



Numb suppression promoted the pathogenesis of SiHa cells by stimulating Notch and Hedgehog signaling pathways

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Background: Cervical cancer is a life-threatening disease prevalent in the female population. Although many important advances have taken place in the diagnosis and treatment of cervical cancer, its mechanisms have not been fully elucidated to date. The aim of this study was to clarify the functional role of Numb in human cervical squamous carcinomas.

Methods: The expression and location of Numb in SiHa cell lines was detected by Western blotting and immunofluorescence. Numb gene knockdown using shRNA plasmid and a Numb transfection system were used to assess its function *in vivo* and *in vitro*. Twenty-five normal cervical epithelium, 60 CIN, 203 squamous carcinoma and 21 lymph node tissue specimens of the cervix tissue were collected from clinical specimens. The expression of Numb protein was detected by Western blotting and immunofluorescence, and data on clinical variables were collected by retrospective chart review. The mechanistic studies of the Numb regulation progression were identified by Western blotting.

Results: Ectopic expression of Numb confers cervical squamous carcinomas with malignant features including malignant proliferation, migration and invasion. Meanwhile, Numb repression in tumor cells dramatically accelerated the progression of cervical squamous carcinomas in xenograft mouse model. Furthermore, our data revealed that Numb inhibition promotes malignant transformation of cervical squamous carcinoma through stimulation of Notch and Hedgehog-dependent pathways. Clinical evidence also showed that in squamous cervical cancer with different lymph node metastases, Numb expression was significantly lower in the lymphatic metastasis group than in the no lymph node metastasis group.

Conclusions: Collectively, our findings indicated that ectopic expression of Numb was an important factor in the progression of cervical cancer and that low Numb expression levels can be used as an indicator of the prognosis of squamous cervical cancer.

Keywords: Cervical cancer; Numb; Notch; prognosis

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Introduction

Cervical cancer is the prevalent life-threatening disease diagnosed in the female population today. Cervical cancer still the commonest cause of cancer deaths among women in developing countries with large geographic variations in cancer incidence and mortality rates (1-5). Detection of cervical cancer at an early stage is associated with excellent survival. However, patients with advanced cervical cancer had a higher mortality, especially in developing countries. The ratio between incidence and mortality from cervical cancer remains high due to lack of access to appropriate anti-cancer therapies (6-8).

Previous study indicated that human papillomavirus infection has been the major causes in most cervical cancer cases and genetic alterations play the key role for tumor genesis and progression (9-11). Recent advances in the biology of cervical cancer revealed genetic changes is common in cervical carcinogenesis and metastasis (12-14). Genetic alteration due to point mutation and chromosome translocation has been extensively studied in cervical cancer (15-17). Recent research strategies keep insight into tumor suppressors. Meanwhile, significance has been grown to study the utility of these changes as biomarkers to determine disease progression as well as use them as therapeutic targets (18-21).

The human Numb gene is located at chromosome 14q24.3 which was known as the “cell fate determinant” and its encoded protein is the first signal protein which was found to regulating cell asymmetric division (22-24). The current

research on Numb is mainly focused on the development of the nervous system (24-27). Numb is involved in the directed differentiation of neural precursor cells. It is asymmetrically distributed into two daughter cells during cell division (28-30). More recently, studies have shown that asymmetric division of cell fate determinants can affect cell proliferation and differentiation (31-34). Which implied the function of Numb may be related to tumorigenesis or progression.

Here, we investigated the involvement of Numb in development of cervical squamous carcinoma progression. In the study, we showed that repression of Numb in cervical squamous carcinoma cells lead to malignant progression *in vitro* and *in vivo*. Further mechanistic studies identified the Numb reduction promoted cervical squamous carcinoma malignant progression through Notch and Hedgehog pathways. Meanwhile, the clinical evidences we provided were in correspondence with our experiments of Numb function. In summary, our data provided mechanistic insight into Numb reduction-induced malignant transformation, enhance our understanding of the events in progression of cervical squamous carcinoma and establish Numb reduction as a viable predictor to the prognosis of cervical squamous carcinomas. We present this article in accordance with the ARRIVE reporting checklist (available at <https://gpm.amegroups.com/article/view/10.21037/gpm-23-30/rc>).

Methods

Materials, cell lines and agents

HaCaT (ATCC[®]PCS-200-011TM) and SiHa (ATCC[®]HTB-35TM) cells were from the American Type Culture Collection (ATCC; Rockville, MD, USA) and these cells were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Caoyuan Iyve, Huhht, China). YOYO-1, Hoechst 33342, were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Horseradish peroxidase (HRP)-labeled goat anti-rabbit, anti-mouse secondary antibodies or anti-Numb antibody were provided by Cell Signaling Technology (Notch1, 70109; Hes, 11988; Shh, 2207) (Danvers, MA, USA) and Gene Tex (Smo, GTX128298) (California, USA). All other chemicals were purchased from Sigma-Aldrich (Shanghai, China) and used without further purification.

Cervical tissue samples

Between January 2019 and October 2021, we collected 25 normal cervical epithelial tissue specimens, 60 CIN tissue

Highlight box

Key findings

- Numb inhibition promotes malignant transformation of cervical squamous carcinoma through stimulation of Notch and Hedgehog-dependent pathways.

What is known and what is new?

- The current research on Numb is mainly focused on the development of the nervous system.
- We found that ectopic expression of Numb was an important factor in the progression of cervical cancer and that low Numb expression levels can be used as an indicator of the prognosis of squamous cervical cancer.

What is the implication, and what should change now?

- Numb reduction is an important contributor to cervical cancer progression, rationalizing low expression level of Numb may be a predictor to the prognosis of cervical squamous carcinomas.

specimens, 40 cervical invasive cancer tissue specimens and 15 cervical cancer lymph node tissue specimens. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Medical Ethics Committee of West China Second Hospital of Sichuan University (No. 2020126) and informed consent was obtained from all individual participants. Cervical cancer tissue chips CR806 and CR2085 were purchased from Xi'an Elena Biotechnology Co., Ltd. 163 cases of cervical squamous carcinoma and 6 cases of lymph node metastasis of cervical squamous carcinoma were involved in the analysis.

Preparation of SiHa^{Numb-} cell line

First, a custom shRNA plasmid was designed to target human Numb gene (Open Biosystems). We screened one of the four shRNAs achieving 90% knockdown efficiency by RT-PCR. The sequence of Numb interference sites: CACATTTACTGAAATGTAA. Subsequently, the lentiviral transduction was performed as described (35). Briefly, 293 T cells were co-transfected with pMD2.G, pPAX2 (Addgene), and pGIPZ shRNA plasmids. For infections, cells were incubated with viral supernatants in the presence of 8 µg/mL polybrene. After 48 h, the infected cells were selected with puromycin (2 µg/mL) for SiHa^{Numb-} clones for 3–4 days and confirmed by western blot before experiments.

Xenograft experiments in vivo

Female Balb/c nude mice (4–5 weeks) were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). The animals were used for anti-tumor tests *in vivo*, which were housed at a controlled temperature of 20–22 °C, relative humidity of 50–60% and 12 h light-dark cycles. Five-week-old female nude mice were obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China). All animal experiments were carried out in accordance with the Guide for Care and Use of Laboratory Animals, approved by the Ethics Committee of Sichuan University (Chengdu, China). SiHa and SiHa^{Numb-} tumors were established by subcutaneous injection of 1×10^7 cells in the right flank of mice. Tumor length and width were measured every 4 days and tumor volume (TV) was calculated using the following formula: $TV = 0.5 \times \text{length} \times \text{width}^2$. At the end of the experiment, mice were sacrificed. Solid tumors were removed and processed for immunohistochemical analysis. Mice were killed by cervical dislocation after deep

anesthesia with 2% isoflurane. All animal work was approved by Sichuan Animal Care (Chengdu, China). All mice were treated humanely throughout the experimental period.

Western blot analysis

For Western blotting analysis, the cells and samples were harvested, and then resuspended in RIPA. After incubation on ice for 1 h, the cell lysates were centrifugated for 15 min at 15,000 ×g. The protein concentration was determined by BSA protein assay kit and ~50 µg protein was applied to 10% SDS-PAGE gels and incubated with antibodies including Numb, Nothc1, Hes1, Shh and Smo at 4 °C overnight. Blots were developed with HRP-conjugated secondary antibodies and the protein bands were visualized using a chemiluminescence (ECL) detection system.

Immunofluorescence determination

Based on the results of Western blotting, we further confirmed the expression of Numb protein in cervical epithelial tissues at different pathological stages by immunofluorescence histochemical staining.

To assess the Numb level in mice tumor, we investigated Numb staining by the labeled streptavidin-biotin method. The primary antibody was rabbit anti-mouse polyclonal antibody Numb (BD, USA) and the secondary antibody was biotinylated goat anti-rabbit immunoglobulin (Abcam, USA).

Following the Immunofluorescent analysis protocol, frozen sections 4–5 µm thick of tumor tissue were fixed in cold acetone, washed with PBS, stained with Numb (1:200, BD, USA) overnight at 4 °C, washed twice with PBS, and incubated with a Cy3-conjugated second antibody (1:1,000, Abcam, USA) for 40 min. Toto-3/DAPI was added to stain nucleus (1:500). In the Numb staining tests, all the five tumors in each group were sectioned. In tumor tissue sections, five equal-sized fields were randomly chosen and analyzed. The expression of Numb was evaluated by calculating the average number of small Numb-positive cells (red) in five randomly selected tumor areas in each tumor sample by two independent investigators in a blinded fashion under a fluorescence microscopy.

Wound healing, Transwell invasion assay

The wound healing assay was performed using SiHa and SiHa^{Numb-} cells. SiHa and SiHa^{Numb-} were wounded by scratching with pipette tips and washed with PBS. After 24 h,

images were taken by an Olympus digital camera. The migrated cells were quantified by manual counting, and the percentage of inhibition was expressed using untreated cells at 100%.

Transwell invasion assay was conducted as described previously with some modifications. The filter of the Transwell plate (Millipore) was coated with 50 μ L Matrigel (BD Bio-sciences). After Matrigel polymerization, the bottom chambers were filled with DMEM medium containing 20% FBS and the top chambers were seeded with 100 μ L DMEM medium (without FBS) and SiHa and SiHa^{Numb-} (4×10^4 cells per well) and were allowed to migrate for 24 h. Cells on the upper side of the filter were removed. Cells located on the underside of the filter were fixed with methanol and stained with 0.5% crystal violet, then, migrated cells were quantified by manual counting and photographed under a light microscope. The percentage of migrated cells inhibition was expressed on the basis of medium-treated cells as control wells.

Preparation of SiHa^{Numb-}

Short-hairpin RNAs (shRNAs) can be transduced to suppress gene function by RNA interference, which can model the impact of aberrant expression of target gene. By introducing shRNA targeting Numb into cervical squamous carcinoma cells SiHa, we can study the biological behavior alternation of SiHa. shRNA targeting Numb were linked to puromycin resistance, allowing cells transduced with shRNA to be selected by puromycin *in vitro*.

***In vitro* proliferation analysis**

The SiHa and SiHa^{Numb-} cells were analyzed by EdU detection kit to detect the cell proliferation. All experiments were performed at least in triplicate.

Statistical analysis

Statistical analysis was performed by one-way ANOVA unless otherwise specified. Significant differences between groups were indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Results

The Numb expression is different in tumor and normal cells

We first investigated the expression of Numb in squamous

carcinoma of cervix cell lines SiHa, and non-tumorigenic keratinocytes HaCaT by western blot (*Figure 1A*) and immunofluorescence (*Figure 1B*). As shown, the Numb expression is obviously lower in SiHa than it in the HaCaT cells. Interestingly, the Numb expression in the HaCaT cells was well distributed (*Figure 1B*), while the expression in SiHa nucleus decreased significantly which was mainly detected in cell membrane and cytoplasm (*Figure 1B*).

Numb suppression endowed malignant features of cervical squamous carcinoma cells in vitro

Because of the different Numb expression between SiHa and HaCaT cells, we want to identify how Numb influence the cell proliferation, migration and invasion. We used RNA interference to characterize Numb functions *in vitro*. In this approach, various genetic elements are introduced into cervical squamous carcinoma cells SiHa by lentiviral mediated gene transfer. We obtained SiHa^{Numb-} cells verified by the protein expression of Numb and observed a striking suppression of Numb on SiHa^{Numb-} cells (*Figure 1C*). Subsequently, we investigated the alteration of biological behavior including cell proliferation, migration and invasion. The SiHa^{Numb-} cells displayed higher cell proliferation rate than SiHa cells as shown using EDU assay (*Figure 1D*, * $P < 0.05$). Meanwhile, as shown in *Figure 1E-1H*, SiHa^{Numb-} cells exhibited enhanced invasion and migration by Transwell test and wound healing assay. These observations indicated that SiHa^{Numb-} cells expressed malignant traits in response to Numb suppression compared with SiHa cells, in terms of enhanced cell proliferation, migration and invasion (* $P < 0.05$).

Numb suppression aggravated tumor malignance progression in xenograft mouse model in vivo

To investigate the role of Numb in tumor progression *in vivo*, we transplanted equal numbers of cervical squamous carcinoma cells SiHa or SiHa^{Numb-} into the back flanks of nude mice and monitored their growth. All mice were sacrificed at day 28, whereas group with SiHa^{Numb-} cells increased tumor size and weight, with a growth enhancement compared with SiHa groups *in vivo* (*Figure 2A-2C*). Immunohistochemistry analysis showed that the expression of Numb in malignant tumors formed by SiHa^{Numb-} cells was lower (* $P < 0.05$) (*Figure 2D*). Collectively, our observations indicated that suppression of Numb in SiHa cervical squamous carcinoma cells dramatically accelerated

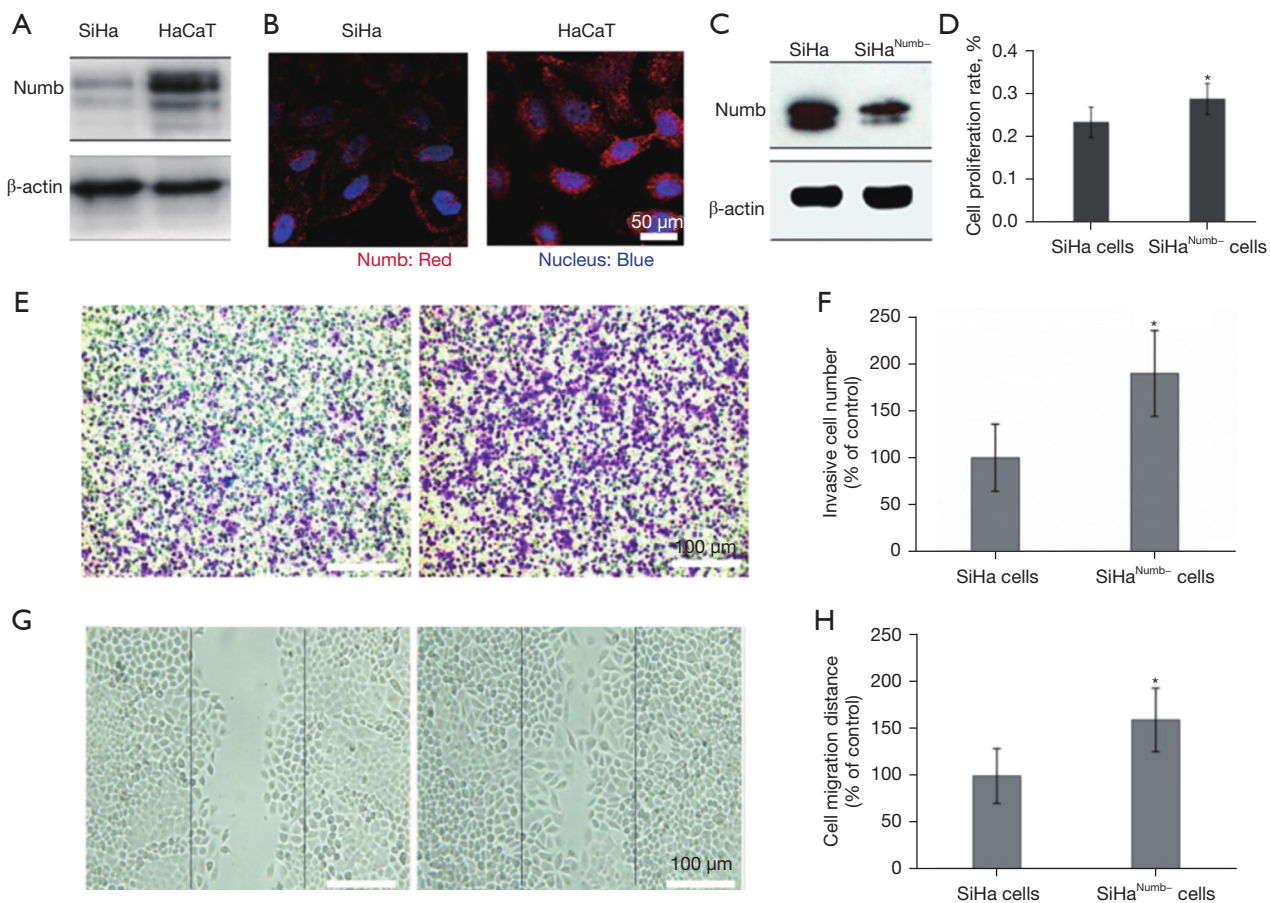


Figure 1 Assay the influence of Numb on the biological behavior of cervical cancer cell SiHa. The Numb protein expression in HaCaT and SiHa cell lines by Western blot (A) and immunofluorescence (B). Numb protein expression was decreased significantly after RNAi in cells by Western blotting (C). The shRNA-Numb SiHa stable cell lines were selected by puromycin. The different cell proliferation rate was showed in D. The different of cell invasion was showed by Transwell test (stained with 0.5% crystal violet) (E,F) and the cell migration was observed by Wound healing assay (G,H). *, $P < 0.05$.

tumor growth and Numb supposedly function as tumor suppressor gene.

Suppression of Numb upregulated Notch1, Hes1, Shh and Smo expression

The aforementioned findings prompted us to investigate how Numb suppression in tumors accelerated malignant progression. To test this, we examined using by western blot the protein levels of components involved in tumor-related pathways. As shown in *Figure 3A,3B*, depletion of Numb in cervical squamous carcinoma cells SiHa resulted in a statistically significant increase of Notch1 and Hes1 protein compare with SiHa cells. Interestingly, a similar effect on Shh and Smo were observed after Numb depletion

(*Figure 3C-3E*, $*P < 0.05$).

Correlations between Numb expression and clinicopathological characteristics

To further assess the clinical relevance of our findings in cervical cancer, we investigated the Numb expression of clinical samples collected from patients suffering from cervical cancer with different clinicopathological characteristics. CIN is an intraepithelial neoplasia of the uterus and classified from CIN I to CIN III grades by histology characteristics which represent a transitional stage from normal tissue to tumors of cervical squamous carcinoma. Therein, the grade of CIN III is severe dysplasia with undifferentiated neoplastic cells, also be referred to as

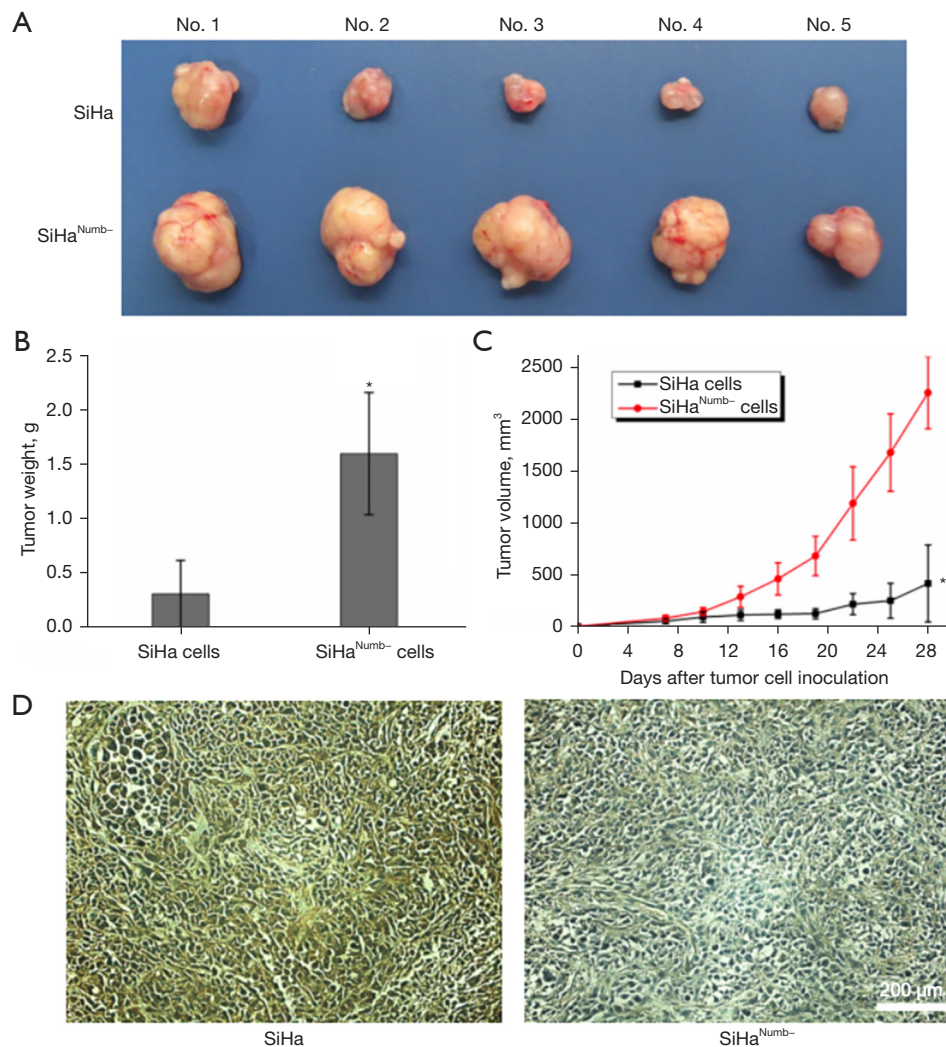


Figure 2 *In vivo* analysis of tumorigenesis. (A) Representative pictures of SiHa and SiHa^{Numb-} tumor in nude mice. (B,C) Tumor weight and tumor volume of SiHa and SiHa^{Numb-}. (D) Immunohistochemistry analysis of the expression of Numb in SiHa (left) and SiHa^{Numb-} (right). *, $P < 0.05$.

cervical carcinoma in situ. In comparison with normal and CIN III group, the Numb expression in cervical squamous carcinoma was significantly lower than that in the two groups (Figure 4A). As shown in comparison of Numb expression between normal group, CIN group and cervical squamous carcinoma group, statistical analysis indicated that Numb protein was reduced in tumors compared with normal tissues and the expression of Numb gradually decreased from normal group to tumor group by western blot (Figure 4B). As shown in the comparison of Numb expression between cervical squamous carcinomas with different clinical stages, the expression of Numb protein in stage IIB and above was significantly lower than that in

group I-IIA, and the difference was statistically significant ($*P < 0.05$) (Figure 4C). In addition, as shown in comparison of Numb expression between cervical squamous carcinomas with different pathological grades, from high differentiation to poor differentiation, the expression of Numb gradually decreased with the increase of pathological grade. There was a statistically significant difference in the expression of Numb between the three groups ($*P < 0.05$). Compared with the highly differentiated group, the moderately differentiated group and the poorly differentiated group, the expression of Numb was significantly decreased, and the difference was statistically significant ($*P < 0.05$). Similarly, immunofluorescence revealed that the levels of

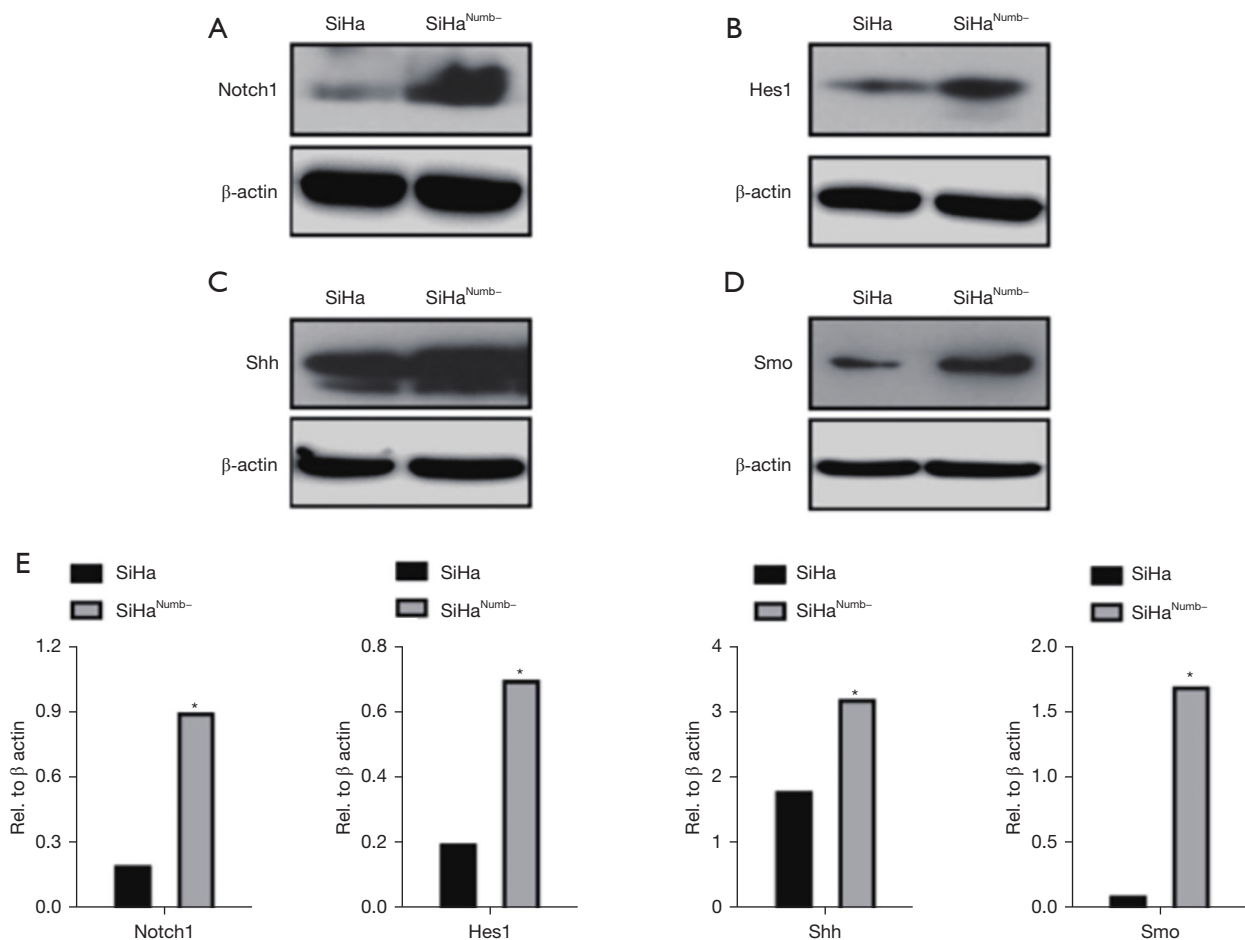


Figure 3 Numb suppression upregulated the Notch and Hedgehog signaling pathways. The expression of Notch 1 (A), Hes 1 (B) in Notch signaling pathway; and Shh (C) and Smo (D) protein in Hedgehog signaling pathway in SiHa and SiHa^{Numb-} cells were detected by Western blotting assays; (E) the quantification of Western blot. *, P<0.05.

Numb protein gradually decreased with the increase of pathological grade (Figure 4D). As shown in comparison of Numb expression between cervical squamous carcinomas with different lymph node metastasis, the expression of Numb in the lymphatic metastasis group was significantly lower than that in the no lymph node metastasis group (*P<0.05) (Figure 4E). The clinical data indicated low expression level of Numb may be a significant putative predictor to the prognosis of cervical squamous carcinomas.

Discussion

The study of the relationship between Numb and cancers was initiated by the P. P. Di Fiore laboratory. During embryonic development, Numb and Notch played antagonistic roles in asymmetric cell division, and enhanced

Notch signaling pathways had been shown to be associated with tumorigenesis (36). Therefore, the Numb-Notch axis may be one of the causes of tumor genesis.

Previous studies found that the expression of Numb decreased in many tumors (37-39). These findings indicated that lack of Numb expression was closely related to tumor genesis or progression. In breast tumors, deletion of Numb resulted in excessive activation of Notch and inhibition of TP53 activity, both of which are responsible for the transformation. Thus, Numb was a tumor suppressor in breast cancer, and its functional inhibition promoted tumorigenesis by activating the oncogenic pathway (Notch) and inhibiting the tumor suppressor pathway (TP53) (40,41). In non-small cell lung cancer, Numb expression was also frequently lost, and similarly activation of the Notch pathway can also be observed. In this case, the inability to

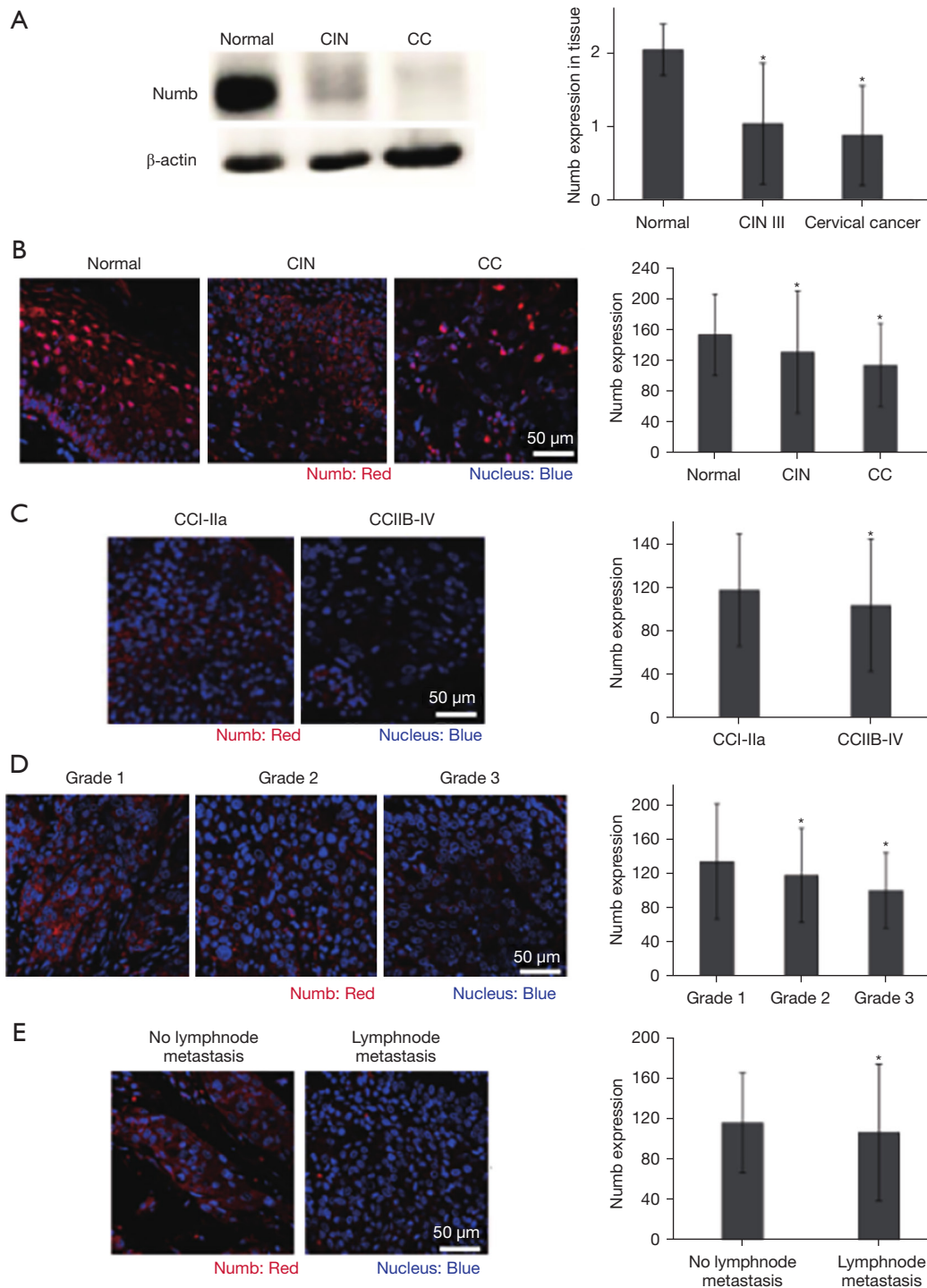


Figure 4 Correlations between Numb expression and clinicopathological characteristic (A) Western blot analysis of the Numb expression in human normal tissue, CIN III and CC tissues. Immunofluorescent staining under confocal microscopy of Numb (B, left) and statistical analysis between normal human cervical tissue group (normal), CIN group and CC group (B, right); Numb in cervical cancer stage I-IIa and stage IIb-IV (C, left) and quantitative analysis (C, right); Numb (D, left) and quantitative analysis of Numb during three histopathological grades (D, right). Well differentiated (grade 1), moderately differentiated (grade 2), and poorly differentiated group (grade 3); Numb (E, left) and quantitative analysis of Numb between no lymph node metastasis group and lymph node metastasis group (E, right). *, $P < 0.05$. CC, cervical cancer; CIN, cervical intraepithelial neoplasia.

demonstrate a correlation with prognosis may be due to the confounding influence of additional, Numb-independent mechanisms causing Notch activation in these tumors, which was also supported by the fact that poor prognosis was associated with Notch activation (42). In breast and non-small cell lung cancers, loss of Numb expression due to exaggerated ubiquitination and ensuing degradation, despite the absence of genetic alterations in the NUMB locus (29,42). In these tumors, the genetic lesions upstream of Numb are still unknown. It may be present in a component of the ubiquitination/de-ubiquitination machinery, although studies of E3-ligands in breast cancers had not found alterations immediately consistent with the Numb status of these cancers. It had been shown that reduced Numb levels was also associated with poor prognosis in salivary gland cancers, although the exact mechanism of these tumors was unknown (22). It was further found that Numb was methylated by lysine methyltransferase Set8, which led to the increased Numb-p53 ubiquitination degradation and affected apoptosis ability (38). As a component of adhesion junction, Numb can also regulate cell adhesion and migration and participates in the ubiquitination of p53 and Gli1 (the main effector of Hedgehog signal), this affected the stability of GLI1 and inhibited Hedgehog signaling through a mechanism very similar to that of Notch signaling attenuation in mammals. In addition, Numb, expressed in granulocyte progenitors, inhibited the Hedgehog pathway, leading to its differentiation. As the mechanism between Notch and Hedgehog involves normal developmental programs and tumorigenesis, Numb may serve as a master regulator of both basic and closely integrated morphogenetic systems (43,44).

Previous studies have reported that Numb may play a role in tumorigenesis. However, the possible roles of Numb in the progression of cervical cancer have rarely been investigated. In this research, we found that a decrease in Numb was associated with malignant features of cervical squamous carcinoma, including malignant proliferation, migration and invasion *in vitro*, and greatly accelerated the progression of cervical squamous carcinoma in an *in vivo* xenograft mouse model (Figures 1,2). Mechanistic studies revealed that Numb inhibition stimulated Notch- and Hedgehog-dependent malignant transformation and promoted the progression of cervical squamous carcinoma (Figure 3). Clinical evidence also supported the involvement of loss of Numb function in the progression of cervical squamous carcinomas (Figure 4). Our findings identified Numb reduction as an important contributor to cervical

cancer progression, rationalizing low expression level of Numb may be a predictor to the prognosis of cervical squamous carcinomas.

Conclusions

In summary, we proposed that the low expression of Numb promoted cervical squamous carcinoma cells proliferation, migration and invasion, greatly accelerated the progression of cervical squamous carcinoma in a xenograft mouse model. Furthermore, our data also showed that Numb inhibition promoted malignant transformation of cervical squamous carcinoma through stimulation of Notch- and Hedgehog-dependent pathways. What's more, clinical evidence also supported that loss of Numb function promoted the progression of cervical squamous carcinoma. Our findings provided a new mechanism for understanding the deterioration of cervical squamous carcinoma cells and helped develop therapeutic strategies for treating cervical carcinoma.

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Footnote

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from July 2023 to June 2024. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Medical Ethics Committee of West China Second Hospital of Sichuan University (No. 2020126) and informed consent was obtained from all individual participants. All animal experiments were carried out in accordance with the Guide for Care and Use of Laboratory Animals, approved by the Ethics Committee of Sichuan University (Chengdu, China).

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