Neuropilin-1 down-regulation impairs cell migration and induces the differentiation of human tongue squamous cell carcinoma cells

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Abstract
Objective
Our previous study demonstrated that neuropilin-1 is useful for the prognosis of tongue squamous cell carcinoma. In this study, we aimed to explore the role of neuropilin-1 in the differentiation of tongue squamous cell carcinoma.

Methods
Forty primary tongue squamous cell carcinoma samples were subjected to immunostaining for neuropilin-1. The relationship between neuropilin-1 expression and differentiation was analysed. A specific small hairpin ribonucleic acid (shRNA) targeting neuropilin-1 was transfected into the tongue squamous cell carcinoma cell line squamous cell carcinoma-25 to knockdown neuropilin-1 expression. Scratch and transwell assays were performed to examine the cell migration and invasion ability after neuropilin-1 was knocked down. Real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blot were used to detect the efficiency of neuropilin-1 knockdown and the expression of differentiation markers.

Results
We found a significant correlation between the high neuropilin-1 expression level and the poor histopathological differentiation of tongue squamous cell carcinoma (p = 0.026). Neuropilin-1 was expressed at a high level in squamous cell carcinoma-25 cells. After neuropilin-1 knockdown via shRNA, cell migration and invasion were significantly reduced as demonstrated by scratch and transwell assays (p < 0.01). In addition, Western blotting and RT-PCR demonstrated that neuropilin-1 knockdown increased E-cadherin and decreased fibronectin and vimentin expression in squamous cell carcinoma-25 cells.

Conclusion
Neuropilin-1 knockdown may inhibit the migration and invasion of tongue squamous cell carcinoma by inducing a more differentiated cancer state. Considering the high level of neuropilin-1 expression in tongue squamous cell carcinoma, it may serve as a useful marker for differentiation and as a target for the treatment of tongue cancer.

Introduction
Neuropilin-1 (NRP-1) is a 130 kDa single-spanning transmembrane glycoprotein, which was first identified in the optic tract of Xenopus laevis¹. NRP-1 was originally identified as a receptor for semaphorin 3A (SEMA3A), which promotes axon repulsion in the developing nervous system²–⁴. It was recently demonstrated that NRP-1 acts as a co-receptor for vascular endothelial growth factor-A (VEGF-A) and semaphorin, promoting vascular development with vascular endothelial growth factor receptor 2 (VEGFR2) or mediating neuronal guidance with plexin, respectively⁵–⁸.

NRP-1 is expressed not only in endothelial cells (ECs) and normal tissue but also in numerous cancers including breast, prostate, lung, pancreas, gastric and colon cancer⁹–¹⁴. Several reports have revealed that higher level of NRP-1 expression is significantly related to advanced tumour progression and poor prognosis¹⁵. Stephenson et al. found that NRP-1 is highly expressed in human breast cancer tissue compared with the limited NRP-1 expression found in normal breast tissue¹⁶. In addition, the high level of NRP-1 expression is highly correlated with high lymphatic metastasis in patients with breast cancer. Moreover, it was demonstrated in vitro that breast cancer cell lines with a high metastatic potential appear to have a higher level of NRP-1 expression than that in breast cancer cell lines with a low metastatic potential¹⁷. Ochiimi et al. also reported that high level of NRP-1 expression in colon cancer positively correlates with prognosis, and NRP-1 knockdown decreased the migration and increased the apoptosis of human colon cancer cell lines. In our previous study¹⁸, we demonstrated that NRP-1 expression was significantly elevated in human tongue cancer tissue compared with normal tongue tissue. The expression ratio of SEM3A/NRP-1 was significantly correlated with the prognosis of tongue cancer. In this study, we further investigated the relationship between NRP-1 expression and histopathological...
differentiation of human tongue squamous cell carcinoma (SCC) samples and the effect of NRP-1 on the regulation of cell migration and differentiation of SCC25 cells in vitro by knocking down NRP-1 expression.

Materials and methods
Cell lines and tissue collection
The human tongue SCC cell line SCC25 was purchased from American Type Culture Collection (ATCC, VA, USA) and maintained at 37°C in a humidified atmosphere of 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) F12 supplemented with 10% foetal bovine serum (FBS), 600 μg/mL L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Forty primary tongue SCC biopsy specimens were obtained from the Department of Oral Pathology and the Department of Oral and Maxillofacial Surgery in the Stomatological Hospital of Jiangsu Province, Nanjing Medical University. All patients were diagnosed from 2000 to 2006, and none had been treated with any type of tumour-specific therapy before surgery. The histological SCC characteristics were classified into well, moderately and poorly differentiated groups (G1–G3) according to the criteria proposed by the World Health Organization.

Immunohistochemistry (IHC)
IHC for NRP-1 was performed on formalin-fixed, paraffin-embedded tissues cut into 4 μm serial sections. Endogenous peroxidase was blocked with 3% hydrogen peroxide. The sections were processed by conventional microwave heating in 0.01 M sodium citrate retrieval buffer (pH 6.0) for 12 min with phosphate-buffered saline (PBS, pH = 7.2), and the sections were developed using 3,3'-diaminobenzidine and counterstained with haematoxylin. All negative controls were prepared as described above in the absence of the primary antibody. Specific immunoreactive staining was not observed in any of the control sections.

Immunoreactivity was semi-quantitatively evaluated using the staining distribution score. The proportion score was defined as follows: 0 represented 0%–10%; 1 represented 10%–30%; 2 represented 30%–60%; and 3 represented 60%–100%. To analyze the clinicopathological characteristics, the immunoreactive sections were divided into two groups: low immunoreactivity, which was defined as having a proportion score of ≤2, and high immunoreactivity, which was defined as having a proportion score of >2.

Lentiviral plasmid construction and transfection
Using a small interfering RNA (siRNA) selection tool, three small hairpin RNAs (shRNA) targeting NRP-1 were selected. The sequences were as follows: sh-NRP-F1: CCG GGG ACA GAG ACT GCA AGT ATC TTC TCG TCA ATA CTT GCA GTC TCT TGT CTT TTT TG; sh-NRP-R1: AAT TCT AAA AAA GGA CAG AAG CAG AGT CTT AAG TGG CTT AAG ACA AGA TAC TGG CAG TCT CTG TCC; sh-NRP-F2: CCG GGA CCC ATA CCA GAG AAT TAC TCG AGT AAT TCT CTG TGG GTC TTT TTG; sh-NRP-R2: AAT TCA AAA AGA CCC ATA CCA GAG AAT TAC TCG AGT AAT TCT CTG TGG GTC TTT TTG; sh-NRP-R3: AAT TCA AAA AGA CCC ATA CCA GAG AAT TAC TCG AGT AAT TCT CTG TGG GTC TTT TTG; and sh-NRP-R3: AAT TCA AAA AGA CCC ATA CCA GAG AAT TAC TCG AGT AAT TCT CTG TGG GTC TTT TTG. The sequences were then incubated with a rabbit monoclonal anti-NRP-1 antibody (1:100; Abcam, Cambridge, MA, USA) overnight at 4°C and subsequently incubated with a goat anti-rabbit (1:5000; Abcam, Cambridge, MA, USA) antibody for 30 min at 25°C. The sections were then washed three times for 3 min with phosphate-buffered saline (PBS, pH = 7.2), and the sections were developed using 3,3'-diaminobenzidine and counterstained with haematoxylin. All negative controls were prepared as described above in the absence of the primary antibody. Specific immunoreactive staining was not observed in any of the control sections.

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Real-time Reverse transcription polymerase chain reaction (RT-PCR)
Quantitative RT-PCR (qRT-PCR) for epithelial cadherin (E-cadherin), vimentin and fibronectin transcripts in SCC25 cell lysate was performed using the PrimeScript RT reagent kit following the manufacturer’s instructions (TaKaRa Bio, Shiga, Japan). The primer sequences were as follows: E-cadherin: 5'-AAC ACC GAG AGA GG-3’ (sense) and 5'-CTG ACT GAG CTA GGC AAA-3’ (antisense); vimentin: 5'-CGA GTA CCG GAG ACA GGT AGA GCA-3’ (sense) and 5'-TAG CAG CTT CAA CAA CAA GTG-3’ (antisense); fibronectin: 5'-GTA CGC TCA TGA GCC AAC-3’ (sense) and 5'-CTG GCC TCC AAA GCA GTA G-3’ (antisense); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-GAA GGT GAA GGT CCG AGT C-3’ (sense) and 5'-GAG ATG ATG ATG GGA TTC C-3’ (antisense). The results of qRT-PCR were represented as Ct values, where Ct is a fraction defined as the cycle number at which the sample fluorescent signal passes a given threshold above the baseline. The ΔCt is the difference in the Ct values derived from specific genes after comparison with GAPDH.

Western blot analysis
Western blotting was performed as previously described using antibodies against E-cadherin (1:1000;
Bioworld; USA), fibronectin (1:1000; Bioworld; USA), vimentin (1:1000; Bioworld; USA) and β-actin (1:5000; Bioworld; USA).

**Scratch assay**
SCC25 cells, which were transfected with shRNA-NRP-1 or shRNA-scramble, were grown to 80%–90% confluence in 12-well plates. A scratch was made in the centre of the monolayer. Detached cells were removed by gentle washing with PBS, and fresh medium was added. After 24 h, the width of the scratch was monitored by microscopy to determine the extent of migration into the scratched space.

**Transwell assay**
A total of $2 \times 10^5$ SCC25 cells, which were transfected with shRNA-NRP1 or shRNA-scramble, were suspended in 100μL DMEM-F12 medium without FBS and seeded in the upper chamber of 24-well plates. The lower chamber contained 500μL DMEM-F12 supplemented with 5% FBS. After 8 h of incubation, the inserts were washed three times with PBS, fixed with 4% polyoxymethylene for 30 min and stained with 0.05% crystal violet staining solution. The number of migrating cells was counted using microscopy, and 10 randomly chosen fields were counted per insert.

**Statistical analysis**
The means of the experimental groups were compared with those of the negative controls using Student’s t-test. A ratio analysis was performed using the chi-square or Fisher’s exact test. A p value of $\leq 0.05$ was considered statistically significant.

**Results**
**NRP-1 expression correlates with poor histopathological differentiation**
NRP-1 expression was mainly detected in the cell membrane and cytoplasm (Figures 1a–1c). Among the 40 tongue SCC samples, 13 (32.5%) exhibited low expression, and among these, 10 (77%) were well differentiated. However, in the high-expression group, only 9 (33%) were well differentiated and 16 (60%) were moderately differentiated. The NRP-1 expression level significantly correlated with the differentiation of tongue SCC, with higher NRP-1 expression level corresponding with poor histopathological differentiation ($p < 0.05$) (Table 1).

**NRP-1 is elevated in human tongue SCC cells**
NRP-1 expression in SCC25 cells was detected using immunofluorescent cell staining and Western blot. NRP-1 was mainly expressed in the cytoplasm and membrane (Figures 1d–1f). PBS was used as the negative control (Figures 1g–1i), and the nuclei were stained with 4′,6-diamidino-2-phenylindole. While NRP-1 was highly expressed in SCC25 cells, which is similar to that found in human tongue SCC, it was poorly expressed in normal tongue tissue as demonstrated by Western blot (Figure 2a).

**NRP-1 knockdown using shRNA in SCC25 cells**
SCC25 cells were transfected with shRNAs targeting different sequences in human NRP-1. Cells that were transfected with shRNA1 demonstrated significant down-regulation of NRP-1 expression as determined by Western blot (Figure 2b) and RT-PCR (Figure 2c). The down-regulation efficiency was up to 65%.

**NRP-1 down-regulation suppressed the migration of SCC25 cells**
Scratch and transwell assays were used to detect the migration of SCC25 cells. In the scratch assay, there were...

Table 1  Relationship between NRP-1 expression and pathological differentiation of tongue squamous cell carcinoma

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<th>Variable</th>
<th>No.</th>
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<th>Moderate</th>
<th>Poor</th>
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<td>16</td>
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NRP-1, neuropilin-1

Figure 2: Down-regulation of neuropilin-1 (NRP-1) expression by small hairpin RNA (shRNA) in squamous cell carcinoma (SCC25) cells. While NRP-1 was highly expressed in SCC25 cells and human tongue SCC, it had a low expression in normal tongue tissue (a). NRP-1 mRNA levels were measured by real-time reverse transcription polymerase chain reaction using total RNA isolated from different transfectants. The knockdown efficiency was approximately 65% when SCC25 cells were transfected with shRNA1 (b). Western blotting was performed to detect the NRP-1 knockdown efficiency in the different transfectants (c).

NRP-1 mediates SCC25 cell differentiation

We next determined whether NRP-1 knockdown could affect tumour cell differentiation. We found that the expression level of the epithelial marker E-cadherin was increased, while the expression levels of the mesenchymal markers fibronectin and vimentin were decreased in the NRP-1 knockdown SCC25 cells (Figure 4). These results indicated that SCC25 cells exhibit a more differentiated phenotype after NRP-1 knockdown.

Discussion

NRP-1 is expressed in various types of cells and plays important roles in angiogenesis development and neuronal guidance. In recent years, an increasing number of studies have reported the importance of NRP-1 in cancer. In agreement with reports of the high NRP-1 expression level in several other cancers, we hypothesized that NRP-1 may play an important role in the biological behaviour of human tongue SCC. IHC analysis demonstrated that the NRP-1 expression level was significantly correlated with histopathological differentiation, and the high NRP-1 expression level in tongue SCC corresponded with poor histopathological differentiation. We first established the relationship between the NRP-1 expression level and histopathological differentiation. We then questioned whether the high NRP-1 expression level plays an important role in maintaining the poorly differentiated phenotype of tongue SCC.

To investigate the role that NRP-1 may play in mediating the differentiation of tongue SCC, we down-regulated NRP-1 expression in SCC25 cells using lentiviral plasmids containing shRNA sequences targeting NRP-1 mRNA. The treated cells demonstrated a 65% reduction in the NRP-1 expression compared with the control group. Scratch and transwell assays exhibited a significant reduction in the cell migration of NRP-1 knockdown SCC25 cells, indicating that NRP-1 may play an essential role in mediating the migration and invasion of SCC25 cells. A recent report mentioned that NRP-1 may be an indispensable component of the molecular cell migration signalling network in a variety of cells. NRPs were reported to be involved in the epithelial mesenchymal transition (EMT) in several studies. For example, miR-320a was down-regulated in...
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All authors contributed to the conception, design, and preparation of the manuscript, as well as read and approved the final manuscript. All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

required to determine the exact mechanisms involving NRPs in tongue SCC differentiation.

This is the first study to report the role of NRP-1 in the biological behaviour of human tongue SCC. We demonstrated that the expression of NRP-1 was upregulated in human tongue SCC tissues and cell lines, indicating that NRP-1 may play an important role in the tumourigenesis of human tongue SCC. After the down-regulation of NRP-1 in SCC25 cells, there was a reduction in cell migration and induces the differentiation of human tongue squamous cell carcinoma cells. Head Neck Oncol. 2012 Sep 9;4(2):54.

**Figure 4:** Neuropilin-1 (NRP-1) knockdown induced the differentiation of squamous cell carcinoma (SCC25) cells. After NRP-1 was knockeddown, the mesenchymal markers fibronectin (FN) and vimentin (VIM) decreased, while the epithelial marker E-cadherin (E-cad) increased in SCC25 cells (a, b). *, p < 0.05; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**References**


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