

NRP1 knockdown promotes the migration and invasion of human neuroblastoma-derived SK-N-AS cells via the activation of β 1 integrin expression

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Abstract. Neuropilin 1 (NRP1) is a transmembrane glycoprotein, which regulates many aspects of cellular function by functioning as co-receptor of various ligands. Recent studies have suggested that NRP1 promotes tumorigenesis, not only by activating the growth of tumor vessels, but also by activating the growth or migration of tumor cells themselves. The present study was performed to elucidate the roles of NRP1 in the development and/or progression of neuroblastoma (NB). In contrast to previous observations in various types of cancer, the analysis of public datasets indicated that lower levels of NRP1 expression were significantly associated with a shorter survival period of patients with NB. Consistent with this finding, wound-healing assay and Matrigel invasion assay revealed that NB cells in which NRP1 was knocked down exhibited increased migratory and invasive abilities. Further analyses indicated that β 1 integrin expression was markedly increased in NB cells in which NRP1 was knocked down, and NB cells in which β 1 integrin was knocked down exhibited decreased migratory and invasive abilities. The results presented herein indicate that NRP1 exerts tumor suppressive effects in NB, at least in part by regulating the expression of β 1 integrin.

Introduction

Neuroblastoma (NB) arising from neural crest cells within the sympathetic nervous system is the most common extracranial

solid malignant tumor in childhood (1). This tumor generally occurs in young children, with a median age of 17 months at diagnosis, and it accounts for 15% of all pediatric oncological deaths (1,2). NB exhibits marked heterogeneity as regards biological characteristics and clinical features. For example, NBs occurring in patients younger than 12 months of age usually regress or mature into a benign ganglioneuroma spontaneously, while the majority of cases are associated with an aggressive phenotype and a poor prognosis when they occur in patients at 18 months or older. Although marked improvements have been made for patients with lower-grade NBs, the 5-year survival rate of patients with high-risk NB remains <40% (1).

A number of genetic aberrations in NBs, such as aneuploidy, the amplification of oncogenes or allelic loss and mutations, have been reported to be associated with clinical outcome (3). Among these, the amplification of the proto-oncogene MYCN is one of the few predictive markers of a poor prognosis (4). NBs with MYCN amplification exhibit an aggressive phenotype and resistance to chemotherapy, and patients with NB harboring the amplification are classified as a high-risk group. In addition to the MYCN aberration, the gain of chromosome 17q (5) and the deletion of chromosome 1p or 11q (6) have also been shown to be associated with a poor prognosis of patients with NB. Gain-of-function mutations in the anaplastic lymphoma kinase (ALK) gene have been observed in most cases of familial NB and in some sporadic NB cases (7). Nevertheless, the above-mentioned genomic abnormalities are lacking in a significant number of malignant NBs.

Neuropilin 1 (NRP1) is a transmembrane glycoprotein known to function as a co-receptor for many types of ligand, including semaphorin 3A and 4A (SEMA3A, SEMA4A) (3,8) and vascular endothelial growth factor (VEGF) (9). As NRP1 has the ability to modulate the activity of a number of extracellular ligands, it is involved in a wide range of physiological and pathological processes.

Numerous studies have demonstrated that NRP1 is frequently overexpressed in a variety of tumors, such as leukemia (10), gastric cancer (11), hepatocellular carcinoma (12) and osteosarcoma (13). In addition, an elevated NRP1 expression is generally

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associated with a poor prognosis in many types of tumor (12-15). For example, a high NRP1 expression level has been reported to be associated with an advanced stage and lymph node invasion in pancreatic cancer (14).

The ability of NRP1 to enhance VEGF receptor 2 activity in response to VEGF-A suggests that one of the most important roles of NRP1 in tumor development is its role in angiogenesis (16). NRP1 has been found to be expressed in blood vessels in >98% of carcinomas derived from the breast, colon and lung (17). Based on these observations, NRP1 has been identified as a potential target for anti-angiogenic therapies. In addition to tumor vessels, NRP1 is known to be expressed in a variety of cancer tissues (17), and recent studies have indicated that NRP1 regulates tumor cell functions in an angiogenesis-independent manner. A previous study using an esophageal cancer cell line indicated that NRP1 activates cell proliferation by inducing p65 transcription via CREB activation (15). It has also been reported that the knockdown of NRP1 in gastric cancer cells results in cell cycle arrest caused by p27 upregulation, and in a reduced cell migratory ability via the inhibition of focal adhesion kinase phosphorylation (11).

However, the role of NRP1 in NB has not yet been elucidated. Although it has been reported that NRP1 is expressed at higher levels in NB tissues compared to normal adrenal tissues, it has also been reported that the expression levels of NRP1 are higher in early-stage than in late-stage NB (18). Consistent with this observation, in this study, the investigation of public datasets of global gene expression analysis obtained from the R2 platform (<http://r2.amc.nl>), indicated that a higher level of NRP1 expression was closely associated with a longer survival period of patients with NB (Fig. 1). These results suggest that NRP1 may function to suppress the malignant progression of NB.

In the present study, we performed a functional analysis of NRP1 to determine its role in the development and/or progression of NB, and to elucidate the molecular mechanisms underlying its functions.

Materials and methods

Cell lines and culture conditions. The following human NB-derived cell lines were used in this study: The SK-N-SH (HTB-11), SK-N-AS (CRL-2137) and SH-SY5Y (CRL-2266) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), NB69 cells (RCB0480) were from Riken Cell Bank (Ibaraki, Japan) and Kelly cells (EC92110411-F0) were from DS Pharma Biomedical (Osaka, Japan). The Kelly and NB69 cells were cultured in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with heat-inactivated fetal bovine serum (FBS; Nichirei Bioscience, Tokyo, Japan) at a final concentration of 10% (Kelly) or 15% (NB69). The SK-N-SH and SH-SY5Y cells were cultured in MEM supplemented with 10% FBS, 0.1 mM non-essential amino acids (Thermo Fisher Scientific, Waltham, MA, USA) and 5 mM sodium pyruvate (Thermo Fisher Scientific). The SK-N-AS cells were cultured in DMEM (Nacalai Tesque) supplemented with 10% FBS. All the media contained 100 IU/ml of penicillin (Life Technologies, Carlsbad, CA, USA) and 100 μ l/ml of streptomycin (Life Technologies). The cells were maintained at 37°C in a CO₂ incubator with a

controlled humidified atmosphere composed of 95% air and 5% CO₂.

Analysis of cell viability. The SK-N-AS cells were seeded in 96-well plates at a density of 1x10⁴ cells per well, and immediately transfected with control siRNA, NRP1 siRNA, or with β 1 integrin siRNA (Thermo Fisher Scientific) using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. The target sequence of the NRP1 siRNA was 5'-AGCAAAGAAGGTTT-3' and that of the β 1 integrin siRNA was 5'-CCGTAGCAAAGGAACA GCA-3'. As a control siRNA, Silencer Select Negative Control #1, whose target sequence information is not available, was used. Cell viability was measured by WST8 assay using Cell Count Reagent CF (Nacalai Tesque) immediately after the cells were attached to the plate bottom, or at 24, 48 and 72 h following transfection.

Matrigel invasion assay. The SK-N-AS cells were seeded in dishes 6 cm in diameter at a density of 5x10⁵ cells per dish, and immediately transfected with control siRNA, NRP1 siRNA, or β 1 integrin siRNA, as described above. At 24 h after seeding, the cells were removed from the plate using trypsin, and seeded into cell culture inserts (Falcon, Durham, NC, USA) coated with human fibronectin (Sigma-Aldrich, St. Louis, MO, USA) at a density of 7.5x10⁴ cells/300 μ l, and the inserts were placed in 24-well plates for cell culture inserts (Falcon) filled with 700 μ l of medium/well. After 48 h, the non-invading cells on the upper surface of the membrane were removed, and the invading cells were fixed with methanol and stained with Giemsa (Muto Pure Medicals, Tokyo, Japan) for 1 min at room temperature, followed by washing with PBS. The number of invading cells in 5 microscopic fields was counted for each membrane under a light microscope at x200 magnification. All of the analyses were performed in triplicate.

Wound-healing cell migration assay. The SK-N-AS cells were seeded in 24-well plates at a density of 2x10⁵ cells per well, and immediately transfected with control siRNA, NRP1 siRNA, or β 1 integrin siRNA, as described above. At 48 h following transfection, cell layers were wounded using a Cell Scratcher Scratch stick (AGC Techno Glass, Shizuoka, Japan) and the medium was replaced with fresh medium. After 48 h, the cells were photographed by phase-contrast microscope Leica DM IL (Leica, Wetzlar, Germany), and the widths of the wounded areas were measured at 3 places in each sample. All of the analyses were performed in triplicate.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the cells using RNeasy mini kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. For cDNA synthesis, 500 ng of total RNA was reverse transcribed using an iScript cDNA synthesis system (Bio-Rad Laboratories, Hercules, CA, USA). qPCR was performed using a SYBR Premix Ex Taq™ system according to the manufacturer's recommendations (Takara, Shiga, Japan). A mixture of cDNA derived from total RNA of SK-N-AS cells was used as a reference. Subsequently, a dilution series of the cDNA mixture was prepared and used in qPCR as the templates to obtain a standard curve for each gene, and then

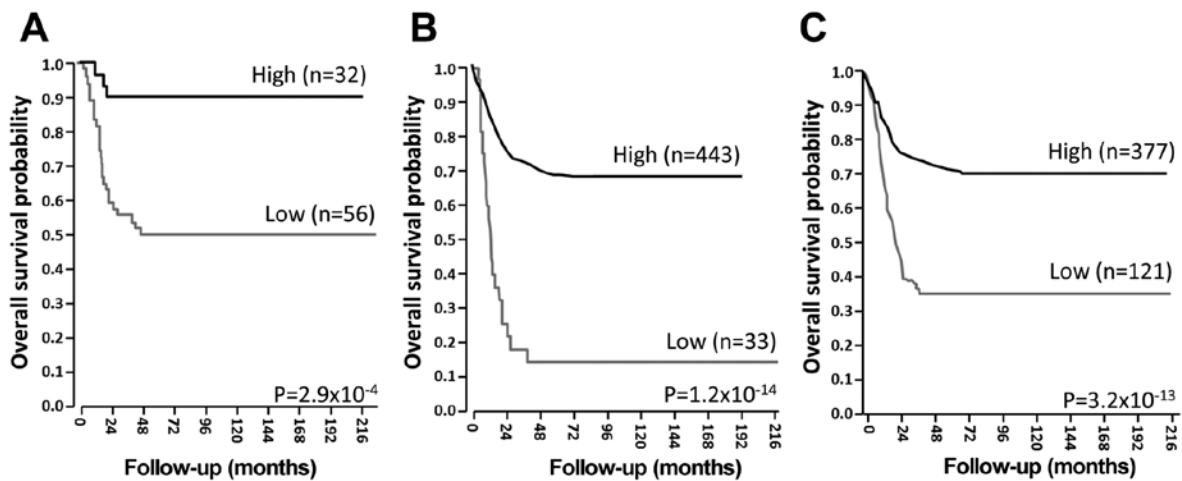


Figure 1. Elevated expression level of neuropilin 1 (*NRP1*) is associated with a favorable prognosis in patients with neuroblastoma. Kaplan-Meier survival analyses were performed based on 3 independent public microarray datasets: (A) GSE16476, (B) GSE45547, and (C) GSE49710.

the expression levels of each genes were estimated by extrapolation from a standard curves. Three independent measurements were taken. The primer sets used for qPCR-based amplification were as follows: *NRP1* sense, 5'-ATGCGAATGGCTGATT CAGG-3' and antisense, 5'-TCCATCGAAGACTTCCACG TAG-3'; β 1 integrin sense, 5'-CATCCCTGAAAGTCCCAA GTG-3' and antisense, 5'-TACCAACACGCCCTTCATTG-3'; and *GAPDH* sense, 5'-TCACCAGGGCTGCTTTAAC-3' and antisense, 5'-TGACGGTGCCATGGAATTTG-3'. The house-keeping gene *GAPDH* was used as an internal reference. All of the PCR reactions were carried out with an initial denaturation for 2 min at 94°C followed by 40 cycles of 94°C for 5 sec and 60°C for 30 sec using Thermal Cycler Dice TP800 (Takara).

Immunoblotting. The cells were lysed in RIPA buffer containing protease inhibitor cocktail (Nacalai Tesque) and phosphatase inhibitor cocktail (Nacalai Tesque). Following the sonication of the lysates, protein concentration was measured using Bio-Rad DC kits (Bio-Rad Laboratories). Cell lysates (20 μ g of protein) were separated by 4-12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted onto Immobilon-P membranes (Millipore, Billerica, MA, USA) by the wet transfer method. The membranes were then blocked with Blocking-one (Nacalai Tesque) overnight at 4°C, and incubated with rabbit monoclonal antibodies (Cell Signaling Technology, Beverly, MA, USA) to *NRP1* (D62C6; 3725P), vimentin (D21H3; 5741P), N-cadherin (D4R1H; 13116P), E-cadherin (24E10; 3195P), matrix metalloproteinase (MMP)2 (D8N9Y; 13132S), MMP9 (D603H; 13667S), β 1 integrin (D2E5; 9699S), focal adhesion kinase (FAK) (D2R2E; 13009S), FAK phosphorylated at Y397 (D20B1; 8556S), PI3K (p85) (19H8; 4257P), or with rabbit polyclonal antibody to phosphorylated PI3K (p85; 4228P) (all from Cell Signaling Technology), or with rabbit polyclonal antibody to *GAPDH* (ab9485, Abcam, Cambridge, UK) at 4°C. All the antibodies were diluted 500-fold for the reaction. After 24 h of incubation, the membranes were washed with Tris-buffered saline containing 0.1% Tween-20 (TBS-T), followed by incubation with 2,000-fold diluted horseradish peroxidase-conjugated secondary antibody for rabbit IgG (NA934-1ML,

GE Healthcare Life Sciences, Buckinghamshire, UK), for 1 h at room temperature. The membranes were then washed extensively with TBS-T, and treated with Chemi-Lumi-One Super (Nacalai Tesque) to visualize immunoreactive signals using LAS4000 (Fujifilm, Tokyo, Japan).

Analysis of the amount of filamentous actin (F-actin) and globular actin (G-actin). The SK-N-AS cells were seeded in dishes 10 cm in diameter at a density of 1×10^6 cells per dish, and immediately transfected with control siRNA or with *NRP1* siRNA, as described above. At 48 h after seeding, cell lysates were collected by using the G-actin/F-actin In Vivo Assay kit (Cytoskeleton, Denver, CO, USA), and G-actin and F-actin were separated by using an ultracentrifuge according to the manufacturer's instructions. In brief, cell lysates in lysis and F-actin stabilization buffer were centrifuged at $100,000 \times g$, 37°C for 1 h, and precipitated F-actin was dissolved in F-actin depolymerization buffer. As a positive control, phalloidin, which can drive actin polymerization, was added to the cell lysate. The amount of G-actin and F-actin was analyzed by immunoblotting.

Statistical analysis. Statistical analyses were performed using the Student's t-test. Data are presented as the means \pm SD from at least 3 independent experiments. In all analyses, a value of $P < 0.05$ was considered to indicate a statistically significant difference. To generate survival curves for the overall survival of patients with NB, 3 independent microarray datasets, GSE16476, GSE45547 and GSE49710, were obtained from the R2 platform (<http://r2.amc.nl>). Using this platform, survival curves were calculated according to the Kaplan-Meier method, and analyzed by the log-rank test followed by adjustment with Bonferroni's test.

Results

A lower *NRP1* expression level is closely associated with the poor prognosis of patients with NB. To examine the clinical significance of *NRP1* in the development and/or progression of NB, Kaplan-Meier survival analysis was performed

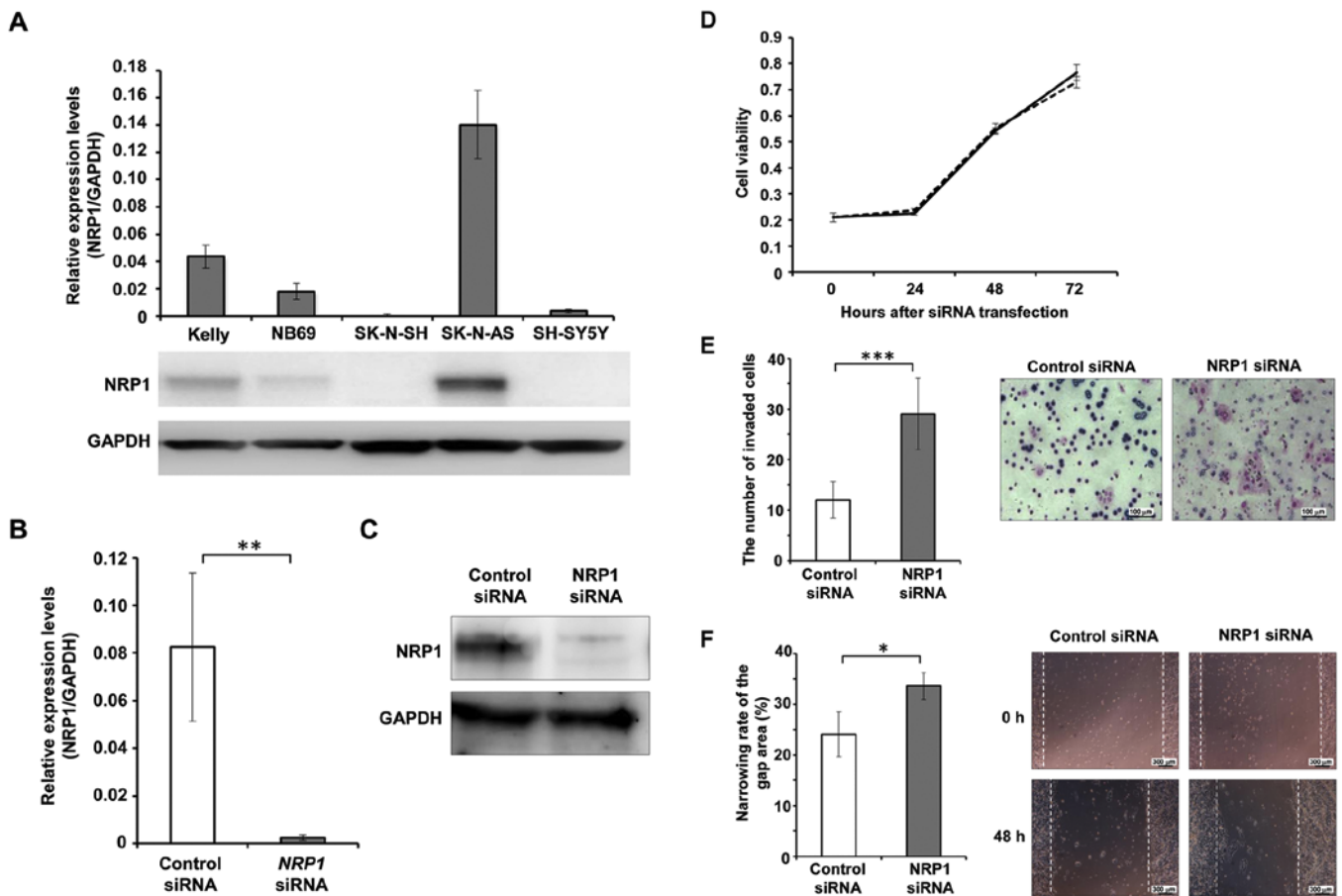


Figure 2. Knockdown of neuropilin 1 (*NRPI*) enhances the invasive and migratory ability of the SK-N-AS cells. (A) *NRPI* mRNA and protein expression levels in neuroblastoma (NB) cell lines. (B and C) SK-N-AS cells were transfected with *NRPI* siRNA or with control siRNA. At 48 h after transfection, total RNA and cell lysates were prepared and analyzed by (B) RT-qPCR and (C) immunoblotting. ** $P < 0.01$. (D) Cell viability was measured by WST8 assay at the indicated time-points following transfection with *NRPI* siRNA (solid line) or control siRNA (dashed line). (E) Matrigel invasion assay. SK-N-AS cells transfected siRNA were seeded on invasion chambers coated with fibronectin. After 48 h, the invading cells were fixed and stained, and the number of stained cells was counted as described in the Materials and methods. The means \pm SD from experiments performed in triplicate are presented. *** $P < 0.001$. Representative images of the membranes with invading cells are shown. (F) Wound-healing assay. At 48 h after transfection with siRNA, the cell layer was scratched, and the medium was replaced with the fresh medium. The wound closure ratio was calculated by the formula: (width at 0 h - width at 48 h)/width at 0 h. The means \pm SD from experiments performed in triplicate are presented. * $P < 0.05$. Representative images of the wounded area are shown.

utilizing public microarray datasets. As shown in Fig. 1, a lower NRPI expression level was significantly associated with a shorter survival period of patients with NB. The results were confirmed in all 3 independent datasets, suggesting that NRPI may have a suppressive effect on the malignant progression of NB.

The siRNA-mediated knockdown of NRPI enhances the invasive and migratory ability of NB cells. To clarify its function in NB cells, the expression levels of NRPI in NB-derived cell lines were analyzed. Among the 5 cell lines examined, the SK-N-AS cells exhibited the highest expression of NRPI at both the mRNA and protein level (Fig. 2A). Thus, we then performed the siRNA-mediated knockdown of NRPI in the SK-N-AS cells (Fig. 2B and C), and observed no significant differences in cell viability between the cells in which NRPI was knocked down and the control cells at 24, 48 and 72 h following transfection (Fig. 2D).

Matrigel invasion assay was also performed to evaluate the effects of NRPI depletion on cell invasive ability. The invasive ability of the cells in which NRPI was knocked down was

significantly higher than that of the control cells. At 24 h after cell seeding in the invasion chamber, the cells in which NRPI was knocked down exhibited significantly greater numbers of invading cells compared to the controls (Fig. 2E).

Wound-healing assay was performed to evaluate the effects of NRPI knockdown on cell migratory ability. At 48 h after scratching the cell layer, the wound closure ratio of the cells in which NRPI was knocked down was significantly greater than that in the control cells (Fig. 2F).

Expression of $\beta 1$ integrin is upregulated in cells in which NRPI is knocked down. To elucidate the mechanisms underlying the regulation of cell invasion and migration by NRPI, we examine the expression levels of molecules that can affect the invasive capacity and motility of the cells. First, we analyzed the proteins involved in epithelial-mesenchymal transition (EMT), which is a significant process for cancer cells to gain migratory and invasive abilities. However, the expression levels of EMT-related proteins, such as vimentin, N-cadherin and E-cadherin, were not altered by NRPI knockdown (Fig. 3A). We then analyzed the expression levels

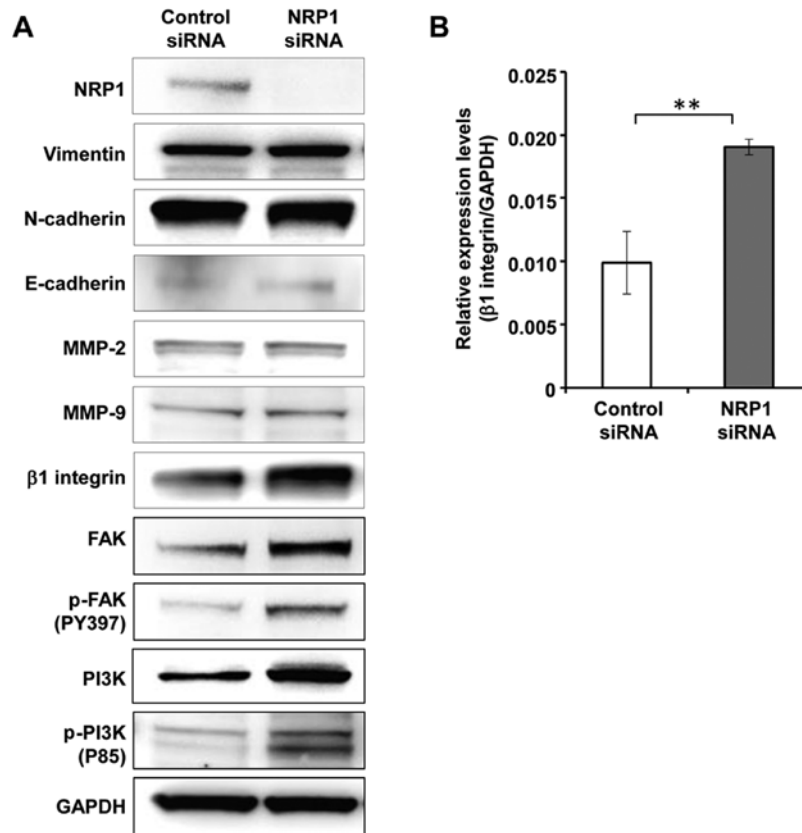


Figure 3. Knockdown of neuropilin 1 (*NRP1*) upregulates the expression level of $\beta 1$ integrin and activates its downstream signals. SK-N-AS cells were transfected with NRP1 siRNA or with control siRNA. At 48 h following transfection, cell lysates and total RNA were prepared and analyzed by (A) immunoblotting or (B) RT-qPCR. The means \pm SD from experiments performed in triplicate are presented. **P<0.01.

of MMPs, which are required for cells to digest extracellular matrix proteins. Again, no clear differences were observed in the amounts of MMP2 or MMP9 between the cells in which NRP1 was knocked down and the control cells (Fig. 3A). In contrast, $\beta 1$ integrin was strongly induced in NRP1 knock-down cells compared to control cells (Fig. 3A). To determine whether $\beta 1$ integrin expression is regulated by NRP1 at the transcriptional level, the mRNA expression level of $\beta 1$ integrin was measured by RT-qPCR. As shown in Fig. 3B, the mRNA expression level of $\beta 1$ integrin was significantly higher in the cells in which NRP1 was knocked down than in the control cells.

We also found that the levels of downstream molecules of $\beta 1$ integrin, such as FAK and PI3K, were increased and these molecules were activated in the cells in which NRP1 was knocked down (Fig. 3A). The analysis of the amounts of F-actin and G-actin demonstrated that the cells in which NRP1 was knocked down exhibited a markedly reduced F-actin formation compared to the control cells (Fig. 4). These results indicated that the induction of $\beta 1$ integrin mediated by NRP1 knockdown resulted in the alteration of actin fiber organization via the activation of its downstream signal.

The siRNA-mediated knockdown of beta1 integrin suppresses the invasive and migratory abilities of NB cells. To confirm that the integrin signal mediates the effects of NRP1 knockdown, we examined the phenotype of SK-N-AS cells after the silencing of $\beta 1$ integrin (Fig. 5A and B). No obvious differences were

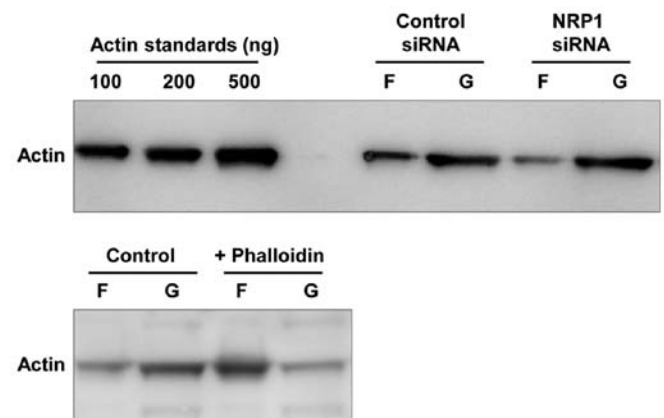


Figure 4. Cells in which neuropilin 1 (*NRP1*) is knocked down exhibit reduced amounts of F-actin. The SK-N-AS cells were transfected with *NRP1* siRNA or with control siRNA. At 48 h after transfection, cell lysates were prepared and analyzed by immunoblotting to detect F-actin and G-actin. As a positive control, extracts of the cells treated with phalloidin, which drives actin polymerization, were also analyzed (lower panel). F indicates F-actin, and G is for G-actin.

observed in cell viability between the cells in which $\beta 1$ integrin was knocked down and the control cells at any time-point after siRNA transfection (Fig. 5C). On the other hand, the cells in which $\beta 1$ integrin was knocked down exhibited a significantly decreased invasive ability compared to the control cells 24 h after cell seeding in the invasion chamber (Fig. 5D). The cell migratory ability was also shown to be suppressed in the cells

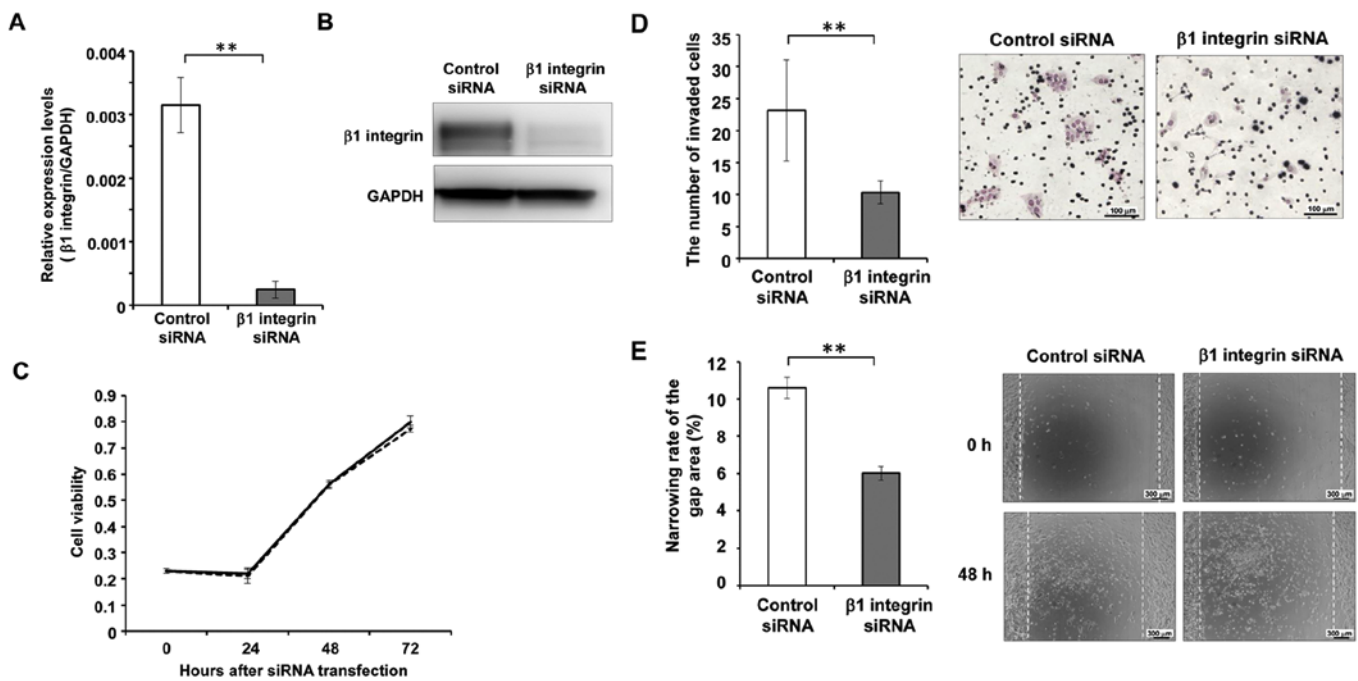


Figure 5. Knockdown of $\beta 1$ integrin suppresses the invasive and migratory ability of SK-N-AS cells. (A and B) SK-N-AS cells were transfected with $\beta 1$ integrin siRNA or with control siRNA. At 48 h after transfection, total RNA and cell lysates were prepared and analyzed by (A) RT-qPCR and (B) immunoblotting. $**P < 0.01$. (C) Cell viability was measured by WST8 assay at the indicated time-points following transfection with $\beta 1$ integrin siRNA (dashed line) or control siRNA (solid line). (D) Matrigel invasion assay. SK-N-AS cells transfected with siRNA were seeded on invasion chambers coated with fibronectin. After 48 h, the invading cells were fixed and stained, and then the number of stained cells was counted as described in the Materials and methods. The means \pm SD from experiments performed in triplicate are presented. $**P < 0.01$. Representative images of the membranes with invading cells are shown. (E) Wound-healing assay. At 48 h after transfection with siRNA, the cell layer was scratched, and the medium was replaced with the fresh medium. The wound closure ratio was calculated by the formula: (width at 0 h - width at 24 h)/width at 0 h. The means \pm SD from experiments performed in triplicate are presented. $**P < 0.01$. Representative images of the wounded area are shown.

in which $\beta 1$ integrin was knocked down. The wound closure ratio of the cells in which $\beta 1$ integrin was knocked down was significantly lower than that of the control cells at 48 h after wounding the cell layer (Fig. 5E).

Discussion

In the present study, we analyzed public datasets and demonstrated that a decreased expression of NRP1 was associated with the shorter survival length of NB. The NB cells in which NRP1 was knocked down exhibited markedly enhanced invasive and migratory abilities, suggesting that NRP1 may have a tumor suppressive function in NB.

Neuropilin, consisting of NRP1 and its homolog NRP2, is a transmembrane protein that functions as a co-receptor for several ligands, such as VEGF, semaphorin and transforming growth factor (TGF)- $\beta 1$, and enhances their signals (19). Under physiological conditions, NRPs are known to play significant roles in angiogenesis, the development of the nervous system and immunity. Almost all previous reports on malignancies have demonstrated that NRP1 is highly expressed in cancer tissues compared to normal tissues, and/or patients with a higher expression of NRP1 in cancer tissues exhibit a shorter survival period than those with lower NRP1 expression levels (10-15). As many of the ligands for NRPs are relevant to angiogenesis, the acceleration of tumor vessel formation is one of the most likely mechanisms for the tumor-promoting effect of NRPs (16). Indeed, it has been reported

that the co-expression of NRP1 and VEGF2 in endothelial cells and melanoma cells promotes vascular formation (20). In addition, NRPs are known to affect tumor cells directly, activating cell growth and migration (6,15). In breast cancer, NRP1 plays a key role in mammosphere formation, which is one of the typical features of breast cancer stem cells, via activating the NF- κ B signal (21). The ability of NRPs to bind to TGF- $\beta 1$ and its receptors suggests that NRPs can promote tumor metastasis (22). Based on these observations, NRPs have been proposed to be candidate therapeutic target for malignancies.

The findings of the present study indicate that NRP1 has a tumor suppressive function in NB, which contradicts the findings of previous reports on NRP1 in other types of cancer. Among the molecules relevant to cell invasion and/or motility, we found that $\beta 1$ integrin was significantly upregulated following NRP1 knockdown. The integrins are a family of transmembrane receptors through which cells adhere to the extracellular matrix, regulating cell proliferation and migration. The integrin signal has been known to induce the autophosphorylation of FAK followed via the activation of downstream signals, such as PI3K and F-actin formation (23,24). On the other hand, it has been reported that reactive oxygen species produced upon integrin receptor activation oxidize actin and this modification results in disassembly of the actin-myosin complex (25). It has been also shown that the oxidation of actin causes the prevention of actin polymerization (26). These cytoskeleton dynamics induced

by integrin signal is necessary for cell ability of migration and invasion. Our results indicated that FAK and PI3K were upregulated and activated in NRP1-depleted cells (Fig. 3A). In addition, NRP1-depleted cells demonstrated an obviously reduced amount of F-actin compared to control cells (Fig. 4). These observations suggested that the decreased expression of NRP1 resulted in an enhanced cell invasion and motility via the induction of $\beta 1$ integrin expression. The observation that $\beta 1$ integrin silencing suppressed cell invasion and migration supports this suggestion.

It is worth noting that $\beta 1$ integrin expression was upregulated at the transcriptional level following the knockdown of NRP1. Although several studies have demonstrated that the activity of integrins can be regulated by semaphorins via its receptors, including NRPs and plexins (27-29), there are few reports available regarding the regulation of integrin expression by semaphorins/NRPs. In a previous study using a breast cancer cell line, cells treated with SEMA3A exhibited an increased expression of $\alpha 2$ and $\beta 1$ integrin at the transcriptional level (30). As NRP1 should be activated by SEMA3A, this report was in contrast with the findings of the present study. It has been reported that the expression of $\beta 1$ integrin is regulated by the homeobox family gene, HOXD1, in endothelial cells (31), hypoxia-inducible factor in fibroblasts (32), or fork head box M1 in breast cancer cells (33). A detailed analysis indicated that the $\beta 1$ integrin promoter contains binding sites for these transcription factors (31-33). To elucidate the mechanisms through which NRPs regulate the expression of integrins, we are currently planning a study to evaluate whether there is a crosstalk between NRP signals and the above transcription factors.

Intriguingly, the present findings suggested that therapeutics to inhibit the function of NRP1 can promote the malignant alteration of NB, even though NRP1 is assumed to be a promising therapeutic target for many other tumors. The reason why NRP1 exerts tumor suppressive effects against NB cells in contrast to the findings in other types of cancers remains unclear. NRP1 and its ligand, SEMA3A, are involved in axonal guidance during nervous system development (27,34,35). As NB is derived from neural crest progenitor cells in the sympathetic nervous system and differentiated NB cells exhibit a neuron-like morphology with elongated dendrites, we speculated that the knockdown of NRP1 may inhibit the neuron-like phenotype of NB cells and result in undifferentiated, malignant properties. Further analyses are warranted to evaluate this possibility.

In conclusion, the present study indicated that a decreased NRP1 expression level was associated with a poor prognosis of patients with NB, and that the silencing of NRP1 results in the promotion of the migratory and invasive activities of NB-derived SK-N-AS cells, along with the upregulated expression of $\beta 1$ integrin. These results indicate that NRP1 exerts its tumor suppressive effects by reducing $\beta 1$ integrin expression in NB.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YI, KS and KF planned the experiments. YI and KF performed the experiments. YI, TK and KF interpret experimental data. SY designed and tested the primers for real-time PCR. TH, ENM, YW, RH, HK and SU have maintained the NB cells and confirm the identity of these cells. YW, NF and MS performed statistical analysis of the data. KF and YI wrote the paper. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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