NLRR1 enhances EGF-mediated MYCN induction in neuroblastoma and accelerates tumor growth in vivo

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Abstract

Neuronal leucine-rich repeat protein-1 (NLRR1), a type-1 transmembrane protein highly expressed in unfavorable neuroblastoma (NB), is a target gene of MYCN that is predominately expressed in primary NBs with MYCN amplification. However, the precise biological role of NLRR1 in cell proliferation and tumor progression remains unknown. To investigate its functional importance, we examined the role of NLRR1 in epidermal growth factor (EGF) and insulin growth factor-1 (IGF-1)-mediated cell viability. We found that NLRR1 positively regulated cell proliferation through activation of ERK mediated by EGF and IGF-1. Interestingly, EGF stimulation induced endogenous MYCN expression through Sp1 recruitment to the MYCN promoter region, which was accelerated in NLRR1-expressing cells. The Sp1 binding site was identified on the promoter region for MYCN induction, and phosphorylation of Sp1 was important for EGF-mediated MYCN regulation. In vivo studies confirmed the proliferation-promoting activity of NLRR1 and established an association between NLRR1 expression and poor prognosis in NB. Together, our findings indicate that NLRR1 plays an important role in the development of NB and therefore may represent an attractive therapeutic target for cancer treatment.
Introduction

Neuroblastoma (NB) is one of the most common extracranial malignant tumors that develop in children; they arise from neural crest cells and are mostly found in the adrenal medulla or along the sympathetic chain (1). NB exhibits clinical and biological heterogeneity, ranging from rapid progression associated with metastatic spread and poor clinical outcome to occasional, spontaneous, or therapy-induced regression or differentiation into benign ganglioneuroma (2,3). Different subsets of NB show various distinct genetic features, including DNA ploidy, MYCN amplification, allelic loss of the distal part of chromosome 1p, and gain of chromosome 17q (4). Amplification of the MYCN gene usually distinguishes a subset of NB with poor prognosis (1), and recovery of children with high-risk NB remains low, providing a compelling reason for better understanding of the molecular mechanisms that can be targeted to treat this disease (5,6). MYCN transgenic mice develop NB, which implicates that MYCN can maintain the tumorigenic state, supporting the importance of the MYCN gene as a potential therapeutic target (7-9). However, the precise mechanism of MYCN regulation and the functional correlation with other proteins in the progression of NB are still elusive.

NLRR1 is a type I transmembrane protein with extracellular leucine-rich repeats, and belongs to the mammalian neuronal leucine-rich protein family (NLRR1 to NLRR5) (10-13). We previously reported that mRNA expression levels of NLRR1 are significantly higher in unfavorable NB (12). We further reported that NLRR1 protein expression is higher in MYCN-amplified primary NBs than in non-amplified tumors, and that MYCN can transcriptionally upregulate NLRR1 (14). We also found that overexpression of NLRR1 promoted NB cell proliferation and inhibited cellular apoptosis upon serum starvation (14). NLRR family proteins have also been considered as cell adhesion or signaling molecules, and mouse NLRR3 functions in epidermal growth factor (EGF)-mediated activation of ERK (15).

EGF signaling was reported to be involved in NB cell proliferation via the activation of ERK and AKT (16). Insulin growth factor-I (IGF-I) stimulation was also reported to enhance NB cell proliferation, and is involved in the induction of MYCN expression through mitogen activated protein kinase (MAPK) (17). MAPK kinase (MKK) proteins are crucially important in several cellular events, including proliferation, survival and differentiation (18,19). Several stimuli activate MKKs, which is followed by activation of MAPKs, including ERK, JNK, and p38 MAPK. Abnormalities in MAPK pathways, especially mutations of proteins of these signaling cascades, have been reported in about 20% of all human cancers (20, 21). Several reports suggest that MKK/ERK signaling has an important role in tumorigenesis and metastasis (22-24). However, the precise role of MKK/ERK signaling in the development of NB and its functional relationship with MYCN oncogene are still unknown. NLRR1 is a possible regulator of growth factor signaling, and may play a crucial role in MYCN-amplified NB to form aggressive tumors. In the present study, we report that EGF promotes ERK activation and is involved in MYCN induction via recruitment of Sp1 to the MYCN promoter. Overexpression of NLRR1 enhanced MYCN induction by activating ERK signaling, while knockdown of NLRR1 suppressed ERK phosphorylation and MYCN induction upon EGF treatment. In vivo studies in nude mice showed significant tumorigenic activity of NLRR1. Our present findings collectively indicate that NLRR1 accelerates growth factor signaling to induce MYCN, and plays a positive feedback loop with MYCN to induce aggressive tumor progression in NB.

Materials and Methods

Cell Lines, Transfection and Reagents
Human NB-derived SK-N-BE and SH-SY5Y cells were collected from CHOP cell lines (Philadelphia, PA, USA) and were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 50 µg/ml penicillin, and 50 µg/ml streptomycin (Invitrogen). Cells were cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. For transient transfection, SK-N-BE cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Cell Proliferation Assays**

SK-N-BE and SH-SY5Y cell proliferation was evaluated using the tetrazolium compound WST-8 (Cell Counting Kit-8 Dojindo Laboratories, Japan). Cell proliferation was determined according to the manufacturer’s instructions.

**RNA Extraction and RT-PCR**

Total RNA was prepared from the indicated cells using the RNeasy mini kit (Qiagen, CA) according to the manufacturer’s protocol, and reverse-transcription was performed. The specific primers used were as follows: MYCN, 5’-CTTCGGTCCAGCTTCTCACT-3’ and 5’-GTCCGAGCGCTTCAATTGTTT-3’; NLRR1, 5-GCAGCTTTTCAACTTGAGAA-3 and 5-TGCAGGCACTTTGACTGAAC-3; VEGF, 5’-AAGGAGGAGGCAATGATC -3’ and 5’-ATCTGCATGGTGATGGT -3’; Sp1, 5’-TGAGGAGGAGGCAATGATC -3’ and 5’-CACACACTCTCCACC -3’; GAPDH, 5’-ACCTGACCTGGCTCTAGAA-3’ AND 5’-TCCACCACCTGTGCTGA -3’. GAPDH expression was measured as an internal control.

**Immunoblotting**

Cells were collected and washed with phosphate-buffered saline (PBS). Whole cell lysates were prepared by incubating cells in lysis buffer containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM ethyleneglycol tetraacetic acid, 50 mM β-mercaptoethanol, 1% Triton X-100, a commercial protease inhibitor mixture (Sigma), and phosphatase inhibitor mixture (Sigma), for 30 minutes on ice, and subjected to brief sonication for 10 sec at 4 °C, followed by centrifugation at 15,000 rpm at 4 °C for 10 minutes to remove insoluble materials. Protein concentration was measured using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer’s instruction. Equal amounts of protein (50 µg) were separated by 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred onto PVDF membranes (Immobilion-P, Millipore, Bedford, MA). PVDF membranes were then blocked with Tris-buffered saline containing 5% nonfat dry milk and 0.1 % Tween 20 at room temperature for 1 h. After blocking, the membranes were incubated at 4 °C overnight with anti-MYCN (Ab-1, Oncogene), anti-EGFR (Rockland, Gilbertsville, PA), anti-actin (20-33; Sigma) and other antibodies against ERK1/2, phospho-ERK, IGF1R, phospho IGFR1, Akt, phospho-Akt, and phospho-EGFR were purchased from Cell Signaling Technology (Boston, MA, USA). After incubation with primary antibodies, membranes were incubated with horseradish peroxidase-coupled goat anti-mouse or anti-rabbit IgG secondary antibody (Cell Signaling Technology, Boston, USA) for 1 h at room temperature. Immunoblots were visualized using ECL detection reagents according to the manufacturer’s instruction (Amersham Biosciences).

**Construction of Luciferase Reporters**

A luciferase reporter construct driven by the MYCN promoter was generated by using the following primer sets: MYCN (-221/+21), 5’- GAGCTCCAGCTTTCAGCCTTTC -3’ and 5’- GAGCTCCAGCCAGCATGACTGTC -3’. Underlined sequences indicate
Sac1 enzyme recognition sites. Then, the PCR product was inserted into the Sac1 site of pGL3basic vector. Mutation of the two putative Sp1 binding sites was performed using a site-directed mutagenesis kit (Promega) using the following primer sets: mut-1 (-221/+21), 5'-ACAGCCCCCTTCTCCTCCC(A)G(A)CCC(A)CCCGG -3' (sense), 5'-GGGAGAGAAGGGGCTGTGGCGCA -3' (antisense); mut-2 (-221/+21), 5'-ATGAAATCAGGAGGGC(A)G(A)GGTAAAG -3' (sense), 5'-CCCTCCTGATTTCATAAAAATCA -3' (antisense). Underlined sequences represent putative Sp1 binding sites, and the mutated nucleotides are marked by brackets.

Luciferase Reporter Assays

SK-N-BE cells were plated in 12-well plates at a density of 50,000 cells/well, and transiently transfected with reporter constructs driven by the MYCN promoter (200 ng) and pRL-TK Renilla luciferase reporter plasmid (20 ng). After the indicated time periods, cells were collected and washed with PBS, and their luciferase activities were measured using a luciferase reporter assay system (Promega, Madison, WI, USA). Each experiment was performed at least three times in triplicate.

siRNA Transfection

A mixture of two siRNAs with antisense sequences of 5’-UCUUGGUUGAGCUGUAGTT-3’ and 5’-UUGUGGACACUCACUAUUCT-3’ were designed to target human NLRR1 (TAKARA, Japan). Control siRNA was purchased from Ambion (Cat 4635). SK-N-BE cells were transfected with 20 nM of the indicated siRNAs using Lipofectamine RNAiMAX (Invitrogen).

ChIP Assays

Before collection, cells were cross-linked with 1% formaldehyde in medium for 10 min at 37 °C. Chromatin immunoprecipitation (ChIP) was performed following the protocol provided by Upstate Biotechnology. In short, cross-linked chromatin was prepared from cells and sonicated to an average length of 200-800 nucleotides, precleaned with protein A-agarose beads pretreated with shared salmon sperm DNA, and immunoprecipitated with rabbit anti-E2F1 (KH95, Santa Cruz) and rabbit anti-Sp1 (DAM1718081, Upstate/Millipore) antibodies conjugated with protein A-agarose. The immunoprecipitates were eluted with 100 μl elution buffer (1% SDS and 0.1 M NaHCO3). Formaldehyde-mediated cross-links were reversed by heating at 65 °C for 4 h, and the reaction mixtures were treated with proteinase K at 45 °C for 1 h. Precipitated DNA and control input DNA were purified using a QiAquick PCR purification kit (Qiagen, Valencia, CA, USA). Purified DNA was amplified by PCR using the following primer set: 5’-CAGCTTTGCAGCCTTCTC-3’ and 5’-GTCCAGACAGATGACTGTC -3’ targeting the MYCN core promoter region (-221, +21).

Animals and Tumor Xenograft Studies

Male BALB/c athymic (nu/nu) mice (5–6 weeks old) were purchased from Japan SLC, and maintained under specific pathogen-free conditions strictly following the Chiba Cancer Center Research Institute guidelines. Stably-expressing NLRR1 and mock SH-SY5Y cell lines were established by transfection followed by selection with G418 at a concentration of 600 μg/ml for about 6–8 weeks. Single colonies were picked to confirm the ectopic expression of NLRR1. Three NLRR1-expressing clones were used for the tumor xenograft studies, and three mock-transfected single clones were used as negative controls. A total of six groups of mice (seven mice in each group) received subcutaneous injections of 1×10⁷ cells dissolved in 100 μl PBS. The length and width of each tumor was recorded each week at the indicated time periods. Tumor volume was calculated according the following formula: [(length × (width)²)/2 (25,26). Survival curves were generated using the Kaplan-Meier method using SPSS software. Log-rank tests were performed to calculate the P-value between the two survival curves.
Immunohistochemical staining

To prepare the cryosections, tumors were fixed in 4% paraformaldehyde, washed with sucrose solution, embedded in OCT compound, frozen, and sectioned at 10 µm thickness. Sections were air-dried, washed with TBS, blocked in Mouse on Mouse blocking solution (MOM; PK-2200, Vector Laboratories) with 5% goat serum and 2% bovine serum albumin, and then treated with MOM diluent. Sections were then incubated with anti-NLRR1 (TB776, affinity purified, MBL) and anti-Ki-67 (mouse monoclonal 556003, BD) antibodies. MOM anti-mouse IgG and rabbit Alexa 488 were used as secondary antibodies followed by Fluorescein Avidin CY3 (Vector Laboratories). DAPI was used to stain the nuclei.

Four mm thick paraffin tissue sections of the mock and NLRR1 tumors were subjected to immunohistochemistry (IHC). After deparaffinization antigen retrieval was performed by boiling with 0.1 M citrate buffer (pH 6.0) using microwave at 800 W for 10 min. The primary antibody for NMYC (Ab-1, Oncogene), p-ERK (4376, Cell signaling), and ERK (4695, Cell signaling) was used at 1:100 dilutions followed by the standard protocol of Cell signaling. Secondary biotinylated universal antibody from Vector Laboratories was applied at a dilution of 1:400. Reactivity was visualized with an avidin-biotin complex immunoperoxidase system using diaminobenzidine as the chromagen and Hematoxylin as the counterstain (Vector Laboratories).

Results

NLRR1 enhances EGF/IGF-mediated cell proliferation in neuroblastoma cells

Our previous report showed that NLRR1 is a direct transcriptional target of MYCN in NB, and is associated with cell proliferation and survival (14). However, the mechanism by which NLRR1 regulates cell proliferation was still unknown. Another member of leucine rich repeat protein family, NLRR3, has been associated with the activation of ERK (15), and EGFR activation was reported to accelerate NB cell proliferation (16), suggesting the possibility that NLRR1 might activate EGFR to induce cell proliferation. To test this hypothesis, we first investigated NB cell proliferation upon EGF and IGF-I treatment. Consistent with their proliferative role (16,17), both EGF and IGF-I also accelerated the proliferation of SK-N-BE cells (Fig. 1A). Similar data were also observed in NB SH-SY5Y cells (data not shown). To determine whether NLRR1 promotes EGF- and IGF-1-mediated cell proliferation, we transiently transfected SK-N-BE cells with NLRR1 expression plasmid and treated them with EGF and IGF-I. Interestingly, ectopic expression of NLRR1 enhanced the cell proliferation mediated by EGF and IGF-I (Fig. 1B). Overexpression of NLRR1 in SH-SY5Y showed similar promotion of proliferation (data not shown). To confirm the role of NLRR1, we used an RNA interference approach to knock down endogenous NLRR1. The data suggested that knockdown of NLRR1 suppressed the EGF- and IGF-1-mediated cell proliferation (Fig. 1C). Collectively, the data suggested that NLRR1 enhances cell proliferation mediated by EGF and IGF-I.

NLRR1 enhances EGF and IGF-mediated activation of ERK

Because ERK is an important kinase that is often regulated by EGF and IGF-1 growth factors to induce cell proliferation, we were interested to determine whether NLRR1 affected ERK phosphorylation upon EGF and IGF-I treatment. We examined the activation of ERK in NLRR1-overexpressing cells. Interestingly, ERK phosphorylation was enhanced compared with the mock-transfected cells (Fig. 2A). The increased phosphorylation of EGFR was observed in the NLRR1 over-expressing SK-N-BE cells upon EGF
stimulation (Fig. 2A). Similar data was also observed in SH-SY5Y cells (data not shown). We also employed siRNA studies to further elucidate the role of NLRR1 in EGF and IGF-1 signaling. Consistent with the overexpression study, we observed a reduction in ERK phosphorylation in NLRR1 knockdown cells upon both EGF and IGF-1 treatment compared with the control siRNA-transfected cells (Fig. 2B). Interestingly, phosphorylation of EGFR and IGFR was found to be decreased in NLRR1 knockdown cells (Fig. 2B). To elucidate whether the activation of ERK is important for cell proliferation, we employed the MEK1/2-specific inhibitor, U0126 (27), in the cell proliferation assays. For the cell proliferation assays, we have used the minimum concentration of U0126 (5 µM) required to inhibit ERK activation in the cells (Fig. S1). The data showed that EGF- and IGF-I-mediated proliferation of NLRR1-overexpressing SK-N-BE cells was inhibited upon U0126 treatment (Fig. 2C).

**EGF stimulation induces MYCN via ERK**

IGF-I stimulation induces endogenous MYCN expression in NB cells via MAPK activation (17). Therefore, it is possible that EGF can induce MYCN because EGF treatment also activates MAPK. To investigate this hypothesis, we treated SK-N-BE cells cultured in serum-free medium with increasing amounts of EGF. Consistent with our hypothesis, both mRNA and protein levels of MYCN were induced upon EGF treatment (Fig. 3A). Time course experiments were also employed to confirm that EGF can induce MYCN. MYCN was found to be induced at 6 h after EGF treatment (Fig. 3B). Under our experimental conditions, EGF treatment successfully phosphorylated EGFR (Supplementary Fig. S1) and ERK (data not shown). We also observed that EGF stimulation induced MYCN expression in other NB cell lines, SH-SY5Y and NLF (data not shown). To verify our experimental data that EGF induces MYCN, we used VEGF as positive control, which was reported to be induced by EGF (28). AG1478 is a specific inhibitor for EGFR, and is sufficient to block EGFR-mediated activation of ERK (29,30). We have confirmed the effect of the optimum concentration of AG1478 to inhibit EGF-mediated phosphorylation of EGFR in the cells (Fig. S1). Therefore, we used the optimum concentrations of AG1478 to elucidate that EGF-mediated MYCN induction is dependent on EGFR-ERK signaling. Consistently, MYCN induction by EGF was successfully blocked in AG1478-pretreated SK-N-BE cells (Fig. 3C left). We also used U0126 to prove that EGF-mediated MYCN induction is dependent on ERK (Fig. 3C, right panel). These data collectively suggest that EGF-mediated MYCN induction is dependent on the EGFR-ERK pathway in NB cells.

**EGF enhances MYCN transcription via recruitment of Sp1 to the MYCN promoter**

To confirm the EGF-mediated MYCN transcription, we generated a luciferase reporter plasmid containing a MYCN genomic fragment spanning positions -221 to +21 (Fig. 4A, left panel), where +1 represents the transcriptional initiation site. This promoter region contains both Sp1 and E2F1 transcriptional element sites (31, 32). We also used the empty control vector pGL3basic to compare the EGF responses. SK-N-BE cells were transiently transfected with pGL3basic and pGL3-MYCN(-221,+21) together with Renilla luciferase reporter plasmid. The data demonstrated that EGF stimulation significantly enhanced the promoter activity of MYCN gene at 6 h (Fig. 4A, right panel): pGL3-Basic reporter constructs did not respond to EGF treatment (Fig. 4A, right), suggesting that EGF treatment is specific to the MYCN promoter. MYCN has been reported to be transcriptionally regulated by two major transcription factors, Sp1 and E2F1 (31,32). To determine the regulatory mechanism of EGF-mediated MYCN transcription, we performed ChIP assays. Both antibodies against Sp1 and E2F1 used for ChIP assays pulled down the specific endogenous
proteins (data not shown). Twelve hours after EGF stimulation, chromatin DNA from SK-N-BE cells was cross-linked and processed for ChIP assays. Sp1-derived pulled-down chromatin was amplified by a specific primer targeting the MYCN core promoter region (-221 to +21) in the EGF-treated cells (Fig. 4B top panel), suggesting that EGF enhances recruitment of Sp1 to the MYCN promoter. However, no change in E2F1 recruitment was observed between the EGF-treated and untreated cells (Fig. 4B, bottom panel).

To identify the critical Sp1 binding region required for the transactivation of MYCN, we mutated the Sp1 binding elements (Fig. 4C, left panel) by PCR reactions, as described in the Materials and Methods section. SK-N-BE cells were transiently transfected with wild type MYCN promoter as well as the mutated luciferase constructs. Promoter assays showed that the pGL3MYCN (mut-1) construct had significantly ($P < 0.001$) less promoter activity (Fig. 4C, middle panel) than the wild type and pGL3MYCN (mut-2) constructs. Furthermore, we transiently transfected the wild type, as well as the Sp1 site 1-deleted constructs, into the SK-N-BE cells to determine the effects on EGF. The data showed that the deletion construct failed to respond to EGF treatment (Fig. 4C, right panel), suggesting that the Sp1 binding element 1 is important for EGF-mediated transactivation of MYCN.

To check whether Sp1 is critical for the expression of MYCN, we knocked down Sp1 using siRNAs. Consistently, MYCN expression was suppressed in cells transfected with siRNAs against Sp1 (Supplementary Fig. S2A). Both siRNAs against Sp1 reduced MYCN promoter activity (Supplementary Fig. S2B). Cell proliferation was also suppressed in the Sp1 knockdown cells (Supplementary Fig. S2C). It has been reported that EGF stimulation induces phosphorylation of ERK, and that this phosphorylation event might be important for Sp1 phosphorylation (33) and recruitment to the target gene promoter. Similarly, our results also showed that EGF treatment resulted in phosphorylation of Sp1, and that this phosphorylation event was inhibited in U0126- and calf intestinal phosphatase (CIAP)-treated cells (Supplementary Fig. S3A). CIAP treatment is reported to block recruitment of phospho-transcriptional factors on genomic DNA (35). We also used Mitramycin-A (Mit-A), a well-known Sp1 inhibitor (34). EGF-mediated MYCN induction was successfully inhibited in cells pretreated with Mit-A (Supplementary Fig. S3B) and CIAP (Supplementary Fig. S3C). Consistently, CIAP treatment also reduced the recruitment of Sp1 to the MYCN promoter (Supplementary Fig. S3D). Collectively, our results suggest that Sp1 recruitment to the MYCN promoter can enhance the transactivation of MYCN upon EGF treatment.

**NLRR1 enhances MYCN induction**

ERK is reported to induce phosphorylation of Sp1 (33). In our present findings, NLRR1 promotes phosphorylation of ERK upon EGF treatment, suggesting that NLRR1 might induce MYCN. Therefore, we overexpressed NLRR1 in SK-N-BE cells and found that endogenous MYCN was effectively induced in a dose-dependent manner (Fig. 5A, left panel). NLRR1-mediated MYCN induction was inhibited in U0126- and AG1478-pretreated cells (Fig. 5A, right panel), suggesting that NLRR1 induced MYCN via EGFR-ERK signaling. Ectopic expression of NLRR1 in cells accelerated MYCN induction upon EGF treatment compared with that of mock-transfected (empty pcDNA3.1 vector) cells (Fig. 5B, left panel). Consistent with the overexpression study, knockdown of NLRR1 suppressed MYCN induction, suggesting that NLRR1 accelerates EGF-mediated MYCN induction. To determine whether NLRR1 enhances Sp1 recruitment, we performed ChIP assays. The data shows that overexpression of NLRR1 in SK-N-BE cells increased Sp1 recruitment to the MYCN promoter, which was further accelerated upon EGF treatment (Fig. 5C).

**Stable expression of NLRR1 in cells accelerates tumor growth in nude mice**

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NLRR1 is highly expressed in aggressive MYCN-amplified NB (14), suggesting the possibility that NLRR1 might have a potent tumorigenic role. NLRR1 overexpression enhanced the colony formation ability of SH-SY5Y cells (Supplementary Fig. S4A). Consistent with our previous observation that ectopic expression of NLRR1 enhanced cell proliferation and inhibited apoptosis, NLRR1-stably expressing SH-SY5Y clones proliferated faster than mock stable clones (Supplementary Fig. S4C). Furthermore, MYCN expression was upregulated in NLRR1 stable clones compared with mock clones (Supplementary Fig. S4B), suggesting that NLRR1 has oncogenic potential. To elucidate the tumorigenic activity of NLRR1, we performed tumor xenograft studies in nude mice using NLRR1-stably expressing SH-SY5Y clones. Significant enhancement of tumor growth was observed in mice bearing NLRR1-expressing xenografts compared with the mock-expressing xenografts (P < 0.01; Fig. 6A). Mice of each group were sacrificed when they become morbid, and the survival curve was analyzed using the Kaplan-Meier method. The survival of mice with NLRR1-expressing xenografts was significantly shorter compared with mice bearing mock xenografts (P = 0.003; Fig. 6B). Immunohistochemical data showed that NLRR1-expressing tumors had increased numbers of Ki-67-positive cells and decreased number of TUNEL positive cells compared with mock tumors (Fig. 6C), indicating that there were more proliferative cells in the tumors derived from NLRR1-expressing clones. To see the consistence in vitro finding of NMYC induction by NLRR1, we performed IHC assays using the tumor xenografts. Induction of NMYC and p-ERK was found in NLRR1 tumors whereas total ERK was unchanged in both tumors (Fig. 6D). Collectively, our data suggest that NLRR1 induces NMYC in vivo and has potent tumorigenic roles.

Discussion

Upregulated MKK-ERK signaling is known to be involved in the genesis of several cancers (18-21). Multidrug-resistant human NB cells have 3- to 30-fold more cell surface EGFRs than the drug-sensitive parental cells (36), indicating that EGFRs might play an important role in the aggressiveness of human NB. EGF stimuli activated MAPK in NB (16) and IGF-1 enhanced NB cell proliferation via activation of MAPK (17). NLRR family proteins are considered to modulate cellular signaling, especially that of MAPKs (15). Although it was reported that overexpression of NLRR1 enhances cell proliferation and inhibits cellular apoptosis in NB, our previous study did not rule out the involvement of NLRR1 with EGF and IGF-1 signaling. Here, we first report that ectopic expression of NLRR1 also enhanced EGF- and IGF-1-mediated cellular proliferation, which is inhibited in cells treated with MEK1/2 inhibitor. This suggests that NLRR1-mediated promotion of proliferation is at least in part dependent on activation of ERK. However, the method by which NLRR1 enhances EGFR and IGFR signaling still needs to be clarified. Another NLRR family protein, NLRR3, was reported to induce phosphorylation of ERK in response to EGF (15), suggesting the possibility that NLRR1 which is 54% homology to NLRR3 might also induce ERK phosphorylation. Our results propose that NLRR1 is important in enhancing ERK phosphorylation in cells upon EGF and IGF stimuli. Interestingly, NLRR1 affects p-ERK and p-AKT in different extent (Fig. 2B) which depends on cell lines (data not shown). The reason behind this selectivity is unknown and need to be addressed further in the future.

ERK is known to phosphorylate many transcriptional factors, including Sp1 (33,37). Moreover, Sp1 and E2F1 are reported to transcriptionally regulate MYCN in NB (31,32). Therefore, there might be an important link between the activation of ERK and MYCN induction, which is further supported by the evidence that IGF-1 can induce MYCN via activation of MAPK (17). However, in this report of EGF-1-mediated MYCN induction, the involvement of Sp1 or E2F1 was not explained. Here, we report that EGF
induces MYCN via the EGFR-ERK pathway. Several stimuli, including retinoic acid and transforming growth factor (TGF-β) repress the MYCN gene, and are associated with recruited Sp1 and E2F1 and their other co-factors (31, 38). By using ChIP assays, we observed that Sp1 but not E2F1 was recruited to the MYCN promoter in response to EGF. However, it has been reported that the presence of Sp1 is not always sufficient to activate a transcriptionally silent MYCN gene (32). Therefore, we have further investigated whether Sp1 is important for MYCN induction in our experimental conditions. siRNA-mediated knockdown of Sp1 reduced MYCN expression and also inhibited cell proliferation, suggesting that Sp1-mediated MYCN induction regulates cell proliferation. Using luciferase reporter assays, we identified the responsive Sp1 binding element in the promoter region for MYCN induction. Sp1 phosphorylation is a prerequisite for the interaction with genomic DNA (33). Therefore, we also investigated whether EGF induces Sp1 phosphorylation and enhances its recruitment to the MYCN promoter. Western blotting data showed that EGF treatment enhanced phosphorylation of Sp1, and the MEK1/2 inhibitor U0126 inhibited this phosphorylation event, supporting the previous report that ERK induces Sp1 phosphorylation (33). Using the dephosphorylating agent CIAP, we further confirmed that phospho-Sp1 is involved in the transactivation of MYCN by recruitment to the genomic DNA of the MYCN promoter. Collectively, this evidence suggests that EGF induces MYCN through phospho-Sp1 recruitment to the MYCN promoter. However, MYCN induction by IGF-1 was not investigated in the present study, but we speculate that it might be by the same mechanism as EGF because IGF-1 has been reported to activate MAPK in NB.

Another new finding of our current study is that MYCN is induced after ectopic expression of NLRR1 in NB cells. Under our experimental conditions, EGF-mediated MYCN induction was accelerated in NLRR1-overexpressing cells and inhibited in NLRR1-knockdown cells, suggesting that NLRR1 enhances MYCN induction and activation of ERK. Additionally, NLRR1 overexpression enhanced Sp1 recruitment to the MYCN promoter. We previously reported that NLRR1 transcriptionally regulated by MYCN is highly expressed in aggressive NB (14). NLRR1 was also found to be highly expressed in MYCN-amplified tumors compared with that of MYCN non-amplified NB. Therefore, we suggest that MYCN directly regulates NLRR1, and that NLRR1 further induces MYCN through the activation of the EGFR-ERK cascade, suggesting a positive feedback loop between NLRR1 and MYCN that might lead to aggressive NB. To this notion, we further determined the in vivo tumorigenic activity of NLRR1 in MYCN non-amplified SH-SY5Y cells, which are well-known to form tumors in nude mice (39,40). Our results show that SH-SY5Y cells stably-expressing NLRR1 rapidly proliferate in culture medium and in nude mice, and promote decreased survival rates among inoculated mice, presumably by induced endogenous MYCN.

In conclusion, our results provide significant evidence that NLRR1 enhances ERK signaling to induce MYCN, which might play major roles in the progression of aggressive NB. Taking note that MYCN-highly expressing tumors are often resistant to anti-tumor therapies, new drug discovery blocking NLRR1-mediated ERK signaling to control MYCN expression might be an attractive choice in treating aggressive NB.

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Figure Legends

Figure 1. **NLRR1 promotes EGF/IGF-mediated cell proliferation.** A, Quantification of SK-N-BE cell proliferation following EGF (left) and IGF (right) stimulation for the indicated time periods using WST-8 assays. The data are represented as mean ± SD. B, Ectopic expression of NLRR1 in SK-N-BE cells was confirmed by immunoblotting (upper panel). NLRR1-expressing SK-N-BE cells were treated with EGF (left) and IGF (right) for the indicated time periods, and proliferation was measured by WST-8 assays. C, NLRR1 knockdown by transfection with siRNA was confirmed by immunoblotting (upper panel). Growth curve of SK-N-BE cells transfected with control siRNA and siRNA against NLRR1 in the presence of EGF (left) and IGF (right) was measured by WST-8 assays. For all WST-8 assays (A, B and C), cells were cultured in 2% serum-containing medium, and growth factors were used at a concentration of 50ng/mL.

Figure 2. **NLRR1 enhances EGF and IGF-mediated phosphorylation of ERK.** A, SK-N-BE cells were transiently transfected with mock (pCDNA3) and NLRR1-expressing plasmid. Forty-eight hours after transfection, cells were starved with serum-free medium for 12 h and then stimulated with EGF and IGF for 10 min. Whole cell lysates was used for immunoblotting with specific antibodies (lower panel). NLRR1 overexpression was confirmed by immunoblotting of the same lysates (upper panel). B, siRNA-mediated knockdown of NLRR1 suppresses phosphorylation of ERK mediated by EGF and IGF (lower panel). The experimental conditions are similar to those in panel A. Knockdown efficiency was confirmed by immunoblotting (upper panel). C, SK-N-BE cells transfected with NLRR1-expressing plasmids were pretreated with MEK1/2-specific inhibitor (U0126) and then cultured in the presence or absence of EGF and IGF; cell proliferation was measured by WST-8 assays.

Figure 3. **EGF stimulation induces endogenous MYCN.** A, Serum-starved SK-N-BE cells were treated with increasing doses of EGF for 12 h. Total mRNA was used in RT-PCR to check the expression of MYCN and VEGF (upper panel). GAPDH was checked as an internal control. Whole cell lysates were used in immunoblotting to detect the proteins (lower panel). B, MYCN was induced by EGF in a time-dependent manner. Serum-starved SK-N-BE cells were treated with 50 ng/mL of EGF. Total RNA and whole cell lysates were collected at the indicated time points for RT-PCR (upper panel) and western blotting (lower panel) assays to check the expression level of MYCN. C, SK-N-BE cells were cultured in serum-free medium for 12 h with or without different concentrations of AG1478 (left) and U0126 (right). Cells were then treated with EGF (50 ng/mL) for 12 h, and whole cell lysates were used to check MYCN protein levels by immunoblotting. Actin was checked as an internal control.

Figure 4. **EGF enhances Sp1-mediated transactivation of MYCN.** A, MYCN core promoter region (-221 to +21) cloned in pGL3Basic luciferase vector (left) was transiently transfected into SK-N-BE cells for 36 h, followed by culture in serum-free medium for 12 h. Cells were then treated with EGF (50 ng/mL) for the indicated time periods, and luciferase assays were performed to quantify the relative promoter activity. B, SK-N-BE cells were cultured in serum-free medium for 12 h, followed by treatment for 12 h with EGF (50 ng/mL). Cross-linked chromatin was isolated from the cells and precipitated with Sp1- and E2F1-specific antibodies or with normal rabbit serum (NRS). C, Sp1 site 1-deleted MYCN core promoter failed to respond to EGF stimulation. Two Sp1 binding
sites on the MYCN core promoter region were deleted by site-directed mutagenesis (left). Relative activity of the deleted promoter constructs were measured by luciferase assays in SK-N-BE cells 24 h after transfection (middle). SK-N-BE cells were transiently transfected by the two Sp1 site deleted constructs, followed by culture in serum-free medium for 12 h and then treatment with EGF (50 ng/mL). Twelve hours after EGF treatment, firefly luciferase activities were determined (right). Renilla luciferase was used as an internal control to standardize the transfection efficiency of the luciferase vectors.

Figure 5. NLRR1 induces MYCN in NB cells. A, SK-N-BE cells were transiently transfected with increasing amounts of NLRR1-expressing plasmids. Forty-eight hours after transfection, immunoblotting was performed to detect NLRR1, p-ERK, total ERK and MYCN expression (left). EGFR and ERK inhibitors prevent MYCN induction in the NLRR1-overexpressing cells. SK-N-BE cells were transiently transfected with NLRR1 expression plasmids for 48 h and then treated with or without AG1478 (20 μM) and U0126 (10 μM). Twelve hours after treatment, whole cell lysates were prepared. Immunoblotting data shows the expression of NLRR1, p-ERK, total ERK and MYCN (right). B, NLRR1 enhances MYCN induction upon EGF treatment. Twenty-four hours after ectopic expression of NLRR1, SK-N-BE cells were cultured in serum-free medium for 12 h followed by EGF (10 ng/mL) treatment. RT-PCR was performed to check endogenous MYCN expression (left). Forty hours after transfection with control siRNA and NLRR1-siRNA, SK-N-BE cells were serum-starved for 12 h and treated with EGF (10 ng/mL). Twelve hours after EGF treatment, RT-PCR was performed to check the expression of NLRR1 and MYCN (right). C, Crossed-linked chromatin from the SK-N-BE mock- and NLRR1-ectopically expressing cells treated with or without EGF (50 ng/mL) for 12 h was used for pulldown by Sp1-specific antibody. Primers targeting MYCN core promoter (-221, +21) were used to amplify the pulled-down chromatin (left). The recruitment of Sp1 was quantified from the PCR band by image J software and plotted (right). The data represent mean ± SD.

Figure 6. Neuroblastoma cells stably-expressing NLRR1 accelerate tumor growth in nude mice. A, After subcutaneous injection of mock- and NLRR1-stably expressing SH-SY5Y clones into nude mice, tumor volumes were measured on the indicated days. The data represents the mean value. Upper panel shows representative pictures of tumors 70 days after injection. B, Kaplan Meyer survival curve of mice bearing mock- and NLRR1-stably expressing SH-SY5Y xenografts. C, Detection of NLRR1 and Ki-67 expression in the mock- and NLRR1-expressing tumor xenografts (upper panel). TUNEL staining shows that NLRR1 tumor has reduced apoptotic cells (lower panel). D) IHC data shows the staining image of NMYC, p-ERK and ERK in NLRR1 vs control tumors.
Hossain et al., Figure 1

A

- EGF(-)
- EGF(+)

Absorbance vs. Time (days)

B

- Mock
- NLRR1

- NLRR1
- Actin

- EGF

P<0.01

Absorbance vs. Time (days)

C

- si-control
- si-NLRR1

- NLRR1
- Actin

- EGF

P<0.01

Absorbance vs. Time (days)
Hossain et al., Figure 2

A

Mock (pcDNA3) or NLRR1

pcDNA3 or NLRR1

EGF or IGF

EGF

IGF

- EGF - IGF

+ EGF + IGF

p-EGFR

EGFR

p-ERK

ERK

B

control siRNA or siNLRR1

control siRNA or siNLRR1

p-EGFR

p-IGFIR

p-ERK

p-Akt

C

Relative number of cells

control

U0126 (5 µM)

EGF

- +

IGF

- +

Relative number of cells

control

U0126 (5 µM)
Hossain et al., Figure 3

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Hossain et al., Figure 4

**Panel A**

-221  E2F1  Sp1  Sp1  E2F1

+21  Luc.

Fold activation

0  10  20  30  40  50  60  70

P<0.01

0 hr  1 hr  6 hr  12 hr

pGL3-Basic

**Panel B**

-  +

EGF

IP: α-Sp1

IP: NRS

Input

IP: α-E2F1

IP: NRS

Input

**Panel C**

-221  E2F1  Sp1 (site 1)  Sp1 (site 2)  E2F1

+21  Luc.

Fold activation

0  1  2  3  4  5  6  7

Wt

Mut-2

Mut-1

pGL3-Basic

Mut-2

Mut-2+EGF

Mut-1

Mut-1+EGF

Fold activation

2  4  6  8  10  12

P < 0.001

P < 0.001
Hossain et al., Figure 5

A

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EGF -- + +

Mock NLRR1

Relative recruit of Sp1

Input

IP: α-Sp1

IP: NRS

0  10  20  30  40

on April 13, 2017. © 2012 American Association for Cancer Research.
Hossain et al., Figure 6

A

Mock NLRR1

NLRR1 #1
NLRR1 #2
NLRR1 #3

Mock #1
Mock #2
Mock #3

Tumor volume (mm$^3$)

Days after injection

B

Survival percentage

$P = 0.0034$

Mock (n=21)
NLRR1 (n=21)

C

Control NLRR1

ki67

Relative expression

D

Control NLRR1

NMyc

p-ERK

ERK

TUNEL

Relative expression

Control NLRR1
NLRR1 enhances EGF-mediated MYCN induction in neuroblastoma and accelerates tumor growth in vivo

Shamim Hossain, Atsushi Takatori, Yusuke Suenaga, et al.

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