Original Article

NRP1 promotes cell migration and invasion and serves as a therapeutic target in nasopharyngeal carcinoma

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Abstract: Neuropilin 1 (NRP1) promotes tumor growth, angiogenesis, tumor migration, and invasion. Its higher expression is closely related to the metastasis and poor outcome of many cancers. We have reported that NRP1 was expressed at higher levels in highly metastatic cells in comparison to minimally metastatic cells in nasopharyngeal carcinoma (NPC). However, the role of NRP1 in NPC cell migration and invasion is still unclear, and whether it could serve as a potential therapeutic target for patients with NPC still needs further investigation. In this study, our results demonstrated that ectopic expression of NRP1 in S26 and 6-10B cells promoted cell migration and invasion via wound healing and transwell assays. In contrast, knockdown of NRP1 in HONE1, CNE1 and S18 cells through Clustered Regularly Interspaced Short Palindromic Repeats interference (CRISPRi) technology suppressed cell migration and invasion. Moreover, we found that EG00229, a small molecule inhibitor of NRP1, significantly suppressed NRP1-mediated promotion of NPC cells migration and invasion. Mechanistically, we demonstrated that NRP1 promoted migration and invasion by decreasing E-cadherin levels and increasing N-cadherin levels. Collectively, our results showed that NRP1 promotes cell migration and invasion and could function as a promising target for the future treatment of patients with NPC.

Keywords: Neuropilin-1, migration, invasion, nasopharyngeal carcinoma, EG00229

Introduction

Nasopharyngeal carcinoma (NPC) has the highest metastasis rate among head and neck cancers, and it is highly prevalent in South Asia, particularly in southern China [1, 2]. Radiotherapy has considerable effects on NPC patients by improving long-term survival rates [3]. However, local recurrence and distant metastasis remain major issues in the treatment of NPC [4-6]. Previous research has established that the abnormal expression of oncogenes and tumor suppressor genes play an essential role in tumor progression and metastasis [7]. However, the specific mechanisms underlying the metastasis of NPC are still not clear. Therefore, research on the mechanisms of this disease is a valuable area of study to improve the future therapies of NPC.

Neuropilin 1 (NRP1) is a transmembrane glycoprotein that has been shown to be a critical regulator of neural system development, angiogenesis, immunity and tumorigenesis [8-10]. It has been reported that NRP1 is the receptor for semaphorin (SEMA) and vascular endothelial growth factor 165 (VEGF 165) [8, 9, 11]. Abnormal expression of NRP1 is often found in many cancers, including gastric cancer, breast cancer and esophageal cancer, which indicate poor prognosis and cancer metastasis [12-15]. NRP1 regulates tumor migration, invasion and angiogenesis either by acting as a receptor of various cancer factors or by regulating phosphorylation in certain signaling pathways [16-18]. To date, several studies have demonstrated that NRP1 could be a hopeful target for the treatment [19-21]. Our previous study has shown that there is an abnormal expression of
NRP1 promotes cell migration and invasion in nasopharyngeal carcinoma

NRP1 in NPC [22]. However, the understanding of NRP1 in NPC is greatly limited. EG00229, a small molecule inhibitor of NRP1, functions by targeting the b1 domain of NRP1, which in turn activates the VEGF-A165 and TGFβ pathways. A growing body of evidence has shown that EG00229 can affect angiogenesis, cell migration, and invasion [21, 23]. Hence, we hypothesized that EG00229 could be a potentially effective target for NPC treatment.

In this current study, we ascertained the role of NRP1 in NPC cells and also explored the effect of EG00229 as a small molecule inhibitor of NRP1 in NPC cell phenotypes. Our results demonstrate that the overexpression of NRP1 can enhance migration and invasion by decreasing the expression levels of E-cadherin and increasing the expression levels of N-cadherin. Furthermore, our data point out that NRP1 could be an effective target of anti-tumor therapy in NPC.

Materials and methods

Cells and reagents

Cell lines (NP69, 6-10B, 5-8F, HONE1, S26, S18, CNE1, and SUNE1) were provided by the group of Tiebang Kang (Sun Yat-sen University Cancer Center, Guangzhou, China). Cell lines (6-10B, S26, HONE1, CNE1 and S18) were cultured in DMEM (Gibco, Catalog Number: 11965-092) supplemented with 10% fetal bovine serum (Gibco, Catalog Number: 10270-106) and maintained at 37°C in an incubator containing 5% CO₂. All cell lines used in this study were authenticated through short tandem repeat profiling within 6 months of initiating this project.

Plasmids

The full-length cDNA of human NRP1 was cloned into the pSin-puro vector. The oligonucleotides for human NRP1 for CRISPRi technology were GCTGGCGCCCAGGGGAGGTCC, and were added into the BsmBI sites of pLVhU6-sgRNA hU6-cas9-KRAB-T2A-puro (Addgene: #71236). All recombinant plasmids were verified through DNA sequencing.

Antibodies

Anti-actin (Catalog Number: #FB075) antibody was obtained from Nanchang Focus Bioscience Co., Ltd. Anti-E-cadherin (Catalog Number: #14472) and Anti-N-cadherin (Catalog Number: #14215) antibodies were obtained from Cell Signaling Technology.

Stable cell lines

pSin-puro-NRP1, pSin-puro-empty vector, sgRNA-NC, and sgRNA-NRP1 were co-transfected along with pMD2.G and psPAX2 into 293T cells for 48 h. The recombinant viruses were subsequently collected and added to NPC cells, which were cultured with 8 μg/ml polybrene for 24 h. The stable lines were selected by treating the cells with 1 μg/ml puromycin for two weeks (Focus Bioscience Co., Ltd, Nanchang, China).

RNA extraction and qRT-PCR

RNA extraction and qRT-PCR procedures were performed as previously described [24, 25]. Briefly, total RNA was isolated using TRizol reagent (Thermo Fisher Scientific, Catalog Number: 15596026) according to the instructions of the product. First-strand cDNA was synthesized using the RevertAidTM First Strand cDNA Synthesis Kit (Thermo Scientific ,Catalog Number: K1621). The primers employed for amplifying NRP1 and GAPDH were validated. The sequences for the NRP1 primers are as follows: F 5'-ACGTGGAAGTCTTCGATGGAG-3' and R 5'-CACCATGTGTTTCGTAGTCAGA-3'. GAPDH primer sequences are as follows: F 5'-ACACTTGTTCTTCTGGAG-3' and R 5'-GACAAGC-TTCCGTTCTCAG-3'.

Transwell assays

For the transwell migration assay, 7.0×10⁴ cells re-suspended in 300 μl of serum-free DMEM (Gibco, Catalog Number: 11965-092) were added to cell culture inserts, which had 8-μm microporous filter sand no extra cellular matrix coating (Becton Dickinson Labware, Bedford, MA, Catalog Number: 353097). DMEM containing 10% FBS (Gibco, Catalog Number: 10270-106) was then added to the bottom chamber (Becton Dickinson Labware, Bedford, MA, Catalog Number: 353504). After 24 h of incubation, the cells on the lower surface of the filter were fixed, stained, and examined using a microscope. The number of migrated cells in three random optical fields (×100 magnification) from triplicate filters was averaged. For the transwell invasion assay, transwell chambers
NRP1 promotes cell migration and invasion in nasopharyngeal carcinoma

were percolated with ECM Matrix gel solution and solidified at 37°C. After solidification, 1.4 × 10^5 cells re-suspended in 300 μl of serum-free DMEM were added to cell culture inserts. The following steps are the same as the previous migration assay. The number of cells that passed through the membrane was statistically analyzed using \( t \) test.

**Wound-healing assay**

Cells were scratched with a 200-μl pipette tip, and washed with PBS. Cells were supplemented with 10% FBS. The wound closure was photographed at 0, 24, and 48 h. The length of gap was statistically analyzed using \( t \) test.

**Western blotting**

Western blotting procedures were performed as described previously [22, 24]. Briefly, equal amounts of protein were separated by 10% SDS-PAGE. The proteins were then transferred to PVDF membranes. The PVDF membranes were blocked and incubated with corresponding primary antibodies overnight at 4°C. Subsequently, the membranes were incubated with corresponding secondary antibodies for 2 h at room temperature. Specific protein bands were visualized using ECL detection system (Catalog Number: P0018F, Beyotime, China) and exposed to radiographic film (Carestream, Catalog Number 6535876).

**Clinical samples**

This procedure was approved by the Institutional Review Board of The Third Affiliated Hospital of Nanchang University and written informed consent was obtained from the patients prior to sample collection (Number: NCSDYYY-0359). The ages of the patients ranged from 32 to 55 years old. Tissue blocks prepared from NPC tissues and LN metastases were sectioned for performing Q-PCR for NRP1.
NRP1 promotes cell migration and invasion in nasopharyngeal carcinoma

A

Relative expression of NRP1

NC
KD

HONE1
CNE1
S18

B

HONE1
CNE1
S18

NC
KD

oh
24h
48h

Length of the gap (μm)

NC
KD

NC
KD

Migration

D

HONE1
CNE1
S18

NC
KD

Invasion

Cell numbers per field

NC
KD

Cell numbers per field

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NRP1 promotes cell migration and invasion in nasopharyngeal carcinoma

Figure 2. Knockdown of NRP1 inhibits cell migration and invasion of NPC cells. A. HONE1, CNE1 and S18 cells were silenced by CRISPRi technology. The mRNA levels of NRP1 in HONE1, CNE1 and S18 cell lines were determined by qRT-PCR. NC stands for negative control; KD stands for knockdown of NRP1. Data were normalized to the expression of GAPDH. The bars represent the mean ± SEM. Each cell line was analyzed in triplicate. B. Wound healing ability of NC and KD groups in HONE1, CNE1 and S18 cells was determined at 0 hour, 24 hours and 48 hours by wound healing assays. The bars represent mean ± SEM values. The length of the gap was statistically analyzed in triplicate and repeated three times with similar results. *P < 0.05, **P < 0.01. C, D. The migration and invasion abilities of NC and KD groups in both HONE1, CNE1 and S18 cells were measured at 24 hours by transwell assays. The bars represent mean ± SEM values. The number of cells passing through the membrane in each cell was statistically analyzed in triplicate and repeated three times with similar results. *P < 0.05, **P < 0.01.

Figure 3. Overexpression of NRP1 increases migration and invasion of NPC cells. A. The establishment of stable cell lines in S26 and 6-10B cells in which NRP1 was overexpressed was determined by qRT-PCR. Data were normalized to the expression of GAPDH. The bars represent the mean ± SEM values. Each cell line was analyzed in triplicate. B. Wound healing ability of Vector and NRP1 groups in both S26 cells and 6-10B cells was determined at 0 hour, 24 hours and 48 hours by wound healing assays. The bars represent mean ± SEM. The length of the gap was statisti-
NRP1 promotes cell migration and invasion in nasopharyngeal carcinoma

Statistical analysis

All statistical analyses were performed using SPSS for Windows, version 22.0 (SPSS). The figures were performed by GraphPad Prism 5.0. All values from the in vitro assays are expressed as the mean ± SD or mean ± SEM of at least three independent experiments or replicates. P values were calculated using the two-tailed Student’s test. A p value < 0.05 was considered statistically significant.

Results

NPR1 is overexpressed in metastatic NPC tissues and cell lines

In order to determine the key regulators in the metastasis of NPC, we utilized a gene expression microarray to screen and analyze the primary and metastatic tissues of NPC [22]. As shown in Figure 1A, we found that NRP1 had a higher expression in metastatic tissues than in the primary tumor tissues through detection of clinical NPC samples. In addition, we also detected the expression of NRP1 mRNA in NP69 cells and seven NPC cell lines. The result suggested that NRP1 was highly expressed in the highly metastatic cell lines (Figure 1B). Furthermore, we also analyzed the mRNA levels between normal tissues and cancerous tissues in Oncomine database. Results from the Oncomine database revealed that the transcript level of NRP1 gene was overexpression in NPC tissues than in nasopharynx tissues (Figure 1C). The above results indicated that NRP1 was overexpressed in metastatic NPC tissues and cell lines.

Knockdown of NRP1 inhibits cell migration and invasion of NPC cells

Given that NRP1 is overexpressed in HONE1, CNE1 and S18 cells, we evaluated whether knockdown of NRP1 affected NPC cell biologic behavior. Stable knockdown cell lines were constructed in HONE1, CNE1 and S18 cells using CRISRPi technology (Figure 2A). Evaluation of the migratory and invasive effects of NRP1 knockdown was then conducted. As shown in Figure 2B, knockdown of NRP1 inhibited cell wound healing. Transwell assays also showed similar results in that the knockdown of NRP1 significantly suppressed the migration and invasion abilities of NPC cells (Figure 2C and 2D). In sum, these results demonstrate that knockdown of NRP1 has an inhibitory effect on the migration and invasion abilities of NPC cells in vitro.

Overexpression of NRP1 increases migration and invasion of NPC cells

To better understand the function of NRP1, we constructed stable ectopic expression cell lines in 6-10B and S26 cells (Figure 3A). Wound healing assays and transwell assays were conducted to observe the migratory and invasive effects of ectopic NRP1. As shown in Figure 3B-D, overexpression of NRP1 significantly promoted cell migration and invasion. Therefore, these results confirm that NRP1 possesses an oncogenic function and is a contributor of cell migration and invasion.

EG00229, an inhibitor of NRP1, suppresses the invasion and migration of NPC cells

Further experiments were performed to determine whether EG00229, a small molecule inhibitor of NRP1, could effectively suppress cell migration and invasion in NPC cells. Treatment by EG00229 at a concentration of 10 µM, was implemented in the indicated stable cell lines (Figure 3A) for 24 hours. As shown in Figure 4A and 4B, EG00229 decreased wound healing abilities. Transwell assays were additionally conducted to study the effect of EG00229 on NPC migration and invasion. After EG00229 treatment, the quantity of 6-10B and S26 cells that passed through the membrane decreased, which proved that EG00229 could effectively suppress the migration and invasion.

NRP1 decreases expression of E-cadherin and increases expression of N-cadherin in NPC cells

For the purpose of exploring a potential mechanism of NRP1 in inhibiting cell migration and invasion, we investigated the expression levels...
Figure 4. EG00229 suppresses cell migration and invasion targeting NRP1 in NPC. A, B. S26 and 6-10B stable cell lines were treated with EG00229 (10 µM) in complete medium for 24 hours. Wound healing ability of stable cell lines was determined at 0 hour, 24 hours and 48 hours by wound healing assays. The bars represent mean ± SEM. The length of the gap was statistically analyzed in triplicate and repeated three times with similar results. *P < 0.05, **P < 0.01. C, D. Treatment with EG00229 for 24 hours resulted in decreased cell migration and invasion abilities in S26 and 6-10B stable cell lines. The bars represent mean ± SEM. The number of cells passing through the membrane in each well was statistically analyzed in triplicate and repeated three times with similar results. *P < 0.05, **P < 0.01.
NRP1 promotes cell migration and invasion in nasopharyngeal carcinoma

of E-cadherin and N-cadherin in NPC cell lines. It was reported that E-cadherin and N-cadherin play major roles in the migration and invasion of NPC cells [26, 27]. As shown in Figure 5, E-cadherin protein levels were lower in 6-10B and S26 cells that stably expressed NRP1, whereas N-cadherin protein levels were higher. When we decreased NRP1 expression, the opposite results were shown in HONE1 and CNE1 cells lines. After adding EG00229 for 24 hours in 6-10B and S26 stable cell lines, E-cadherin and N-cadherin expression levels were rescued. From the combined data of Figure 5, it was apparent that NRP1 enhanced NPC cell migration and invasion abilities via an abnormal expression of E-cadherin and N-cadherin.

Discussion

Due to much rapid advancement in radiotherapy and chemotherapy, the long-term survival rates of patients with NPC have been greatly improved [3, 28]. Unfortunately, local recurrence and distant metastasis of tumors remain as the main causes of treatment failure [4-6]. The migration and invasion abilities of cancer cells are critical steps in the process of cancer progression and metastasis due to their significant effect on cell mortality. Therefore, clarifying the detailed mechanism of metastasis in NPC is a key point for effective treatment intervention.

Transmembrane proteins (TP), such as TMEM16A and TMEM88, play a versatile role in tumor metastasis and often serve as an effective target for cancer treatment [29, 30]. Our previous study suggests that NRP1, a transmembrane glycoprotein, might be an essential component in the metastasis of NPC [22]. Many studies have reported that NRP1 is upregulated in tumor tissues in comparison to normal controls, and its overexpression is an indication of cancer metastasis in many cancers [12, 15, 31, 32]. NRP1 promotes cell migration and invasion by regulating Epithelial-Mesenchymal Transition (EMT), microRNA expression, and so on [13, 33]. In this study, we investigated the specific role of NRP1 in relation to the migration and invasion of NPC cells and its underlying mechanisms. Our data indicated that NRP1 was overexpressed in NPC cells, indicating a significant improvement in the wound healing abilities of NPC cells. Simultaneously, through transwell assays, we showed that NRP1 remarkably enhanced the capabilities of NPC cell migration and invasion. It is generally known that the escape of tumor cells from the original site to adjacent tissues or other organs of the body requires a variety of biological factors. Many studies have shown that E-cadherin and N-cadherin are crucial factors of the tumor migration and invasion process. Thus, our study investigated E-cadherin and N-cadherin expression levels of 6-10B and S26 cells, which revealed NRP1 overexpression. As expected, our results demonstrated that NRP1 negatively regulated the expression of E-cadherin, whereas it was positively associated with N-cadherin expression.

Consistent with our findings, it has been demonstrated that NRP1 is a strong potential therapeutic target in vivo and vitro [19-21]. In view of the important role of NRP1 in NPC, we considered whether suppressing the expression of NRP1 could inhibit the migration and invasion of NPC cells. Thus, we examined the effect of EG00229 on the migratory and invasive abilities of 6-10B and S26 stable cell lines with NRP1 overexpression. As expected, EG00229
NRP1 promotes cell migration and invasion in nasopharyngeal carcinoma

remarkably decreased cell migration and invasion, as confirmed through wound healing assays and transwell assays. Intriguingly, through western blotting, we found that E-cadherin expression increased, while N-cadherin expression decreased with NRP1 overexpression.

In summary, NRP1 can greatly promote the migratory and invasive abilities of NPC cells via regulation of E-cadherin and N-cadherin levels, namely decreasing E-cadherin expression and increasing N-cadherin expression. At the same time, EG00299 inhibited the ability of NRP1 to promote migration and invasion. Since NRP1 is positively expressed in NPC cells and EG00229 is a specific NRP1 inhibitor, these findings suggest that NRP1 is promising target for NPC treatment. However, there are still many unanswered questions about the exact molecular mechanism of how NRP1 regulates E-cadherin and N-cadherin. Further studies are needed to elucidate these mechanisms to confirm the role of NRP1 as a new target for NPC metastasis.

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Disclosure of conflict of interest

None.

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NRP1 promotes cell migration and invasion in nasopharyngeal carcinoma


