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ORIGINAL RESEARCH

Neuropilin-1 is associated with the prognosis of cervical cancer in Henan Chinese population

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Li Yang[1,*]
Ling Liu[1,*]
Yuan-Hang Zhu[1]
Bing-Bing Wang[2]
Yan-Nan Chen[1]
Feng Zhang[1]
Xiao-An Zhang[3]
Chen-Chen Ren[1]

[1]Department of Obstetrics and Gynecology, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, People's Republic of China; [2]Department of Obstetrics and Gynecology, Yuebei People's Hosptial, Shaoguan 512025, People's Republic of China; [3]Department of Imaging, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, People's Republic of China

*These authors contributed equally to this work

Objective: Neuropilin-1 has been reported to be a valuable diagnostic biomarker in patients with cervical intraepithelial neoplasia (CIN) and early cervical cancer. The aim of this study was to investigate the association between Neuropilin-1 and the prognosis of cervical cancer in Henan Chinese population.

Methods: Tissues were collected in The Third Affiliated Hospital of Zhengzhou University between 2010 and 2012, determining the level and expression of Neuropilin-1 in different cervical lesions by immunohistochemistry. The cell proliferation assay, wound-healing assays and Transwell assay were performed to explore the ability of proliferation, migration and invasion for Hela and Caski cells after NRP-1 was knocked down by shRNA transfection. Western blotting was performed to investigate the role of NRP-1 in endothelial-to-mesenchymal transition (EndMT). Tumor xenografts model was used to evaluate the effect of NRP-1 on the tumor growth.

Results: The expression of NRP-1 was upregulated in the tumor tissues compared with the CIN and normal tissues ($P<0.0001$). The overall survival time of the high NRP-1 expression group was significantly shorter than that of the low NRP-1 expression group ($P<0.0001$); NRP-1-depleted cells had dramatically lower rate of proliferation, migration and invasion compared to control cells (all $P<0.05$). Depletion of NRP-1 significantly suppressed the growth of CaSki xenograft tumor in nude mice.

Conclusions: The current study demonstrated that NRP-1 expression is significantly correlated with the progression of CC. Notably, high NRP-1 expression is correlated with a poorer survival in patients with CC, and has been shown to be an independent prognostic factor.

Keywords: Neuropilin-1, NRP-1, cervical cancer, EMT, proliferation

Introduction

Cervical cancer is the fourth most common cancer and the fourth leading cause of cancer death in women worldwide.[1] The infection of human papillomavirus (HPV) has been recognized as a major cause of cervical cancer,[2] and the routine screening procedures, such as, Pap smear, detection of HPV and vaccine against HPV, have reduced the number of cervical cancer death.[3] However, over 90% of HPV infections are spontaneously cleared within two years,[4] and there exists over-treatment of women with clinically irrelevant high-risk (HR)-HPV infections. Thus, additional triage tests are needed to identify high-risk populations to avoid over-treatment.[5]Therefore, identifying biomarkers that have potential to serve as an indicator for normal biological processes, pathogenic processes or responses to treatment will have the immediate and significant impact on patient outcomes.[6]

Correspondence: Chen-Chen Ren
Department of Obstetrics and Gynecology, The Third Affiliated Hospital of Zhengzhou University, No. 7, Front Kangfu Street, Zhengzhou 450052, Henan Province, People's Republic of China
Email 13783532011@163.com

Xiao-An Zhang
Department of Imaging, The Third Affiliated Hospital of Zhengzhou University, No. 7, Front Kangfu Street, Zhengzhou 450052, Henan Province, People's Republic of China
Tel +8 603 716 690 3982
Fax +8 603 716 690 3982
Email zhangxiaoan09@126.com

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Circulating soluble Neuropilin-1 (NRP-1) has been reported to be a valuable diagnostic biomarker in patients with cervical intraepithelial neoplasia (CIN) and early cervical cancer.[7] The NRP-1 gene locates on chromosome 10p 12 and has 17 exons,[8] encoding a 130–135 kDa transmembrane glycoprotein. NRP-1 has been shown to involve in regulation of vascular endothelial cell migration and angiogenesis and promoting tumor growth, invasion and metastasis.[9] NRP-1 expresses in various types of cancer, such as pancreatic, prostate, lung, ovarian and gastrointestinal cancer.[10–12] However, the roles of NRP-1 in cervical cancer are still poorly understood. In this study, the expression of NPR1 in cervical cancer patients was examined, the function of NPR1 in proliferation and migration of cervical cancer cells was studied and the abilities of NRP-1 in predicting patient outcomes were analyzed.

Materials and methods

Patients and tissue sampling

In total, 135 cervical cancer (CC) cases were clinically and histopathologically diagnosed in The Third Affiliated Hospital of Zhengzhou University between 2010 and 2012. The patients were followed up in an outpatient clinic, and the follow-up period ranged from 4 to 61 months after primary therapy (average: 49.8 months; median: 53.0 months). Tissue samples from non-cancerous cervix and CIN were also collected during the study period, among them, 36 normal tissue samples were obtained from the patients of uterine fibroids, 58 CIN samples were obtained from patients who accepted colposcopy. Patients who died of other causes were excluded from the analysis. All tissues were obtained with the consent of the patients and written informed consent for use of the tissue samples for research purposes. This study was approved by the institutional ethics committee of the Zhengzhou University and this experiment was conducted in accordance with the declaration of Helsinki.

Detection of NRP-1 expression by immunohistochemistry

Immunohistochemical staining was carried out to assess the protein expression of NRP-1 in paraffin-embedded cervical cancer tissues. The procedures were performed with classical protocols. In brief, the paraffin-embedded tissue blocks were cut into 5-μm-thick sections as described previously.[13] Then, the sections were incubated with a rabbit monoclonal anti-NRP-1 antibody (1□100; Abcam) with the Two-Step Histostaining Reagent (ZhongshanGoldenbridge Bio). For semi-quantitative analysis, the score of each tissue specimen was based on staining intensity and distribution scores.[14] The intensity score was defined as 0, negative; 1, weak; 2, moderate; or 3, strong, and the proportion score was defined as 1, 0–25%; 2, 26–50%; 3, 51–75% or 4, >75% positive cells. Based on the immunoreactivity score, the sections were divided into the following two groups: 0–4, low and 6–12, high.[15]

Cell culture and reagents

The human CC cell line HeLa and CaSki were obtained from Cell Bank-Shanghai (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences), and cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin and maintained at 37° C in a humidified incubator containing 5% CO_2. Antibodies used included: NRP-1 (Santa Cruz # sc-7239), CD31 (Cell Signaling # 3528), VE-cadherin (Santa Cruz # sc-6458), N-cadherin (Abcam # ab76057), αSMA (Abcam # ab5694) and β-actin (Santa Cruz # sc-47778) were measured using specific antibodies.

NRP-1 shRNA knockdown

The shRNA target sequences for NRP-1 were CCGGGCT GTGGATGACATTAGTATTCTCGAGAATACTAATGT-CATCCACAGCTTTTTG (TRCN0000063525) and the pLKO.1-shLuc (TRCN0000072243) targeting the luciferase gene was used as a control for RNA interference, were obtained from SIGMA.

Transfections were carried out using ViraDuctinTM lentivirus transduction kit (Cell Biolabs, INC). The target cells were transduced with lentiNRP-1 or Empty (referred to as lentiControl) vectors according to manufacturer's protocols, following transduction, HeLa cells were selected with 1 μg/mL puromycin while CaSki cells were selected with 0.5 μg/mL puromycin, stable cell colons were selected and verified by western blotting.

RNA isolation and real-time PCR

RNA was prepared with RNase Mini kit (QIAGEN) and cDNA was synthesized using cDNA Synthesis Kits (Promega) according to the manufacturer's recommendations. The data are presented as relative expression with the control set to 1. The following primers were used for NRP-1: Forward: 5′ATGGAGAGGGGGCTGCCG3′; Reverse: 5′

CTATCGCGCTGTCGGTGTA3′. β-actin amplification was carried out in parallel to account for loading differences between samples. The following primers were used for β-actin: Forward: 5′TGTTTGAGACCTTCAACACCC3′; Reverse: 5′AGCACTGTGTTGGCGTACAG3′; Specificity of all primers was confirmed by comparing the primer sequence for each gene against the Genbank database.

Western blotting

Cells were lysed and proteins were separated by SDS-PAGE and transferred to PVDF membranes (IMMOBILON) which was then blocked for 1 hr with 5% dry milk in TBST and incubated overnight/4°C for the primary antibody. After 3 washings, membranes were incubated with the secondary antibody for 1 hr, then 3 washings and revealed by chemo-luminescence using the Pierce ECL Western Blotting Substrate (Thermo Scientific).

Scratch assays

Scratch assays were conducted on confluent cells and seeded in six-well plates. After the cells had adhered to the plates, the wells were gently scratched with a sterile 200 μL pipette tip in the central area. Floating cells and debris with PBS were removed, and the culture medium was replaced with a serum-free medium. When cells migrated into the wounds, the area decreased, during which cells were imaged every 12 for 48 hrs under a microscope.

Cell proliferation assay

Cell viability was measured by using CellTiter 96® NonRadioactive Cell Proliferation Assay kit (Promega) according to manufacturer's instructions. Cells were seeded at 5000 per well in 96-well plates, and after the cells cultured to different time-points, the Dye Solution was added into the 96-well plates. The plates containing Dye Solution were incubated at 37°C cell culture incubator for 4 hrs. The Solubilization Solution/Stop Mix was added to the culture plates, and the plates were measured at 570 nm with reference wavelength of 630 nm using a 96-well plate reader.

Transwell migration assay

Transwell chambers coated with GFR Matrigel (BD Biosciences) were used for measurement of cell migration. HeLa or CaSki cells (1×10^3) were grown in serum-free RPMI1640 media in the upper side of the insert. The lower well was filled with RPMI 1640 supplemented with 10% FBS. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 hrs. Cells and Matrigel remnants located on the upper-insert membrane were removed, and invading cells from the lower side were fixed, stained and analyzed according to manufacturer's instructions.

Tumor xenograft model

The CaSki cells were collected to construct a single-cell suspension. PBS was mixed with Matrigel (1: 1) to re-suspend the cells and make the final concentration of the cells 1×10^6 cells/100 μl. Female athymic nu/nu mice (Beijing Vital River Laboratory Animal Technology Co.), 4–6 weeks old, were used in this study. After which 14 nude mice (7 for CaSki control cells and 7 for CaSki NRP-1-sh cells) were evenly assigned into two separate groups. After being anesthetized with ether anesthesia, the nude mice were transplanted with 1×10^6 cells on the back of their respective right hind legs. The 14 nude mice were kept in the same environment and observed every 2–3 days to record the length of the tumor. And tumor volume was calculated by the formula: tumor volume=length×width^2/2.

Statistical analyses

Significant differences were determined from at least two independent experiments performed in triplicate and presented as means ± S.D. using Student's *t*-test, and *P*-values <0.05 were considered to indicate statistical significance.

Results

General information of cervical cancer patients

A total of 135 cervical cancer tissues were obtained from patients (121 married and 14 single), among which there were 91 patients older than 40 years old, and 132 HPV (+) cases. Regarding the International Federation of Gynecology and Obstetrics (FIGO) staging, 117 cases were stages I–IIa, 18 cases were stages IIb–IV. According to the tumor size, 104 cases had ≤4 cm tumor, and 31 had >4 cm tumor. Meanwhile, there were 39 had lymph nodes metastasis. Among the 135 patients, 60 cases were low differentiated; meanwhile, 75 cases were middle and high differentiated (Table 1).

NRP-1 was upregulated in cervical cancer patients

The expression of NRP-1 was significantly upregulated in the tumor tissues compared with the CIN and normal tissues (*P*<0.0001; Figure 1A–C); Meanwhile, the proportion of

Table 1 Comparison of general information among the normal controls, CIN patients and CSCC patients

	Normal control (n=36)	CIN patients (n=58)	CSCC patients (n=135)	*P*
Age (years)				
≥40	28 (77.78)	36 (62.07)	91 (67.41)	0.2840
<40	8 (22.22)	22 (37.93)	44 (32.59)	
Marital status				
Married	35 (97.22)	52 (89.66)	121 (89.63)	0.3507
Single	1 (2.78)	6 (10.34)	14 (10.37)	
HPV (+)	14 (38.89)	56 (96.55)	132 (97.78)	<0.0001
HPV (-)	22 (61.11)	2 (3.45)	3 (2.22)	
FIGO Staging				
I–IIa	/	/	117 (86.67)	/
IIb–IV	/	/	18 (13.33)	/
Lymph nodes metastasis				
Yes	/	/	39 (28.89)	/
No	/	/	96 (71.11)	/
Tumor size				
≤4 cm	/	/	104 (77.04)	/
>4 cm	/	/	31 (22.96)	/
Degrees of differentiation				
Low differentiated	/	/	60 (44.44)	/
Middle and high differentiated	/	/	75 (55.56)	/

Abbreviations: CSCC, cervical squamous cell carcinoma; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus.

high NRP-1 staining patients in FIGO stages IIb–IV and lymph nodes metastasis group were higher than FIGO stages I–IIa and no lymph nodes metastasis group, respectively (Table 2). In addition, the mRNA level of NRP-1 was also significantly higher in cancer patients compared with the CIN and normal people (Figure 1D).

NRP-1 expression is associated with patient survival rate

There were 99 cases in the high NRP-1 expression group, of which 5 cases lost to follow-up and the 5-year overall survival rate was 12.05%. For the low NRP-1 expression group, there were 36 cases, of which 2 cases lost to follow-up and the 5-year overall survival rate was 47.06%. It was found that the overall survival time of the high NRP-1 expression group was significantly shorter than that of the low NRP-1 expression group ($P<0.0001$; Figure 1E).

Silence of NRP-1 inhibited proliferation, migration and invasion of cervical cancer cells

NRP-1 was knocked down by shRNA transfection and the efficiency of knockdown was evaluated by using western blot and qPCR (Figure 2). Hela and Caski cells displayed the same tendencies in regards to cell proliferation. The cell proliferation assay showed that NRP-1-depleted cells had dramatically lower rate of proliferation compared to control cells ($P<0.05$, respectively. Figure 3). Hela and Caski cells also displayed the same tendencies regarding cell migration and invasion. The results of "wound-healing" assays showed that the rate of migration of NRP-1-depleted cells was significantly lower than that of the control groups ($P<0.0001$, respectively; Figure 3A). The Transwell assays showed similar results (HeLa $P<0.001$, CaSki $P<0.0001$; Figure 3B).

Silence of NRP-1 inhibited EndMT

Western blotting demonstrated significant changes in the EndMT markers at protein level in Hela and Caski cells (Figure 3C); the results suggested that NRP-1 could promote EndMT in Hela and Caski cells.

NRP-1 associated with tumor growth in mouse models

Based on the anti-proliferative effect of NRP-1 on cancer cells in vitro, we chose CaSki cells to test the anti-tumor growth efficacy of NRP-1 in mouse models. Nu/nu mice were randomly divided into two groups; one group was inoculated subcutaneously (s.c.) in flank with 1×10^6 CaSki-control cells, while the other group was inoculated with CaSki-NRP-1-sh cells. Tumor growth was monitored and measured every two or three days. Tumor volumes were calculated by the equation V (mm^3)=$a\times b^2/2$, where "a" is the largest diameter and "b" is the perpendicular diameter (Figure 4B). Mice were sacrificed and tumors were collected and weighed (Figure 4C), and photographed after 24 days (Figure 4A). The expression of NRP-1 in tissue was verified by western blotting (Figure 4D). Notably, depletion of NRP-1 significantly suppressed the growth of CaSki xenograft tumor in nude mice.

Discussion

It is known that the growth of new blood vessels is needed for solid tumors to grow and metastasize. Angiogenesis provides nutrients for cancer cell proliferation and removes tumor metabolic waste.[16,17] As an essential

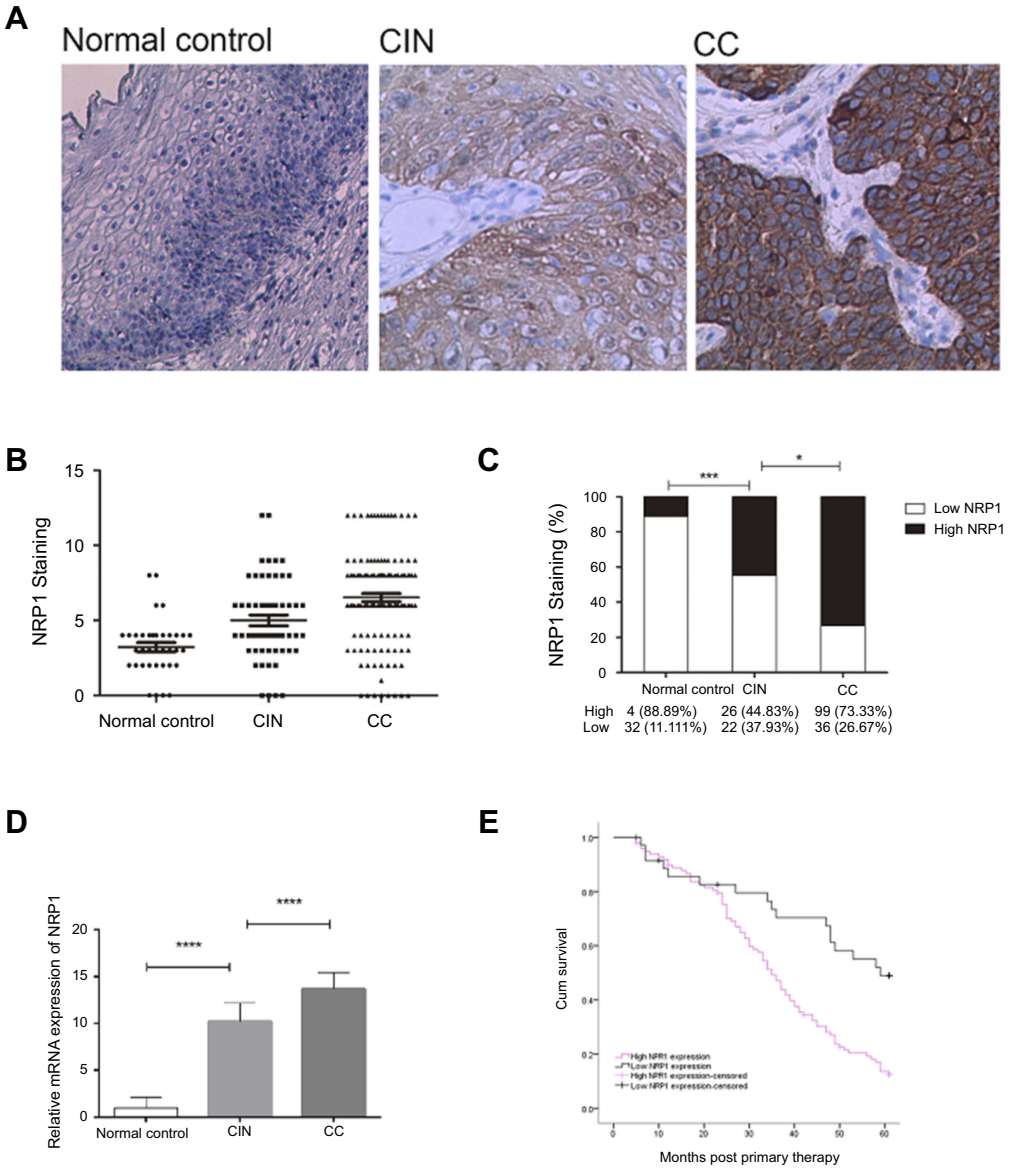


Figure 1 Comparison of NRP-1 expressions among the CC, CIN and normal cervical tissues. (**A**) Immunohistochemistry image of NRP-1 expressions in CC (n=135), CIN (n=58) and normal cervical tissues (n=36) (×400); (**B**) Kruskal-Wallis test for differences in NRP-1 staining among CC, CIN and normal cervical tissues. The mean is depicted as a horizontal line in each group ($P<0.0001$). (**C**) Comparisons of NPR1 expressions among the CC, CIN and normal cervical tissues (3 slices for each specimen to obtain the mean values), NRP-1 expression was increased from normal control to CIN, and CC (normal control to CIN $P<0.001$, CIN to CC $P<0.05$; Chi-square test); (**D**) Comparisons of NRP-1 mRNA expression in CC, CIN and normal cervical tissues, NRP-1 mRNA expression was increased from normal control to CIN, and CC (normal control to CIN $P<0.0001$,CIN to CC $P<0.0001$; *t* test); (**E**) Overall 5-year survival of patients with CC ($P<0.0001$; log-rank test). The CC patients were divided into high NRP-1 expression and low NRP-1 expression groups. A high expression of NRP-1 was strongly associated with a poorer patient survival time.

Abbreviations: NRP-1, neuropilin 1; CC, cervical cancer; CIN, cervical intraepithelial neoplasia; High NRP-1 expression-censored, patients with high NRP-1 expression lost to follow-up; Low NRP-1 expression-censored, patients with low NRP-1 expression lost to follow-up.

player in angiogenesis, VEGF mainly operates by interacting with three receptors: VEGFR-1, VEGFR-2 and NRP-1.[18] NRP-1, by interacting with VEGF, prevents tumor cell apoptosis and regulates angiogenesis, and thus appears as a promising molecular target for the development of anti-angiogenic drugs. NRP-1 overexpression has been reported in a variety of human cancers, including cancer from prostate, kidney, bladder, stomach, colon, pancreas, breast, ovary, lung, liver, nasopharynx and brain.[19–25] In most solid tumors, high expression of NRP-1 is significantly correlated with clinical stage, angiogenesis, node invasion and poor survival. In this study, we demonstrated the increased expression of NRP-1 protein in cervical cancer patients suggesting that the upregulation of NRP-1 may be an early event during the carcinogenesis of cervical cancer.

Table 2 Comparison of NRP1 staining and clinicopathologic characteristics of CC patients

Variables	NRP1 staining		Total	*P*
	Low	High		
All patients (n=135)				
Age (years)				
≥40	18 (20.00)	72 (80.00)	90 (66.67)	0.0132
<40	18 (40.00)	27 (60.00)	45 (33.33)	
Histological types				
SCC	24 (30.77)	54 (69.23)	78 (57.78)	0.2073
ADC	12 (21.05)	45 (78.95)	57 (42.22)	
FIGO Stages				
I–IIa	35 (29.91)	82 (70.09)	117 (86.67)	0.0296
IIb–IV	1 (5.56)	17 (94.44)	18 (13.33)	
Lymph nodes metastasis				
Yes	2 (5.12)	37 (94.87)	39 (28.89)	0.0003
No	34 (35.42)	62 (64.58)	96 (71.11)	
Degrees of differentiation				
Low differentiated	18 (31.58)	39 (68.42)	57 (42.22)	0.2699
Middle and high differentiated	18 (23.08)	60 (76.92)	78 (57.78)	

Abbreviations: CC, cervical cancer; SCC, squamous cell carcinoma; ADC, adenocarcinoma.

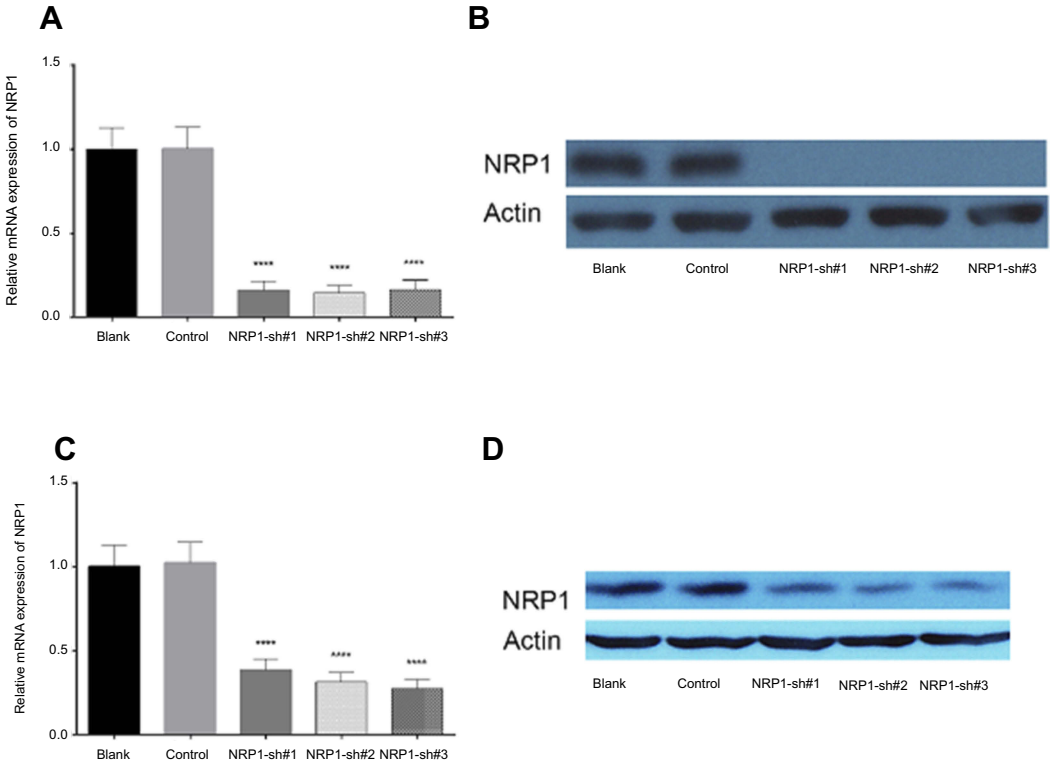


Figure 2 NRP-1 expression in the blank, control and NRP-1-sh groups. (**A** and **C**) Relative expression level of NRP-1 mRNA after lentiviral infection in HeLa (**A**) and CaSki (**C**) cells ($P<0.0001$; *t* test), compared with the control group, respectively; **B** and **D**, NRP-1 protein expression after lentiviral infection in HeLa (**B**) and CaSki (**D**), respectively.

Abbreviation: NRP-1, neuropilin 1.

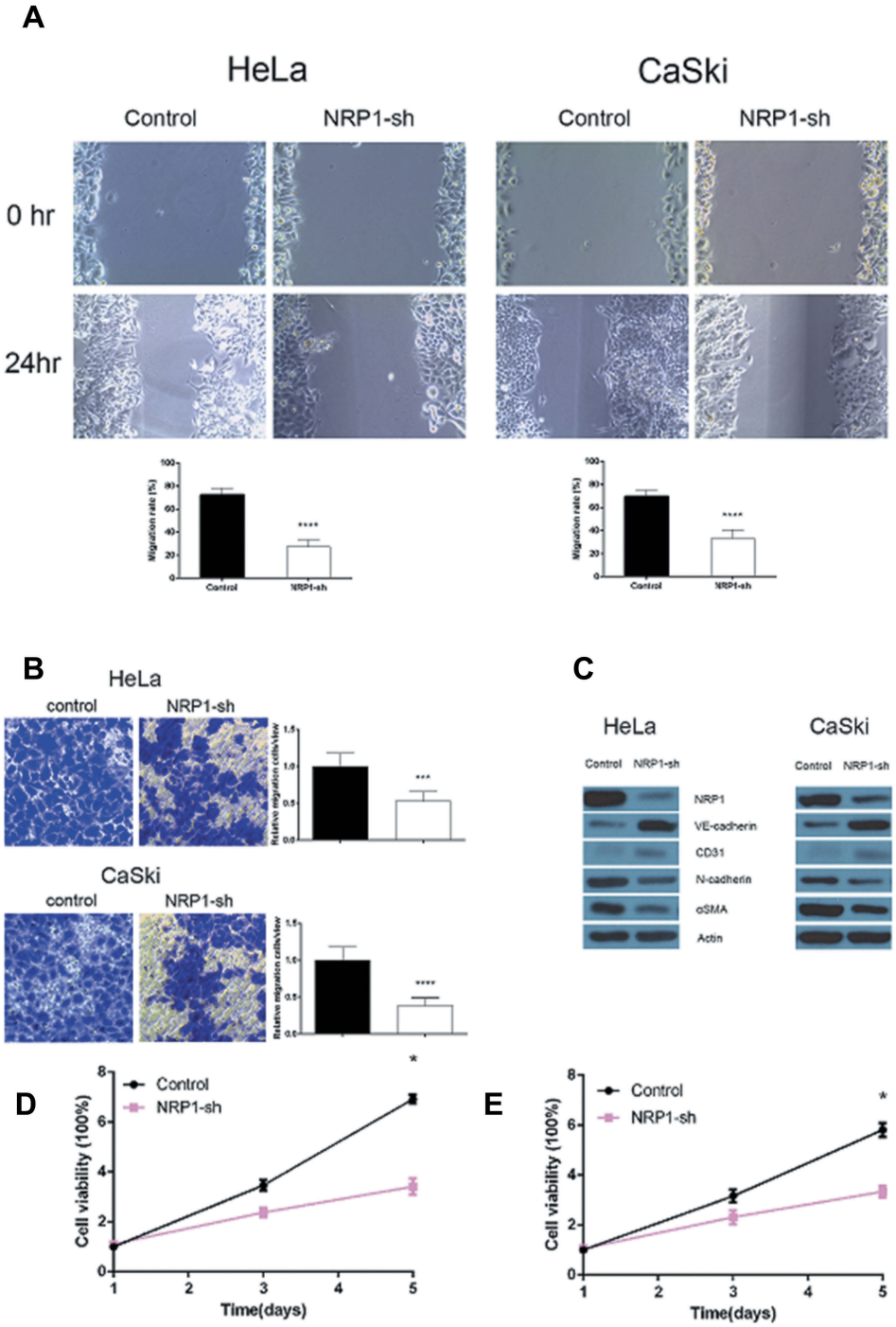


Figure 3 HeLa and CaSki cell migration ability, invasion ability, growth curves in the control and NRP-1-sh groups. (**A**) Images of scratching test of HeLa and CaSki cells, comparison of cell migration ability among the control and the NRP-1-sh groups ($P<0.0001$, respectively; *t* test); (**B**) Images of Transwell assay of HeLa and CaSki cells, comparison of cell invasion ability among the control and the NRP-1-sh groups ($P<0.001$, $P<0.0001$, respectively; *t*-test); (**C**) NRP-1 levels positively correlate with EndMT markers at the protein level in HeLa and CaSki cells; (**D**) Cell growth curve of HeLa cells ($P<0.05$; *t* test); (**E**) Cell growth curve of CaSki cells ($P<0.05$; *t* test).
Abbreviations: NRP-1, neuropilin 1; EndMT, endothelial-to-mesenchymal transition.

It has been demonstrated by preclinical data that anti-NRP-1 antibodies have additive anti-cancer activity in combination with anti-VEGF therapy.[11]However, sometimes, anti-angiogenic hypothesis fails to take into consideration like that in patients, because tumor cells may proliferate in the absence of neo-angiogenesis by modifying and co-opting the existing vasculature.[26] Compared to the main receptors of VEGF, the function of NRP-1 in conjunction with multiple receptors to guide vascular development remains elusive, and it is considered that the role of NRP-1 promote-tumor is not

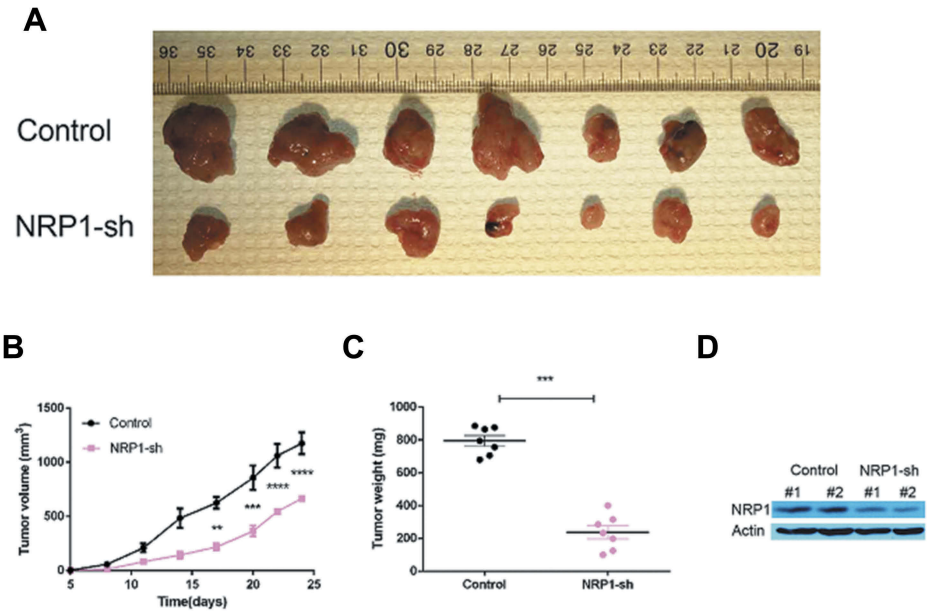


Figure 4 Tumor volume and weight in the control and NRP-1-sh groups. (**A**) Images of tumor in mice inoculated with control and NRP-1-sh CaSki cells; (**B**) Broken line graph of tumor growth of control and NRP-1-sh CaSki cells ($P<0.0001$; two-way ANOVA test); (**C**) Comparison of tumor weight of control and NRP-1-sh CaSki cells ($P<0.001$; *t* test); (**D**) Comparison of NRP-1 protein level in tumor tissue inoculated with control and NRP-1-sh CaSki cells.
Abbreviation: NRP-1, neuropilin 1.

only angiogenesis. In human FG pancreatic cells, overexpression of NRP-1 induce both Erk1/2 and JNK signaling pathways;[27] preclinical data also support a role for NRP-1 in mediating lung and renal cancer cell proliferation and invasion.[28,29]It has been declared that high level expressing of NRP-1 in tumor cells was implicated in cell migration and survival,[30,31] and in this study, when we down-regulated NRP-1 in HeLa and CaSki cells, the results of cell proliferation assay displayed that the proliferative ability of CC cells reduced, and migratory invasion ability also reduced according to the result of Scratch assays and Transwell migration assay. When we injected the NRP-1 knocked down CC cells into mice, the tumorigenic ability reduced compared to the control group in vivo. As we known, EndMT regulates different aspects of tumorigenesis,[32] while it plays an important role in cervical cancer progression and metastasis. In our study, we compared the expression of EndMT markers between NRP-1 knockdown group and control group, and the results showed that the expression changes of EndMT markers associated with the silence of NRP-1 suggested that NRP-1 could change the ability of CC cells through EndMT.

As we know, CC is HPV-associated cancer, and in spite of the routine screening procedures can detect many CC patients in early stage, but Pap smear test has high false positive and false negative results due to operator readings.[33] In addition, HPV nucleic acid detection and biopsy tissue are invasive, require skilled crew, expensive reagents/equipment, sophisticated and concentrated laboratory infrastructure;[34] therefore, rapid, inexpensive and easy-to-use pretesting assays for identifying high-risk populations for further examination are urgently needed in limited resources area, such as Henan province, which is underdeveloped area in China. Unfortunately, none of the biomarkers offer sufficient prognostic value until now, and as such, none serve as an ideal triage method.[35,36] Circulating biomarkers that allow monitoring of fundamental molecular changes in CC may improve the detection of patients who have a high risk of progression in both primary screening and triage settings. In this study, NRP-1 expression is associated with patient survival rate. The prognostic effect of NRP-1 on the overall survival rate of CC patients was investigated, and NRP-1 expression is associated with patient survival rate by comparing the 5-year survival rate of patients using Kaplan-Meier survival curves and the log-rank test, and it is the same tendency in CC mice model. Similar to our results, Shouhua Yang et al[7] reported that both soluble neuropilin-1 (sNRP-1) in circulating and NRP-1 protein in cervical tissues were correlated with CC stage, and sNRP-1 presented a high diagnostic ability of CC and CIN, with a sensitivity of 70.97% and a specificity of 73.68%; therefore, it is a promising hypothesis that sNRP-1 in circulating and NRP-1 in cervical tissues can serve as a possible valuable diagnostic biomarker for CC. Such

findings further implicate NRP-1 as a cell survival factor in CC, demonstrate that NRP-1 may not only serve as a diagnostic biomarker in early stage CC, but also could be a biomarker for cervical progression and prognosis, and NRP-1 represents a promising new target for cancer therapies.

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Disclosure

The authors declare that there are no conflicts of interest in this work.

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