosine kinases (TrkA and EPHB6) and cell surface molecules (CD44, EFNB2, and EFNB3). These five genes in particular have been defined as favorable neuroblastoma genes, because high-level expression of these genes not only predicts favorable outcome of neuroblastoma clinically but also suppresses growth of unfavorable neuroblastoma cells in vitro and in mouse xenograft models (13–17). In addition, when one of the favorable neuroblastoma genes is expressed at high levels, patient disease outcome is favorable (15).

Among the prognostic indicators of neuroblastoma, MYCN amplification is strongly associated with advanced disease stages, rapid tumor progression, and the worst disease outcome (7). MYCN amplification occurs in about 20% to 25% of all neuroblastoma cases, and amplification of MYCN leads to its overexpression at both the mRNA and protein levels (18–22). It has been postulated that high-level expression of the MYCN protein in neuroblastoma results in activation of genes associated with aggressive tumor behavior (23). Nonetheless, the question as to whether MYCN expression is predictive of disease outcome of neuroblastoma remains controversial (13, 19–21, 24–29); thus, the significance of MYCN expression in neuroblastoma has been unclear.

In this study, we have attempted to approach this controversy by analyzing MYCN expression in relation to TrkA expression (a well-established favorable marker of neuroblastoma) in a cohort of 91 neuroblastoma tumor specimens. This approach enabled us to identify a nonlinear relationship between disease outcome of neuroblastoma and MYCN expression, which in turn explains why MYCN expression cannot predict neuroblastoma disease outcome by dichotomous division of the overall study cohort. Interestingly, our clinical observations also suggest that high-level MYCN expression is a favorable feature of neuroblastoma lacking MYCN amplification, including those of advanced stages. In addition, we showed experimentally that forced expression of MYCN resulted in the reduction in the viability of neuroblastoma cells lacking MYCN amplification. Moreover, this growth suppressive effect of MYCN was in part due to apoptosis and the enhancement of favorable neuroblastoma gene expression. Together, these observations suggest that high-level expression of MYCN in neuroblastoma...
lacking MYCN amplification results in a benign phenotype. Thus, the high MYCN expression confers the opposite biological consequence in neuroblastoma, depending on whether or not MYCN is amplified.

Materials and Methods

Primary neuroblastoma tumor samples. The 91 neuroblastoma tumor specimens included those obtained from the Tumor Bank of The Children’s Hospital of Philadelphia, The Tumor Bank of The Pediatric Oncology Group, The Tumor Bank of The Children’s Cancer Group, and Memorial Sloan-Kettering Cancer Center. These included 16 stage I tumors, 16 of stage II, 9 of stage IVS, 19 of stage III, and 31 stage IV tumors. Among these tumors, 2 stage III tumors and 13 stage IV tumors had MYCN amplification. In this cohort, 77% of tumor specimens were from the former Children’s Cancer Group institutions, whereas 23% of them were from the former Pediatric Oncology Group institutions. The median follow-up of this neuroblastoma cohort was 5.0 years, and the overall survival was 69.7%. Four established prognostic factors, including age at diagnosis, stage, MYCN amplification, and TrkA expression, were used to verify the neuroblastoma cohort by single variable Cox regression analysis. The use of human tumor samples for the study was reviewed and approved by the institutional review board.

RNA extraction and quantitative reverse transcription-PCR. Experimental procedures for RNA preparation, reverse transcription, and quantitative reverse transcription-PCR (RT-PCR) have been previously described elsewhere (15, 30, 31). Results of this quantitative RT-PCR were described elsewhere (15, 30, 31). Results of this quantitative RT-PCR were compared by t tests. Survival probabilities in various subgroups were estimated according to the methods of Kaplan and Meier (33). Survival distributions were compared using log-rank tests (34). Cox regression analysis (35) was also used to assess the prognostic significance of variables. P < 0.05 was considered statistically significant.

In vitro experiments. Two human neuroblastoma cell lines (SY5Y and SK-N-AS) were used in this study because they lack MYCN amplification. Transient transfection was carried out by electroporation (120 V, 25 m second square wave) using 0.2-cm cuvettes and a Bio-Rad Xcell electroporator. Five-microgram DNA was used to transfect 10⁷ cells. SK-N-AS cells were also transfected with pBabe (vector control) or pBabe carrying a MYCN-ER fusion construct (gifts from Drs. Antonio Iavarone and Anna Lasorella, Columbia University, New York, NY) by retrovirus transduction. The ER portion of the construct contains a point mutation, which makes the ER only responsive to 4-hydroxy tamoxifen (4-OHT; ref. 36). Stable SK-N-AS transfectants were obtained after the selection with 1 μg/mL puromycin. The MYCN-ER transfectants were further subjected to limiting dilution, and clones with high MYCN-ER expression were identified by Western blot analysis. MYCN protein was detected with the mouse monoclonal antibody, NCM II 100 (37), whereas pro and active forms of caspase-3 were detected by rabbit polyclonal antibodies (AAP113; Stressgen, San Diego, CA). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay (Promega, Madison, WI) was carried out according to the manufacturer’s instructions.

Results

Pattern of MYCN expression in neuroblastoma with respect to tumor stage and age at diagnosis. To gain insight into the prognostic significance of MYCN expression in neuroblastoma, we first examined whether MYCN expression was associated with age or stage in the overall neuroblastoma (n = 91). MYCN expression was associated with tumor stage (P = 0.0042) but not with age at diagnosis (P = 0.5469), although 14 of the 15 MYCN-amplified cases in the group were > 1 year of age. In contrast to MYCN, high TrkA expression was significantly associated with both low-stage tumors (stages I, II, and IVS; P = 0.0006) and younger age (<1 year; P < 0.0001; data not shown). When MYCN-nonamplified neuroblastoma were analyzed separately (n = 76), higher MYCN and TrkA expressions were both associated with the younger age (P = 0.0048 and P = 0.0006, respectively), whereas neither MYCN nor TrkA expression was associated with stage (P = 0.2505 and P = 0.0592, respectively; see Supplementary Data).

Whether MYCN expression predicts outcome of neuroblastoma depends on how the cohort is dichotomized. Previous studies on the prognostic significance of MYCN expression in neuroblastoma have been controversial. We therefore reexamined this issue by performing Kaplan-Meier analysis using different cutoff values. As shown in Fig. 1, whether MYCN expression predicts outcome of neuroblastoma depended on how the cohort was dichotomized. MYCN expression was prognostic when the lowest value among the MYCN-amplified cases was used as the cutoff value (P = 0.0001; Fig. 1A). However, when the median value
was used as the cutoff, MYCN expression was no longer prognostic ($P = 0.2562$; Fig. 1B). In contrast, neuroblastoma with high TrkA expression showed a significant survival advantage over those with low TrkA expression when the median value was used as the cutoff value ($P = 0.0019$; Fig. 1C). Moreover, TrkA expression yielded the same result regardless of the cutoff value that was used to dichotomize the study cohort (data not shown).

**Combination of MYCN and TrkA expression defines three neuroblastoma subsets with distinct survival probabilities.** We previously found that MYCN and TrkA expression (a favorable prognostic indicator of neuroblastoma) correlated with each other in a cohort of low-stage neuroblastoma (38). This observation suggests that by investigating the expression of MYCN in relation to TrkA expression, one may gain a better understanding of the prognostic significance of MYCN expression in neuroblastoma. As shown in Fig. 2A, neuroblastoma expressing very high levels of MYCN but little TrkA were MYCN-amplified cases (shown in red squares). On the other hand, there was a positive linear correlation between MYCN expression and TrkA expression in neuroblastoma lacking MYCN amplification ($r = 0.5664$, $P < 0.00005$; Fig. 2A, blue circles). Based on MYCN-TrkA expressions, we further divided neuroblastoma lacking MYCN amplification into two subsets, shown in green (low MYCN and low TrkA) and blue (high MYCN and high TrkA; Fig. 2B). Thus, this yielded three subsets of neuroblastoma with distinct levels of MYCN and TrkA expression: group 1, group 2, and group 3 (Fig. 2B). It should be noted that no neuroblastoma was found in the area (indicated by the black circle) that represents MYCN nonamplified neuroblastoma expressing high MYCN levels compatible to those in MYCN-amplified cases.

As shown in Fig. 2C, the Kaplan-Meier analysis showed that the survival of group 1 was the worst (5-year survival of 9.3%), whereas group 2 showed a 5-year survival rate of 63.7%. In contrast, group 3 had a 5-year survival rate of 88.1%. Moreover, the differences in a 5-year survival among the three neuroblastoma subsets were statistically significant (Fig. 2C). When the 5-year survival rates of the three neuroblastoma groups were plotted against the mean value of MYCN or TrkA expression of each group (Fig. 2D), it was evident that the level of MYCN expression did not correlate with survival rates of the neuroblastoma subsets, whereas TrkA expression did. In neuroblastoma lacking MYCN amplification, however, neuroblastoma with low MYCN expression (group 2; Fig. 2C and D) was associated with a lower survival rate than that of the MYCN high subset (group 3; Fig. 2C and D), and such a difference in survival rate was statistically significant ($P = 0.0187$; Fig. 2C).
MYCN expression alone can predict disease outcome of neuroblastoma if the cohort is properly stratified. The results shown in Fig. 2C and D suggest that MYCN expression alone would predict neuroblastoma disease outcome if the cohort is properly stratified. In fact, our data revealed that MYCN expression itself was significantly predictive of disease outcome of the entire neuroblastoma cohort by trichotomization (Fig. 3A). Moreover, MYCN expression predicted disease outcome of neuroblastoma lacking MYCN amplification by dichotomization (Fig. 3B). In addition, as shown in Fig. 4, Kaplan-Meier analyses identified trends that low MYCN expression was associated with poor disease outcome of MYCN-nonamplified neuroblastoma of advanced stage (n = 36, P = 0.0060), of age over 1 year (n = 45, P = 0.0673), and of advanced stage and age over 1 year (n = 25, P = 0.0801). These results suggest that high-level MYCN expression is a favorable but not an unfavorable feature of high-risk neuroblastoma lacking MYCN amplification.

Forced expression of MYCN reduced the viability of neuroblastoma cells lacking MYCN amplification by inducing apoptosis and enhancing favorable neuroblastoma gene expression. The above observations collectively suggest that high-level MYCN expression in neuroblastoma lacking MYCN amplification results in a benign phenotype. To test this idea, we transfected two neuroblastoma cell lines lacking MYCN amplification (SY5Y and SK-N-AS expressing little endogenous MYCN) with a MYCN expression construct or a vector control and examined the effect of MYCN on cell growth. As shown in Fig. 5A, an increase in MYCN expression caused a significant reduction in the viability of these neuroblastoma cells, which was accompanied by an increase in apoptosis assessed by caspase-3 activation (Fig. 5B). Moreover, forced expression of MYCN in SY5Y and SK-N-AS resulted in an enhanced expression of favorable neuroblastoma genes (EFNB3 in SY5Y and CD44 in both cell lines; Fig. 5C). Nonetheless, TrkA expression was not increased in these MYCN transfectants (data not shown; see Discussion). It should be mentioned that all the MYCN-transfected cells eventually died off under the selection condition to maintain high MYCN expression levels, and no stable MYCN transfectant was established.

Figure 3. MYCN expression is predictive of the overall neuroblastoma cohort by trichotomization and subsets of neuroblastoma lacking MYCN amplification by dichotomization. A, Kaplan-Meier survival analysis was used to assess the survival probabilities of three neuroblastoma subsets with different MYCN expression levels. The cutoff values of 2 and 1.3 were used to trichotomize the study cohort as indicated (n = 90). The value 2 corresponds to the lowest value among the MYCN-amplified cases, whereas 1.3 corresponds to the MYCN expression value at the cross-section of the two intersecting lines in Fig. 2B. B, neuroblastoma cases lacking MYCN amplification (n = 75) was dichotomized based on the MYCN expression value of 1.3. Survival of each group was assessed by Kaplan-Meier analysis. The log-rank test was used to assess difference in survival of the neuroblastoma subsets.

Figure 4. Low MYCN expression tends to associate with a poor disease outcome of unfavorable neuroblastoma without MYCN amplification. Kaplan-Meier survival analysis was employed to assess survival probabilities of MYCN-nonamplified neuroblastoma with unfavorable features (advanced stage and/or age over 1 year) by MYCN expression. The cutoff value 1.3 was used to dichotomize the study cohort as indicated. A, low MYCN expression was significantly associated with a poor disease outcome of advanced-stage neuroblastoma without MYCN amplification (n = 36). A similar trend was also observed for MYCN-nonamplified neuroblastoma with age over 1 year (n = 40; B) and with age over 1 year and advanced-disease stage (n = 25; C).
To address the effect of sustained high-level MYCN expression in neuroblastoma cells lacking MYCN amplification, we transfected several neuroblastoma cell lines lacking MYCN amplification with pBABE vector or pBABE carrying a MYCN-ER construct. The MYCN-ER is only conditionally active in its transactivation activity (36) when the ER domain is bound by its ligands. Among the cell lines tested, only SK-N-AS gave rise to clones with high MYCN-ER expression after the drug selection and limiting dilution. These results suggest that even the closed conformation of MYCN-ER (with no ligand present) has a marked effect on the viability of the neuroblastoma cells (see below).

As shown in Fig. 6A, the introduction of MYCN-ER caused a significant growth suppression of SK-N-AS cells, and 4-OHT further augmented the growth suppressive activity of MYCN-ER, whereas growth of SK-N-AS cells transfected with pBABE was unaffected by the addition of 4-OHT. These results suggest that the transactivation activity is not absolutely required for the growth suppressive effect of MYCN-ER on MYCN-nonamplified neuroblastoma cells, or alternatively, the MYCN-ER produced in SK-N-AS cells is partially active without ligand binding. Interestingly, there was a significant enhancement of EphB6 and CD44 expression in SK-N-AS transfected with MYCN-ER (Fig. 6B). In the presence of 4-OHT, the enhancement of EphB6 expression was slightly reduced, but the expression of CD44 was further enhanced (Fig. 6B). These results suggest that favorable neuroblastoma genes play an important role in growth suppressive effect of MYCN on neuroblastoma cells lacking MYCN amplification.

**Discussion**

MYCN amplification was identified in neuroblastoma by Schwab et al. over 20 years ago (39). Soon after this discovery, MYCN amplification was confirmed as the most significant prognostic indicator of adverse disease outcome in neuroblastoma (7, 40). Subsequently, a series of studies have been conducted to address the prognostic significance of MYCN expression in neuroblastoma (13, 19–21, 24–29). Conclusions from these studies, however, have been inconsistent to date. This study was undertaken to address this controversy and to gain insight into biological functions of MYCN in neuroblastoma.

As mentioned, the results of Kaplan-Meier analyses indicate that whether MYCN expression predicts neuroblastoma disease outcome depending on which cutoff values are used to dichotomize the neuroblastoma study cohort (Fig. 1A and B). These observations suggest that survival of neuroblastoma patients does not simply correlate with levels of MYCN expression in the overall neuroblastoma population and cast doubt on the previous assumption that MYCN expression correlates with disease outcome of neuroblastoma. Our study provides evidence that this assumption is in fact incorrect (Fig. 2D). The relationship between MYCN expression and disease outcome in the overall neuroblastoma population is nonlinear, and this explains why standard survival analyses, two-arm Kaplan-Meier or Cox regression analysis, cannot yield the correct conclusion as to the prognostic significance of MYCN expression in neuroblastoma. This fact has not been discussed in the
Figure 6. Sustained high-level MYCN expression in neuroblastoma cells without MYCN amplification causes growth suppression and an increase in favorable neuroblastoma gene expression (EPHB6 and CD44). SK-N-AS cells were transfected with pBABE (vector control) or pBABE carrying a MYCN-ER fusion construct by retrovirus transduction. Stable transfectants were obtained after the selection with 1 μg/mL puromycin. The MYCN-ER transfectants were further subjected to limiting dilution, and clones with high MYCN-ER expression were identified by Western blot analysis using a MYCN-specific monoclonal antibody, NCM II 100. A, SK-N-AS/pBABE and SK-N-AS/MYCN-ER cells were grown for up to 4 days in the presence of solvent control (DMSO) or 4-OHT (500 nmol/L), which induced an open (active) conformation of the MYCN-ER fusion protein. Cell growth was monitored by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay. The introduction of MYCN-ER itself caused a significant growth suppression of SK-N-AS cells, and 4-OHT further enhanced growth suppression of SK-N-AS/MYCN-ER cells. B, the expression of favorable neuroblastoma genes was examined in these transfectants with or without the 4-OHT treatments for 6 days.

had a significantly lower survival rate than those with higher MYCN expression. This observation suggests that elevated MYCN expression provides neuroblastoma lacking MYCN amplification with a benign phenotype and may even lead to tumor regression. This may explain why we do not observe cases of neuroblastoma without MYCN amplification expressing very high levels of MYCN (Fig. 2B). In fact, our in vitro studies confirm that elevated expression of MYCN significantly reduced the viability of neuroblastoma cells lacking MYCN amplification through the induction of apoptosis and the enhancement of favorable neuroblastoma gene expression (Figs. 5 and 6). Moreover, pharmacologic augmentation of MYCN in SY5Y cells by proteasome inhibitors was associated not only with growth suppression but also with enhanced expression of favorable neuroblastoma genes EPHB6 and CD44 (44). As previously defined, favorable neuroblastoma genes are genes whose high-level expression predicts favorable neuroblastoma disease outcome and suppresses growth of unfavorable neuroblastoma cells (15, 44). In this regard, it is of interest to note that MYCN can be considered a conditional favorable neuroblastoma gene in neuroblastoma lacking MYCN amplification. In addition, neuroblastoma cell lines without MYCN amplification, which express little or no MYCN, have often been used to assess the biological effect of high-level MYCN expression in neuroblastoma by its ectopic expression via transfection. This operation sensitizes these neuroblastoma cells to apoptosis with or without additional stimuli (45–48), and these results are even thought to be paradoxical (48). However, results of this study are in fact consistent with these previous experimental data.

Based on the positive correlation between MYCN and TrkA expression in neuroblastoma lacking MYCN amplification, one might ask whether forced expression of MYCN could result in an enhancement of TrkA expression in MYCN-nonamplified neuroblastoma cell lines. However, we have not been able to recapitulate this clinical observation in vitro. One possible explanation is that the TrkA promoter in these neuroblastoma cell lines may not be accessible for MYCN-mediated transactivation, because all E-boxes in the TrkA promoter region contain CpG in their cores and thus can be subjected to DNA methylation, which in turn makes them inaccessible for MYCN binding (49). To fully restore the expression of TrkA in these neuroblastoma cells, high-level MYCN as well as demethylation of the TrkA promoter may be required. DNA methylation of E-boxes in the TrkA promoter region also may be a general feature of unfavorable neuroblastoma, from which all neuroblastoma cell lines were derived. On the other hand, the corresponding E-boxes of the group 3 neuroblastoma (Fig. 2) may not be subjected to hypermethylation and therefore be accessible for MYCN-mediated transactivation. Because the group 3 neuroblastoma includes a significantly higher number of younger patients (59.6%) than the group 2 neuroblastoma (16.7%, P < 0.001), DNA methylation of E-boxes may be an age-dependent phenomenon. This explanation is in fact consistent with our observation that in MYCN-nonamplified neuroblastoma, higher MYCN and TrkA expression is associated with younger age of the patients (see Results and Supplementary Data).

The MYCN-transgenic mouse model has been suggested to represent human neuroblastoma, and this model has been employed to explain the effect of an elevated MYCN expression

\[7\] X. Tang and N. Ikegaki, unpublished observation.
on the clinical behavior of human neuroblastoma (50, 51). However, this model only resembles a small fraction of human neuroblastoma cases (i.e., paraspinal stage III neuroblastoma); therefore, one cannot generalize the effect of MYCN expression on human neuroblastoma based solely on this model. In addition, it is likely that MYCN is merely substituting for MYC in the MYCN transgenic mouse model, because human neuroblastoma cases lacking MYCN amplification generally express high levels of MYC but not MYCN. In fact, neuroblastoma cell lines lacking MYCN amplification express high levels of MYC but little or no MYCN with the exception of NBL-S, which expresses relatively high levels of MYCN but not MYC (52).

It is not clear how high MYCN expression exerts such differential effects on MYCN-amplified and MYCN-nonamplified neuroblastoma. However, enhanced expression of favorable neuroblastoma genes by MYCN in MYCN-nonamplified tumors is one possible mechanism. In addition, MYC family proteins are known to promote both cell proliferation and apoptosis depending on the cellular context (53). Perhaps, in neuroblastoma lacking MYCN amplification, the apoptosis-inducing function of MYC is dominant. In contrast, in MYCN-amplified tumors, the balance between the two opposite effects may be shifted towards proliferation through mechanisms that protect these cells from MYCN-induced apoptosis. Taken together, our study suggests that an elevated MYCN expression results in two opposite biological consequences, depending on whether a neuroblastoma has MYCN amplification or not. High MYCN expression in MYCN-amplified neuroblastoma may confer a clinically aggressive phenotype, whereas high MYCN expression in MYCN-nonamplified neuroblastoma gives rise to a benign phenotype, including spontaneous tumor regression, one of the most intriguing characteristics of neuroblastoma.

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