Expression of NLRR3 Orphan Receptor Gene Is Negatively Regulated by MYCN and Miz-1, and Its Downregulation Is Associated with Unfavorable Outcome in Neuroblastoma

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Abstract

**Purpose:** Our previous study showed that expression of NLRR3 is significantly high in favorable neuroblastomas (NBL), whereas that of NLRR1 is significantly high in unfavorable NBLs. However, the molecular mechanism of transcriptional regulation of NLRR3 remains elusive. This study was undertaken to clarify the transcriptional regulation of NLRR3 and its association with the prognosis of NBL.

**Experimental Design:** NLRR3 and MYCN expressions in NBL cell lines were analyzed after induction of cell differentiation, MYCN knockdown, and overexpression. The transcriptional regulation of NLRR3 was analyzed by luciferase reporter and chromatin immunoprecipitation assays. Quantitative PCR was used for examining the expression of NLRR3, Miz-1, or MYCN in 87 primary NBLs.

**Results:** The expression of NLRR3 mRNA was upregulated during differentiation of NBL cells induced by retinoic acid, accompanied with reduced expression of MYCN, suggesting that NLRR3 expression was inversely correlated with MYCN in differentiation. Indeed, knockdown of MYCN induced NLRR3 expression, whereas exogenously expressed MYCN reduced cellular NLRR3 expression. We found that Miz-1 was highly expressed in favorable NBLs and NLRR3 was induced by Miz-1 expression in NBL cells. MYCN and Miz-1 complexes bound to NLRR3 promoter and showed a negative regulation of NLRR3 expression. In addition, a combination of low expression of NLRR3 and high expression of MYCN was highly associated with poor prognosis.

**Conclusions:** NLRR3 is a direct target of MYCN, which associates with Miz-1 and negatively regulates NLRR3 expression. NLRR3 may play a role in NBL differentiation and the survival of NBL patients by inversely correlating with MYCN amplification. 

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Introduction

Neuroblastoma (NBL) is one of the most common malignant solid tumors in children and accounts for 8% of all pediatric cancers (1). NBLs originate from sympathetic precursor neuroblasts derived from the neural crest. NBLs found in patients older than 1 year are usually aggressive and eventually kill the patients despite intensive therapy, whereas those in patients younger than 1 year often regress spontaneously or maturate, resulting in a favorable prognosis (2). We have made extensive efforts to show that TrkA, a high-affinity receptor for nerve growth factor, and TrkB, a receptor for brain-derived neurotrophic factor as well as neurotrophin 4/5, are important key regulators (3–6). However, the precise molecular mechanisms of how NBL becomes aggressive and how the spontaneous regression is induced still remain elusive.

Amplification of the MYCN oncogene is strongly associated with rapid progression of NBL (7). The MYCN amplification occurs in approximately 25% of NBL and is one of the most important prognostic indicators of poor clinical outcome (8–12). MYCN is a nuclear transcription factor and its expression level is well associated with cell proliferation of NBL cells (13, 14). In general, MYCN exerts its biological functions through transcriptional regulation of its target genes in both positive and negative manners. MYCN has an ability to activate its target genes by forming a heterodimer with MAX and binds to the E-box motif, CACGTG, in the proximal promoter region (15–18). On the contrary, MYCN represses the expression of genes, such as...
Translational Relevance

Amplification of MYCN oncogene is strongly associated with rapid progression of neuroblastoma (NBL) and one of the most important prognostic indicators of poor clinical outcome. Our group previously reported that NLRR3 is highly expressed in a favorable subset of NBL but until this work, there was no sound investigation of the function of NLRR3 and its transcriptional regulation. In this study, we found that NLRR3 is a direct target of MYCN but its expression is negatively regulated by MYCN in association with Miz-1. Furthermore, a combination of low expression level of NLRR3 and high expression level of MYCN was strongly correlated with the poor prognosis. These data suggest that the expression pattern of NLRR3, Miz-1, and MYCN plays an important role in defining the clinical behavior of NBLs. The decreased expression of NLRR3 might be one of the key events regulating the aggressive behavior of NBL.

We have identified human neuronal leucine-rich repeat (NLRR) family genes as one of the differentially expressed genes between favorable and unfavorable NBLs, using our unique NBL cDNA libraries (23, 24). The NLRR protein family consists of 3 members, NLRR1, NLRR2, and NLRR3 (23), and belongs to the type y transmembrane protein with leucine-rich repeat (LRR) domains containing 11 or 12 LRRs, an immunoglobulin c2-type domain, and a fibronectin type III domain in its extracellular region. The amino acid sequences of NLRR family proteins are highly conserved in the extracellular domains, and NLRR1 and NLRR3 also possess a conserved stretch of 11 amino acids with 2 clathrin adapter interaction domains and a dileucine-type domain in the short intracellular region (25, 26), which might provide a basis for NLRR function. Our previous reports showed that NLRR1 is a direct transcriptional target of MYCN and that a high expression level of NLRR1 mRNA is associated with a poor prognosis of NBL (23, 27). However, the function of NLRR3 is poorly understood except that mouse NLRR3 expression is increased in the cerebral cortex after a cortical brain injury (28) and that rat NLRR3 may be involved in the regulation of EGF receptor signaling through interaction with clathrins (26).

We have previously reported that high levels of NLRR3 mRNA expression are associated with favorable prognostic factors in NBL (23). In this study, we found that NLRR3 is induced during differentiation of NBL cells. Transcriptional analysis has revealed that NLRR3 is a direct transcriptional target of MYCN, which negatively transactivates it in association with Miz-1. Furthermore, high expression of NLRR3 or Miz-1 and the combination of high expression of both NLRR3 and Miz-1 are significantly associated with a favorable outcome of NBL. On the contrary, the low expression levels of NLRR3 and high expression of MYCN were strongly correlated with a poor prognosis of NBL.

Materials and Methods

Patient population

Eighty-seven patients with NBL were diagnosed clinically and histologically, using a surgically removed tumor specimen according to the International Neuroblastoma Pathological classification (INPC). According to the International NBL Staging System (INSS; ref. 29), 18 patients were diagnosed as stage 1, 11 were stage 2, 20 were stage 3, 33 were stage 4, and 5 were stage 4S. Cytogenetic and molecular biological analysis of all tumors was also carried out by assessing DNA ploidy, MYCN amplification, and TrkA expression. The patients were then treated following the protocols proposed by the Japanese Infantile NBL Cooperative Study (30) and Group for Treatment of Advanced NBL (31), and subjected to survival analysis of the result in a follow-up period of at least 36 months (range, 4–58). The study was conducted under internal review board approval with appropriate informed consent.

Cell lines and transient transfection

Human NBL-derived cell lines, including SK-N-BE, CHP134, IMR32, GOTO, KAN, KP-N-NS, LAN-5, NB-1, NB-9, NLF, RTBM1, SK-N-DZ, TGW, NB69, NBL-S, OAN, SK-N-AS, SK-N-SH, and SH-SYSY cells were obtained from the CHOP cell line bank (Philadelphia, PA) and maintained in a culture condition, using RPMI 1640 supplemented with 10% heat-inactivated FBS (Invitrogen). 100 IU/mL penicillin, and 100 μg/mL streptomycin in a 37°C, 5% CO2 incubator. For the NBL cell differentiation experiment, RTBM1 and SH-SYSY cells were exposed to all-trans retinoic acid (ATRA; Sigma) at a final concentration of 5 μmol/L. For transient transfection, cells were transfected with the indicated expression of plasmids by using a Lipofectamine 2000 transfection reagent (Invitrogen), according to the manufacturer’s recommendations.

RNA extraction and semiquantitative reverse transcriptase PCR

Total RNA was prepared from fresh-frozen tissues of primary NBLs or cultured cells by using Trizol reagents (Life Technologies) or the RNaseasy Mini kit (Qiagen). Reverse transcription was carried out by random primers and Superscript II (Invitrogen), following the manufacturer’s instructions. After reverse transcription, the resultant cDNA was subjected to PCR-based amplification. The sequence of the primer sets were used for PCR amplification, and the resulting cDNA was subjected to PCR-based amplification. The sequence of the primer sets were used for PCR amplification, and the resultant cDNA was subjected to PCR-based amplification. The sequence of the primer sets were used for PCR amplification, and the resulting cDNA was subjected to PCR-based amplification.

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The expression of GAPDH was measured as an internal control.

**Quantitative real-time PCR**

cDNA from primary NBLs and cell lines were subjected to the real-time PCR to quantitate the expression levels of MYCN, Miz-1, and NLRR3 mRNA. TaqMan GAPDH control reagent kit (Perkin-Elmer Applied Biosystems) was used for GAPDH expression and analyzed by an ABI prism 7500 Sequence Detection System (Applied Biosystems). NLRR3 and Miz-1 TaqMan probes were purchased from Applied Biosystems. MYCN mRNA expression was measured by the SYBR green real-time PCR system. The primers and probes used for real-time PCR were listed in Supplementary Table S4.

**Generation of a specific antibody against NLRR3**

The rabbit polyclonal anti-NLRR3 antibody was raised against a mixed synthetic peptide corresponding to amino acid sequences between positions 655 to 670 and 692 to 707 of human NLRR3. The peptide and polyclonal antibody (TB0266) were generated by Medical and Biological Laboratories (Nagoya, Japan). The specificity of the affinity-purified antibody was assayed by immunoblotting.

**Plasmid constructs**

The protein-coding region of Miz-1 was amplified by PCR and inserted into the EcoRI site of pcDNA3.1 (Invitrogen) flanked with a Flag tag. The human NLRR3 promoter region and its 5′ progressive deletion mutant were amplified by PCR and then inserted into the SacI site in the upstream of the luciferase gene of the pGL3-basic plasmid (Promega). All constructs were verified by DNA sequencing. The pUHD-MYCN vector was kindly provided by Dr. M. Schwab (German Cancer Research Center, Heidelberg, Germany).

**Luciferase reporter assay**

SH-SY5Y cells were seeded at a density of 5 × 10⁴ cells/12-well cell culture plate and allowed to attach overnight. The cells were transiently cotransfected with each mutant of the human NLRR3 promoter-driven luciferase reporter and an internal control vector for Renilla luciferase, or a combination of the indicated expression vectors. The total amount of plasmid DNA per transfection was kept consistent with the pcDNA3.1 vector. Both firefly and Renilla luciferase activities were assayed with the Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s instructions. The firefly luminescence signal was normalized on the basis of the Renilla luminescence signal.

**siRNA transfection**

To knockdown endogenous MYCN expression, SK-N-AS, SK-N-BE, and SH-SY5Y cells were transfected with 10 nmol/L of the indicated siRNA purchased from Dharmacon by using LipofectAMINE RNAiMAX (Invitrogen), according to the manufacturer’s recommendations. The list of siRNA sequences used will be provided upon request. Forty-eight hours after transfection, cell lysates were prepared and analyzed for the expression levels of NLRR3 and MYCN by immunoblotting.

**Immunoblot analysis**

The cells were washed twice with ice-cold PBS and then lysed immediately with SDS sample buffer containing 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, and 62.5 mmol/L Tris-HCl (pH 6.8). The protein concentrations were determined by using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories). Equal amounts of cell lysates were separated by SDS-PAGE and electrophoretically transferred onto Immobilon-P membranes (Millipore). The transferred membranes were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween-20 and incubated with appropriate primary antibodies at room temperature for 1 hour followed by incubation with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Cell Signaling Technology Inc.) at room temperature for 1 hour. Immunoreactive bands were visualized by an ECL system (GE Healthcare). The primary antibodies used in this study were as follows: monoclonal anti-MYCN (Ab-1; Oncogene Research Products), polyclonal anti-NLRR3, polyclonal anti-Miz-1 (Santa Cruz Biotechnology), monoclonal anti-GAP43 (9-1E21; Chemicon), and polyclonal anti-actin (20-33; Sigma) antibodies.

**Chromatin immunoprecipitation assays**

A chromatin immunoprecipitation (ChIP) assay was carried out according to the protocol provided by Upstate Biotechnology (Charlottesville). In brief, cells were cross-linked with 1% formaldehyde in medium for 10 minutes at 37°C. Chromatin solutions were prepared and immunoprecipitated with the following antibodies: anti-MYCN, anti-Miz-1, anti-Max rabbit polyclonal antibodies (Santa Cruz Biotechnology), and normal mouse or rabbit serum as a control. The immunoprecipitates were eluted with 100 μL of elution buffer (1% SDS and 1 mmol/L NaHCO₃). Formaldehyde-mediated cross-links were reversed by heating at 65°C for 4 hours, and the reaction mixtures were treated with proteinase K at 45°C for 1 hour. DNAs of the immunoprecipitates and control input DNAs were purified by using a QIAquick PCR purification kit (Qiagen). Purified DNA was subjected to optimized semiquantitative PCR amplification protocol for NLRR3 gene promoter and control regions, using appropriate primer sets (Supplementary Table S4).

**Statistical analysis**

Student t tests were employed to examine the possible association between NLRR3 expression and other prognostic factors. The classification of high and low levels of NLRR3, Miz-1, and MYCN expression was determined on the basis of the mean value obtained from quantitative real-time PCR analysis. Kaplan–Meier survival curves were calculated, and survival distributions were compared by using the log-rank test. Cox regression models were used to search associations along with NLRR3 expression, MYCN
expression, Miz-1 expression, age, MYCN amplification status, INSS, TrkA expression, DNA index, origin, and survival. Statistical significance was considered if \( P \) value was less than 0.05. The statistical analysis was carried out by SPSS Statistical Software release 12.0.

**Results**

**NLRR3 is upregulated during neuronal differentiation**

It has been previously reported that the NBL cell lines exposed to ATRA undergo neuronal differentiation (32), accompanied by a marked decrease in the expression levels of MYCN (33). To examine the possible involvement of MYCN in the regulation of NLRR3 expression, the NBL-derived RTBM1 cells were treated with or without 5 \( \mu \)mol/L ATRA. As previously described (34), RTBM1 cells underwent neuronal differentiation with extensive neurite outgrowth in response to ATRA treatment (Fig. 1A). The induced differentiation was confirmed by the expression levels of GAP43, a marker of neuronal differentiation (35), which increased after ATRA treatment at both mRNA and protein levels (Fig. 1B and C). As expected, MYCN expression was significantly decreased after ATRA treatment and almost diminished at 6 days after treatment. Consistent with our previous observations (23), NLRR3 was markedly upregulated at the mRNA and protein levels during the differentiation process. Similar results were also obtained from ATRA-treated SH-SY5Y cells (Supplementary Fig. S1A and B).

**Inverse correlation between MYCN and NLRR3 expressions**

To further confirm a possible relationship between MYCN and NLRR3, we used MYCN-inducible SHEP21N cells originally derived from NBL (36) and treated with tetracycline to switch off the expression of MYCN. As shown in Fig. 2A, the reduced expression level of MYCN upon tetracycline treatment was confirmed by reverse transcriptase PCR (RT-PCR) and immunoblotting, whereas NLRR3 expression was increased after tetracycline treatment.

![Figure 1. Opposite expression pattern of NLRR3 and MYCN in differentiated RTBM1 cells in response to ATRA. A, ATRA-induced differentiation program in RTBM1 cells. Cells were treated with 5 \( \mu \)mol/L ATRA or left untreated. At the indicated time-periods after treatment with ATRA, neurite outgrowth was examined with a phase-contrast microscope. B and C, RT-PCR and immunoblot analysis for MYCN, NLRR3, and GAP43 in response to ATRA. RTBM1 cells were treated as in A. Total RNA and cell lysates were prepared and processed for RT-PCR (B) and immunoblotting with indicated antibodies (C). For RT-PCR, GAPDH was used as an internal control. For immunoblotting, actin was used as a loading control.](image-url)
To examine whether MYCN and NLRR3 have an inverse functional relationship under these physiologic conditions, siRNA knockdown of the endogenous MYCN was carried out in 2 NBL cell lines, SK-N-AS cells with a single copy of \textit{MYCN} and SK-N-BE cells with \textit{MYCN} amplification. As shown in Fig. 2B, one of the siRNAs against \textit{MYCN}, si-2, efficiently reduced endogenous expression of \textit{MYCN} in both cell lines and resulted in an increased expression of NLRR3. SH-SY5Y cells with a single copy of \textit{MYCN} also showed the similar result after siRNA-mediated knockdown of the endogenous MYCN. SK-N-AS and SK-N-BE cells were transfected with control siRNA (Con.) or with 2 siRNA (si-1 and si-2) against MYCN. At 48 hours after transfection, total RNA and cell lysates were prepared and processed for RT-PCR (left) and immunoblotting with indicated antibodies (right).

### Figure 2

**A**
- Tetracycline (50 ng/mL)
- Time: 0, 6, 12, 24, 48, 72 (h)
- MYCN
- NLRR3
- GAPDH

**B**
- SK-N-AS
  - Con. si-1 si-2
  - MYCN
  - NLRR3
  - GAPDH
  - Actin

- SK-N-BE
  - Con. si-1 si-2
  - MYCN
  - NLRR3
  - GAPDH
  - Actin

**C**
- SH-SY5Y cells
  - MYCN
  - NLRR3
  - GAPDH

*Figure 2. Inverse regulation of MYCN and NLRR3 in various NBL cell lines. A, RT-PCR and immunoblot analysis for MYCN and NLRR3 in SHEP21N cells maintained in the presence of tetracycline. At the indicated time points after the addition of tetracycline (50 ng/mL), total RNA and cell lysates were prepared and processed for RT-PCR (left) and immunoblotting with indicated antibodies (right). B, siRNA-mediated knockdown of the endogenous MYCN. SK-N-AS and SK-N-BE cells were transfected with control siRNA (Con.) or with 2 siRNA (si-1 and si-2) against MYCN. At 48 hours after transfection, total RNA and cell lysates were prepared and processed for RT-PCR (left) and immunoblotting with indicated antibodies (right). C, SH-SY5Y cells were transiently transfected with or without the increasing amounts of the expression plasmid encoding MYCN. Forty-eight hours after transfection, total RNA and cell lysates were prepared and processed for RT-PCR (left) and immunoblotting (right) with indicated antibodies. GAPDH was used as an internal control of RT-PCR and actin was used as a loading control for immunoblotting.*

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\textit{NLRR3} expression is negatively regulated by \textit{MYCN} in NBL cells.

**MYCN represses the promoter activity of NLRR3 in association with Miz-1**

According to the previous reports (19, 20, 37), Myc proteins repress its target genes by forming a complex with Miz-1. Under these conditions at low expression levels of Myc, Miz-1 activates transcription of the target genes by cooperating with other transcriptional cofactors and enhances cell differentiation (20). Therefore, we hypothesized that Miz-1 might be involved in the regulation of NLRR3 expression. To prove this, we examined whether exogenously expressed Miz-1 upregulates NLRR3 expression in SH-SY5Y cells. Figure 3A, left shows that NLRR3 expression was upregulated by overexpression of Miz-1 in the same manner as a positive control, \textit{p15Ink4b} expression, whereas expression of other NLRR family members, \textit{NLRR1}...
and NLRR2, showed no change. The increased expression of NLRR3 protein was also confirmed by Western blot analysis (Fig. 3A, right). This induction of NLRR3 by Miz-1 was also observed in SK-N-AS cells (Supplementary Fig. S3). To determine whether Miz-1 activates the NLRR3 promoter, the region spanning exon 2 and 5'-upstream sequences of the NLRR3 gene (nucleotide −1,020 to +67) was cloned and analyzed for promoter activity by using a luciferase reporter assay. The promoter deletion analysis showed that a nucleotide position between −677 and +67 gives maximum promoter activity (Supplementary Fig. S4A). The core promoter region (−35 to +67) also
showed higher promoter activity than other deletion mutants. In transient cotransfection assays, simultaneous expression of Miz-1 increased the luciferase activities driven by the NLRR3 promoter (−677 to +67; Fig. 3B, left).

On the contrary, overexpression of MYCN resulted in reduced activity of the NLRR3 promoter (Supplementary Fig. S4B). These results suggest that Miz-1 and MYCN together contribute to the transcriptional regulation of the NLRR3 gene. Indeed, the activation of the NLRR3 promoter by exogenous Miz-1 expression in SH-SYSY cells was suppressed by coexpression of MYCN in a dose-dependent manner (Fig. 3B, right). The luciferase activities driven by the core promoter region (−35 to +67) also showed a similar result (data not shown). It was reported that a transcriptional suppression of the MYCN-targeted genes occurs when MYCN forms a complex with Miz-1 and Max (19). To make certain of the physical interaction between MYCN and Miz-1, the whole cell lysates prepared from the SK-N-AS cells cotransfected with MYCN and Miz-1 were subjected to an immunoprecipitation assay. As shown in Fig. 3C, communoprecipitation using either MYCN or Miz-1 antibody confirmed that MYCN and Miz-1 formed a complex in SK-N-AS cells as previously reported in non-NBL cell lines (38). Moreover, ChIP analysis revealed that MYCN, Max, and Miz-1 were recruited onto the same promoter region of NLRR3 (−164 to +67) in SH-SYSY cells (Fig. 3D). Hence, MYCN negatively regulates NLRR3 expression by forming a transcriptional complex with Miz-1 in NBL cells.

**Increased expression of NLRR3 and Miz-1 in favorable neuroblastoma**

In our previous report, NLRR3 is highly expressed in favorable NBLs with a single copy of MYCN as compared with NBLs with MYCN amplification. To evaluate whether the expression pattern of Miz-1, NLRR3, and MYCN observed in NBL cell lines is consistent in primary NBLs, we analyzed expression levels of those 3 genes in 16 favorable (stages 1 or 2, high expression of TrkA and amplification of MYCN) NBL samples by semiquantitative RT-PCR. As shown in Supplementary Fig. S5A, NLRR3 and Miz-1 were expressed at higher levels in favorable NBLs than those in unfavorable tumors, whereas the levels of MYCN expression were predominantly high in the unfavorable tumors. The expression levels of NLRR3 and Miz-1 were also higher in the cell lines with a single copy of MYCN than those with MYCN amplification, indicating evidence of a positive correlation between NLRR3 and Miz-1 expressions and of an inverse correlation between NLRR3 and MYCN expressions (Supplementary Fig. S5B). Those expression patterns were further assessed by immunohistochemistry for NLRR3, MYCN, and Miz-1 in primary NBL tissues (Supplementary Fig. S5C). We carried out immunohistochemical staining on all 11 available paraffin-embedded primary NBL tissues, including 5 NBLs with a single copy of MYCN and favorable histology according to INPC (39), 3 NBLs carrying a single copy of MYCN with unfavorable histology, and 3 NBLs with MYCN amplification and unfavorable histology. As shown in Supplementary Fig. S5C and Supplementary Table S1, the absence of MYCN amplification was associated with strong positive staining of NLRR3 and Miz-1 in all examined samples except one (case 8). All 3 NBLs with MYCN amplification showed weak staining for both NLRR3 and Miz-1.

**Low expression of NLRR3 and Miz-1 is associated with an unfavorable outcome of neuroblastoma**

To evaluate whether a statistically significant relationship exists between the patients’ survival periods and the expression of NLRR3, Miz-1, or MYCN in primary NBLs, we quantitatively measured the expression levels of NLRR3, Miz-1, and MYCN mRNAs in 87 primary NBLs by using the quantitative real-time PCR method. The clinical features of each NBL sample are listed in Supplementary Table S2. As shown in Table 1, high levels of NLRR3 expression were significantly associated with younger age ($P = 0.047$), single copy of MYCN ($P = 0.047$), favorable disease stages ($P = 0.041$), high levels of TrkA expression ($P = 0.042$), and diploid DNA index ($P = 0.003$), but not with tumor origin ($P = 0.933$). A high level of Miz-1 expression was also significantly associated with younger age ($P = 0.004$), single copy of MYCN ($P = 0.004$), favorable disease stages ($P = 0.001$), and high levels of TrkA expression ($P = 0.001$), but not with DNA index ($P = 0.060$) and tumor origin ($P = 0.959$). In contrast, a high level of MYCN expression was significantly associated with MYCN amplification ($P = 0.0001$), advanced disease stages ($P = 0.0031$), low levels of TrkA expression ($P = 0.026$), and tumor origin ($P = 0.028$), but not with DNA index ($P = 0.079$), which is consistent with the previous reports (23, 40, 41). There was also a marginal association with patient age ($P = 0.063$). These results suggest that high expression of NLRR3 and Miz-1 is well associated with conventional prognostic markers predicting a favorable NBL outcome.

To examine whether the expression levels of NLRR3, Miz-1 and/or MYCN have a prognostic significance in primary NBLs, we employed log-rank tests for gene-expression data (Supplementary Table S3). There were significant differences in survival rates in the groups of patients with high and low expression of NLRR3, Miz-1, and MYCN. Patients with high expression of NLRR3 or Miz-1 had a higher survival rate than patients with low expression of NLRR3 or Miz-1, and such a difference in survival rate was statistically significant ($P = 0.0023$ and $P = 0.00060$, respectively). However, a patient with high MYCN expression was associated with a lower survival rate than that of the MYCN low subset ($P < 0.00001$; Supplementary Table S3). Figure 4 shows Kaplan–Meier cumulative survival curves for 87 patients with NBL in terms of expression of NLRR3, Miz-1 and MYCN. High expression of NLRR3 and that of Miz-1 were significantly associated with good survival ($P = 0.0023$ and $P = 0.00060$, respectively; Fig. 4A, left and right). As already known, high expression of MYCN
was strongly associated with a poor prognosis of NBL (P < 0.00001; Fig. 4A, middle). Remarkably, the combination of low levels of both NLRR3 and Miz-1 expressions showed a significantly worse prognosis as compared with the other combination, high NLRR3 and Miz-1 expressions (P = 0.0012; Fig. 4C). Furthermore, the combination of low expression of NLRR3 and high expression of MYCN showed a significantly worse prognosis than the combination of high expression of NLRR3 and low expression of MYCN (P < 0.00001; Fig. 4B). In NBLs with low expression of MYCN, the expression levels of NLRR3 could segregate the prognosis into good and intermediate groups.

The univariate Cox regression analysis shown in Table 2 was employed to examine the individual relationship of each variable to survival. The results in Table 2 showed that NLRR3 expression, MYCN expression, Miz-1 expression, age, MYCN amplification, stage TrkA expression, and origin were of prognostic importance, supporting the results of the log-rank test. Moreover, the multivariate Cox models were fitted to assess the predictive importance of NLRR3 expression for survival after controlling other prognostic factors. The results in Table 2 showed that NLRR3 expression was significantly associated with survival after controlling TrkA expression (P = 0.0212), suggesting that NLRR3 expression was an independent prognostic factor from TrkA expression (Table 2). This suggests that NLRR3 expression is associated with survival after controlling MYCN expression (P = 0.0610), Miz-1 expression (P = 0.1510), MYCN amplification (P = 0.1210), and stage (P = 0.1040), and also supports that NLRR3 expression could serve as a prognostic biomarker for NBL tumors dependent on both MYCN and Miz-1 expression as well as MYCN amplification.

**Discussion**

In primary human NBLs, MYCN is frequently amplified and thereby one of the most important prognostic factors. In this study, we found that NLRR3 is a direct target of MYCN but its expression is negatively regulated by MYCN in association with Miz-1. In primary NBLs, both NLRR3 and Miz-1 are expressed at significantly high levels in favorable NBLs and downregulated in MYCN-amplified aggressive tumors.

In general, favorable NBL cells show more differentiated features than unfavorable cells (30). The treatment of NBL cells with ATRA induces neuronal differentiation accompanied with growth inhibition and reduction of MYCN expression (33). Under such conditions, NLRR3 is induced while MYCN is decreased (Figs. 1 and 2). These results suggest a functional inverse relationship between MYCN and NLRR3 in cellular differentiation and tumor development. In some NBL cell lines, siRNA-mediated knockdown of endogenous MYCN caused NLRR3 induction; conversely, ectopic expression of MYCN resulted in a decreased expression of NLRR3. Hence, the inverse regulatory relationship between NLRR3 and MYCN may be present as a
consequence of MYCN-induced transcriptional downregulation of NLRR3.

MYCN protein is an important regulator of many cellular processes, including growth, proliferation, differentiation, and apoptosis (42). A part of these diverse cellular functions of MYCN may be due to the combined abilities of both activating and repressing transcription of the target genes (42). Transcriptional activation by MYCN occurs via dimerization with its partner protein, Max, and direct binding to specific DNA sequences named E-boxes. MYCN directly binds and stimulates the expression of approximately 4,000 of the E-box containing genes (43). Although heterodimerization of Max with MYCN is necessary to regulate gene expression, the other proteins including Miz-1 may bind to C-terminal MYCN in addition to Max (19, 20, 44). Concurrent binding of these factors redirects the MYCN/Max dimer to noncanonical sites such as the initiator element, where this complex might prevent the efficient binding of basal transcriptional machinery or coactivators necessary for transactivation, resulting in repression of gene expression (38, 44). The dimerization with MYCN switches Miz-1 from a transcriptional activator to a repressor of the target genes, likely by preventing the interaction of Miz-1 with its own coactivator (19, 20). Several studies have shown that Miz-1 binds and activates the promoter of several genes, including p15INK4b and p21CIP1, and the transactivation can be negatively regulated by its association with MYCN (16, 17, 29). Regarding the reduction of NLRR3 expression observed in this study, Miz-1 seems to be a key molecule forming a transcription factor complex with MYCN. Because Miz-1 itself acts as an activator of NLRR3 promoter, NLRR3 expression may be switched on and off through Miz-1 in the absence and presence of MYCN, respectively. Although the expression levels of Miz-1 in unfavorable NBLs are relatively low, its amount still may be enough to act with MYCN to inhibit transactivation of NLRR3 in NBLs.

Figure 4. Real-time PCR analysis for the expression of NLRR3, MYCN, and Miz-1 in 87 primary NBLs. Kaplan–Meier survival curves of patients with NBLs on the basis of higher or lower expression levels of NLRR3 (A, left); MYCN (A, middle); Miz-1 (A, right); NLRR3 and MYCN (B); or NLRR3 and Miz-1 (C). In case of NLRR3 and MYCN survival curve, high NLRR3/high MYCN group was excluded because this group consists of only 2 samples. Relative expression levels of NLRR3 or MYCN or Miz-1 mRNA were determined by calculating the ratio between GAPDH and NLRR3 or MYCN or Miz-1.
Inhibition of cellular differentiation is one of the well-known biological functions of MYCN. Because differentiated NBL cells have a high expression of NLRR3 instead of MYCN, the reduced expression of NLRR3 in undifferentiated, unfavorable NBL cells may propose an important component of the mechanism by which MYCN functions against cell differentiation. As ectopic expression of NLRR3 induced morphologic changes indicative of neuronal differentiation accompanying with neurite outgrowth (data not shown), the downregulation is a new prognostic indicator of unfavorable NBL and may be involved in regulating the biology of the tumor.

Collectively, our present findings suggest that the repression of NLRR3 mediated by MYCN requires an association with Miz-1 and also contributes to the favorable outcome of NBLs. The expression pattern of NLRR3, Miz-1, and MYCN might play an important role in defining the clinical behavior of NBLs. Because NLRR3 is an orphan receptor, the future discovery of its ligand(s) may unveil the molecular mechanism of tumorigenesis, differentiation, and proliferation of NBL. Further investigation is necessary to clarify whether NLRR3 is an important primary cue for developing novel diagnostic and therapeutic strategies against high-risk NBLs.

### Disclosure of Potential Conflicts of interest

No potential conflicts of interest were disclosed.

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**Table 2.** Multiple Cox regression model using NLRR3 expression and dichotomous factors of MYCN expression, Miz-1 expression, age, MYCN amplification, stage, TrkA expression, and origin \( (n = 87) \)

<table>
<thead>
<tr>
<th>Model</th>
<th>Factor</th>
<th>( P )</th>
<th>HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univariate analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>NLRR3 mRNA expression (high vs. low)</td>
<td>0.0041a</td>
<td>0.291 (0.125–0.678)</td>
</tr>
<tr>
<td>B</td>
<td>MYCN mRNA expression (high vs. low)</td>
<td>&lt;0.0001a</td>
<td>5.050 (2.450–10.40)</td>
</tr>
<tr>
<td>C</td>
<td>Miz-1 mRNA expression (high vs. low)</td>
<td>0.0021a</td>
<td>0.212 (0.080–0.561)</td>
</tr>
<tr>
<td>D</td>
<td>Age (&gt;1 vs. &lt;1 y)</td>
<td>0.0161a</td>
<td>0.309 (0.119–0.803)</td>
</tr>
<tr>
<td>E</td>
<td>MYCN amplification (single copy vs. amplified)</td>
<td>&lt;0.0001a</td>
<td>4.628 (2.281–9.387)</td>
</tr>
<tr>
<td>F</td>
<td>Stage (1,2,4s vs. 3,4)</td>
<td>0.0010a</td>
<td>12.66 (3.023–53.09)</td>
</tr>
<tr>
<td>G</td>
<td>TrkA expression (high vs. low)</td>
<td>0.0070a</td>
<td>7.180 (1.714–30.07)</td>
</tr>
<tr>
<td>H</td>
<td>Origin (adrenal gland vs. others)</td>
<td>0.0480a</td>
<td>2.125 (1.005–4.491)</td>
</tr>
<tr>
<td>Multivariate analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>NLRR3 mRNA expression (high vs. low)</td>
<td>0.061</td>
<td>0.424 (0.172–1.041)</td>
</tr>
<tr>
<td>B</td>
<td>MYCN mRNA expression (high vs. low)</td>
<td>0.0011a</td>
<td>3.707 (1.735–7.921)</td>
</tr>
<tr>
<td>C</td>
<td>NLRR3 mRNA expression (high vs. low)</td>
<td>0.151</td>
<td>0.503 (0.198–1.283)</td>
</tr>
<tr>
<td>D</td>
<td>NLRR3 mRNA expression (high vs. low)</td>
<td>0.0301a</td>
<td>0.304 (0.104–0.893)</td>
</tr>
<tr>
<td>E</td>
<td>NLRR3 mRNA expression (high vs. low)</td>
<td>0.0150a</td>
<td>0.347 (0.148–0.814)</td>
</tr>
<tr>
<td>F</td>
<td>Stage (1, 2, 4s vs. 3, 4)</td>
<td>0.0010a</td>
<td>0.384 (0.146–1.013)</td>
</tr>
<tr>
<td>G</td>
<td>TrkA expression (high vs. low)</td>
<td>0.0070a</td>
<td>7.180 (1.714–30.07)</td>
</tr>
<tr>
<td>H</td>
<td>Origin (adrenal gland vs. others)</td>
<td>0.0480a</td>
<td>2.125 (1.005–4.491)</td>
</tr>
</tbody>
</table>

**NOTE:** All variables with 2 categories. HR shows the relative risk of death of first category relative to the second.

*P < 0.05.
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MYCN and Miz-1 Negatively Regulate NLRR3


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