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Notch is a critical regulator in cervical cancer by regulating Numb splicing

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Abstract. Cervical cancer, which is the second most common female malignancy, is characterized by the consistent presence of human papillomavirus. Inappropriate activation of Notch signaling has been associated with various types of cancer; however, the role of Notch in cervical cancer remains unclear. The present study aimed to investigate the role of Notch in cervical cancer. The methods used included the generation of plasmids, viability assays, polymerase chain reaction and western blotting The present findings demonstrated that cervical cancer samples also consistently exhibit abnormal activation of the Notch pathway. The data also indicated that different Numb isoforms may have opposite effects on the proliferation of cervical cancer cells. As a result, the activated Notch signaling pathway regulates the alternative splicing of the Numb gene, which affects the proliferation of the cervical cancer cells. These findings suggest that activated Notch signaling may lead to the development of cervical cancer by regulating Numb splicing. Thus, Numb splice variants may be a potential clinical marker for indicating cervical cancer genesis and development.

Introduction

Cervical cancer, which is the second most common female malignancy in terms of both incidence and mortality, threatens the health of women worldwide (1). Human papillomavirus (HPV) infection was found to be closely associated with cervical neoplasms in 1974 (2). According to the published data, HPV infection has been reported as an indispensable factor in the tumorigenesis and development of cervical cancer (3,4). As cervical cancer screening and diagnostic techniques have improved in recent years, precancerous lesions can be detected and treated early, which helps to decrease the occurrence of cervical cancer. Among these new techniques, combined application of ThinPrep cytology test (TCT) and HPV detection has lead to a dramatic improvement in the detection rate of cervical lesions (5,6). However, the molecular mechanisms of the integration of HPV that induce the malignant transformation of cervical cells remain unclear. Moreover, the signaling pathways involved in HPV induced cervical cancer are yet to be fully understood.

Abnormal Notch signaling has been reported in various diseases, including cancer (7-10). In the published data, Notch has been reported as a potential oncogene which may have important roles in various types of cancer, including colon cancer, pancreas cancer, brain cancer (8,9), acute myeloid leukemia (11) and breast cancer (12). Proliferation of prostate cancer cells has been demonstrated to be inhibited by the overexpression of the active form of Notch1 (13). A similar effect is also found in hepatocarcinoma cells, which is induced by cell cycle arrest and apoptosis due to the constitutive activation of Notch1 (14). Furthermore, Notch1 had also been reported to be a tumor suppressor in Notch-deficient mouse skin, which may act by suppressing the β-catenin signaling pathway (15). The oncogenic and anti-oncogenic role of Notch is still under extensive investigation.

Previous data supports the hypothesis that Notch signalling has an oncogenic or tumor suppressor role in cervical cancer, and the elusive role of Notch signaling has been investigated in other types of cancer (16,17). Notch expression has been demonstrated to be upregulated during the transformation progress from normal cervix tissue to cervical intraepithelial neoplasia (CIN), and Notch is relocated in the cell nucleus after cleavage of the intracellular domain (18). Introduction of Notch siRNA in Caski cells inhibits cell growth (19). However, Notch expression has also been reported to be markedly reduced in the late stages of cervical cancer. This dual pattern of Notch1 expression suggests that this protein may have a tumor-promoting function in the early stages of cervical carcinogenesis, and a suppressive function in the later stages (17). Activated Notch may cooperate with papillomavirus oncogenes during the transformation progress (20) and be induced by HPV16.

In order to further investigate the function of the Notch signaling pathway in cervical cancer, the present study aimed to study Notch signaling in the clinical cervical cancer samples

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and a cervical cancer cell line. The different isoforms of Notch were also investigated, as they may affect the tumorigenesis and development of cervical cancer. The present study was approved by the Ethical Committee of the Affiliated Wuxi Second Hospital, Nanjing Medical University (Wuxi, Jiangsu, China), and written informed consent was obtained from all patients.

Materials and methods

Cervical TCT. A total of 130 female patients between 20 and 60 years old, who underwent cervical lesion screening in the Affiliated Wuxi Second Hospital between March 2012 and February 2014, were selected as research subjects. No patients with vaginal infection were included. None of the patients enrolled in the present study were receiving drugs, nor had they performed sexual intercourse for 3 days prior to the examination (21). All the samples for TCT detection were harvested following the ThinPrep protocols (Hologic, Inc., Marlborough, MA, USA). Generally, a cervical brush was placed in the cervix to collect enough cervical epithelial cells for detection and then the cells were subjected to ThinPrep 2000 analysis for cervical cytology.

HPV detection. Sample preparation was performed using a HPV sampler (Zhichengxinda, Wuhan, China), and samples were subsequently sent for detection. HPV detection was performed via polymerase chain reaction (PCR) amplification, followed by flow-through hybridization genotyping (22). All detection results were statistically analyzed by SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA).

Cervical biopsy. Cervical biopsy was performed in order to evaluate the patients' allotypic variation and epithelial involvement in cervical intraepithelial neoplasia (CIN). CIN samples were classified as follows: Mild dysplasia (CIN I), moderate dysplasia (CIN II) and severe dysplasia (CIN III).

Numb expression plasmid construction. Total RNA were isolated from HeLa cells (American Type Culture Collection, Manassas, VA, USA, treated with oligo$(dT)_{18}$ primers and reverse transcribed. Resulting cDNA were used as the templates for PCR amplification using primers Numb-For and Numb-FLAG-Rev. Subsequently, the DNA fragments containing the full-length open reading frame of four Numb isoforms with the FLAG tag towards the 3' end were cloned into *Xba*I and *Not*I sites of the lentiviral vector, pCDH-CMV-MCS-EF1-Puro (System Biosciences Inc., Palo Alto, CA, USA). Restriction enzymes were purchased from Fermentas (Thermo Fisher Scientific, Inc., Waltham, MA, USA). All primers are purchased from BioSun (Shanghai, China).

Reverse transcription-quantitative PCR (RT-qPCR). Using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), total RNA were extracted from patient tissues and cultured cells, followed by RQ1 DNase (Promega Corp., Madison, WI, USA) treatment to remove genomic DNA. First-strand cDNA was synthesized from 5 μg total RNA using SuperScript III reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) and random hexamers according to the manufacturer's instructions. The resultant cDNA was subjected to qPCR using a SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on a 7500 Fast Real-Time PCR system, with the cycling conditions of 95˚C for 10 min, followed by 95˚C for 20 sec and 68˚C for 1 min, for 40 cycles, and then 68˚C for 5 min, according to the manufacturer's instructions (Applied Biosystems; Thermo Fisher Scientific, Inc.). The ACTB gene was used as the housekeeping control gene. All data were analyzed by ABI 7500 Fast System Software (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Lentiviral packaging and infection. Numb lentiviral expression constructs were generated by inserting the coding region with a FLAG tag on the C-terminal of the pCDH-CMV-MCS-EF1-Puro vector. To package the lentivirus, packaging 293T cells (American Type Culture Collection) were cultured in 10% serum Dulbecco's modified Eagle medium (Gibco; Thermo Fisher Scientific, Inc.) at 70% confluence on P100 plates and were transfected with the lentiviral expression construct combined with two helper plasmids: pVSVG and delta-R-8.2 via calcium phosphate DNA precipitation. Conditioned medium containing the lentivirus secreted by the 293T cells was harvested 48 h after transfection, cleared of debris by low-speed centrifugation (1,500 x *g* for 5 min) and filtered through 0.45-μm filters. To infect the cervical cancer cells, cells were incubated overnight in medium containing lentivirus. Following medium replacement with DMEM containing 10% FBS, the cells were incubated for 36 h, and puromycin was added to the medium to screen for the stably overexpressing cells.

Western blotting. Following protein extraction with RIPA buffer (Thermo Fisher Scientific, Inc.), protein lysates (30 mg/well) were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane (BioRad Laboratories, Inc., Waltham, MA, USA). Following blocking in skimmed milk at room temperature, the membranes were incubated with the following primary antibodies overnight at 4˚C in TBST buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl (0.05%; V/V) Tween-20]: Anti-GAPDH (1:2,500 dilution; cat. no. KC-5G4; Kangcheng Biotechnology Co., Ltd., Shanghai, China), anti-FLAG (1:10,000 dilution; cat. no. F1804; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and anti-Numb (1:1,000 dilution; cat. no. 2733; Cell Signaling Technology, Inc., Danvers, MA, USA). Following washing, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibodies [1:2,000 dilution; cat. no. w4011 (rabbit) and cat. no. w4021 (mouse); Promega Corp.] at room temperature for 1 h. Detection was performed with an enhanced chemiluminescence system (Thermo Fisher Scientific, Inc.) and visualized using an image analyzer (Fujifilm, Life Science, Minato, Tokyo, Japan). Image capture and quantification were performed using the Fujifilm software in the image analyzer (Las4000; Fujifilm)

MTT cell viability assay. MTT assay was used to assess the viability of cervical cells. Cells subjected to different treatments were seeded at a density of 2,000 cells/well in 24-well plates. Following incubation for 24, 48, 72, 96 or 120 h, MTT reagent was added (500 μl/well; 5 μg/μl) to the culture medium

A

Classification	Total	HPV(+)&TCT(+)	HPV(+)	TCT(+)	HPV(-)&TCT(-)
Cervicitis	25	10(40%)	2(8%)	5(20%)	8(32%)
CIN I	40	22(55%)	6(15%)	4(10%)	8(20%)
CIN II	25	18(72%)	3(12%)	4(16%)	0(0%)
CIN III	25	23(92%)	0(0%)	2(8%)	0(0%)
Cervical Cancer	15	15(100%)	0(0%)	0(0%)	0(0%)

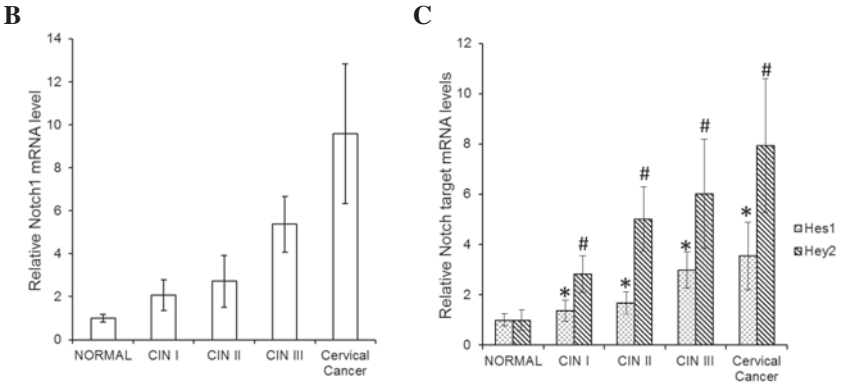

Figure 1. Notch signaling is associated with the occurrence and development of cervical cancer. (A) A total of 130 patients underwent TCT and HPV detection. Total RNA of patient samples were extracted and subjected to reverse transcription-quantitative polymerase chain reaction analysis to detect the mRNA expression levels of (B) Notch and (C) Hes1/Hey2. Data are expressed as mean ± standard deviation of triplicate experiments. *P<0.05 for Hes1 vs. normal group; #P<0.05 for Hey2 vs. normal group. CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; TCT, ThinPrep test.

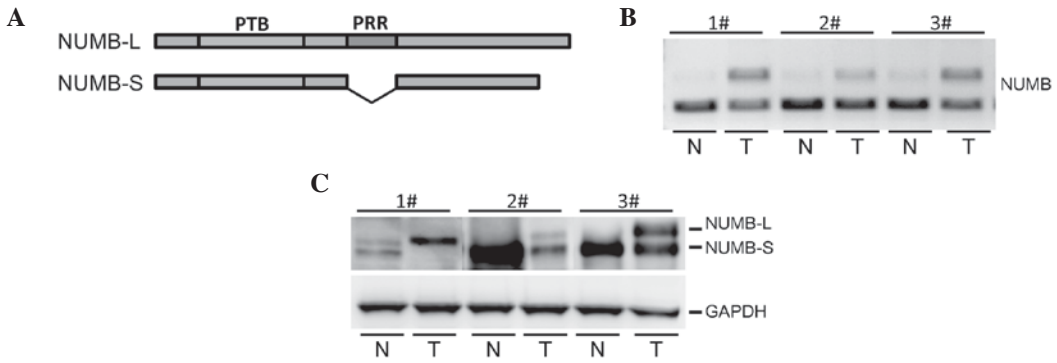

Figure 2. Alternative splicing of Numb is deregulated in cervical cancer. (A) Schematic diagram of Numb isoforms with or without exon 12. (B) Reverse transcription-quantitative polymerase chain reaction analysis and (C) western blot analysis of the splice pattern in three clinical samples of Numb in cervical cancer and adjacent normal tissues. GAPDH served as the loading control. PTB, phosphotyrosine-binding domain; PRR, proline-rich region; L, long isoform; S, short isoform; N, normall tissue; T, tumor tissue.

and incubated at 37˚C for 4 h. Formazan products were solubilized with DMSO, and the optical density was measured at 570 nm using a spectrophotometer (Thermo Fisher Scientific, Inc.). All experiments were performed in triplicate and cells transformed with control lentivirus were treated as controls.

Statistical analysis. All data were analyzed by Student's t-test using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). P<0.05 was used to indicate a statistically significant difference.

Results

Notch overexpression in human cervical cancer. A total of 130 patients underwent TCT and HPV detection; 114 of these 130 patients subsequently received a cervical biopsy for TCT(+) and/or HPV(+). The diagnostic results from the cervical biopsy were as follows: Cervicitis, 25 cases; CIN I, 40 cases; CIN II, 25 cases; CIN III, 25 cases; and cervical cancer, 15 cases. According to the pathological diagnostic results, the percentage of TCT(+) and HPV(+) patients increased with the development of cervical carcinoma (Fig. 1A).

The Notch signaling pathway has important roles in cell fate determination and cell transformation. Inappropriate activation of Notch signaling has been associated with various types of cancer, including glioblastoma, colon, pancreatic, breast and lung cancer (13,22,23). However, the correlation between Notch and cervical cancer development remains unclear.

To investigate whether Notch signaling pathway is involved in occurrence and development of cervical carcinoma, the present study examined the expression levels of the Notch1 and Notch targets (Hes1 and Hey2) via RT-qPCR analysis. The findings demonstrated that the mean Notch1

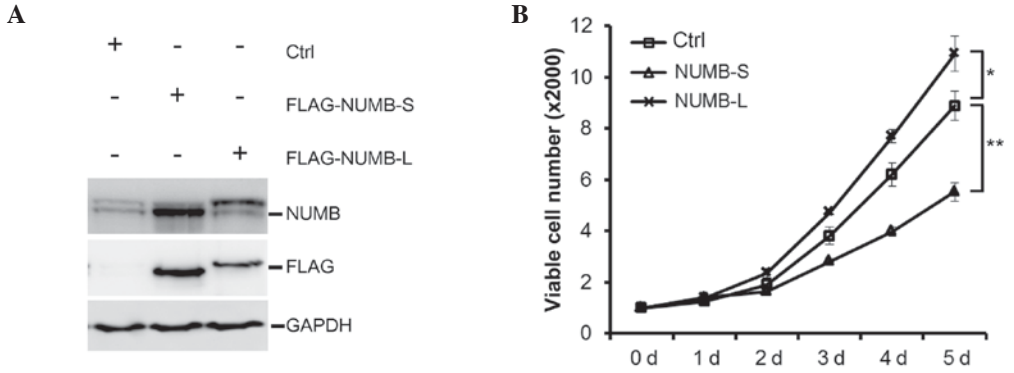

Figure 3. Different Numb isoforms have opposite effects on the viability of cervical cancer cells. (A) Western blot analysis of Numb expression and (B) MTT cell viability assay in HeLa cells stably transduced by lentivirus expressing control, Numb-S or Numb-L. *P<0.05; **P<0.01 (Student's t-test). Error bars represent standard deviation (n=3). Ctrl, control; L, long isoform; S, short isoform.

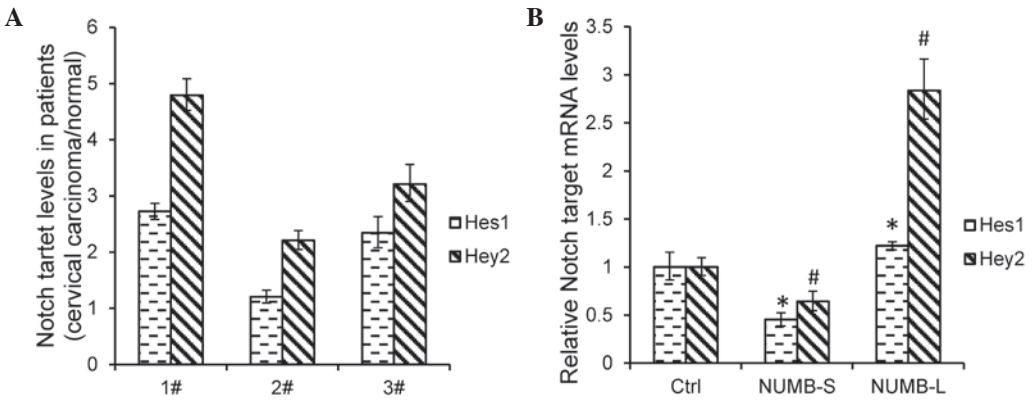

Figure 4. Alternative splicing of Numb regulates Notch signaling pathway in cervical cancer. (A) RT-qPCR analysis of the expression of Notch targets (Hes1 and Hey2) in three clinical samples, as used in Fig. 2. (B) RT-qPCR analysis of the expression of Notch targets, Hes1 and Hey2 in HeLa cells, as described in Fig. 3. *P<0.05 for Hes1 vs. control. $^{\#}$P<0.05 for Hey2 vs. control. RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

mRNA expression levels of each group were notably different (n=5/group); increased expression of Notch1 mRNA was detected in the more malignant tissues, particularly in cervical cancer (Fig. 1B). Moreover, there were significant differences in the mRNA expression levels of Hes1 and Hey2 when comparing CIN and cervical cancer groups with the normal group (P<0.05). Consistent with the expression of Notch1, both Hesl and Hey2 expression levels were increased in cervical cancer tissues, as compared with normal and CIN tissues. Hesl and Hey2 expression levels were also higher in CIN tissues than in normal tissues (P<0.05; Fig. 1C).

Deregulation of Numb expression in cervical cancer. Numb is an evolutionarily conserved signaling adaptor protein and is considered a negative regulator of Notch signaling pathway (24). The human Numb gene contains an alternative exon and is able to generate different isoforms (Fig. 2A). The alternative exon 12 encodes 48 amino acids within the C-terminal proline-rich region (PRR). It has previously been suggested that increased expression of the long isoform containing exon 12 promotes cell proliferation during tumorigenesis (25), whereas the short isoform inhibits cell growth (26). To further investigate the mechanism by which the Notch signaling pathway is altered, Numb expression was investigated in three pairs of cervical squamous cell carcinoma and adjacent histologically normal tissues. Notably, RT-qPCR analysis demonstrated that the alternative splicing of Numb exon 12 was altered, and exon 12 inclusion was markedly increased (Fig. 2B). Moreover, the total amount of the pan-Numb proteins in the carcinoma tissues were unregulated compared with the normal tissues, whereas expression of the long isoform was markedly increased. These results indicate that inclusion of the Numb exon 12 was increased in cervical cancer tissue samples.

Effect of Numb isoforms on cervical cancer cell viability. Previously published data have revealed that differential expression of long (Numb-L) and short (Numb-S) isoforms of Numb may have different functions on cell proliferation and differentiation (27). Therefore, the present study investigated whether increased inclusion of Numb exon 12 may contribute to the development and progress of cervical carcinoma cells. To validate this, MTT assay was performed to monitor cell viability in the cell lines stably expressing control or each of the two Numb isoforms (Fig. 3A). Each cell line was plated at the same density after lentiviral overexpression, and cell viability was measured each day for 6 days after seeding the cells.

After 6 days in culture, a significant increase in cell viability was detected for the HeLa cells transfected with the exon 12 containing the long isoform of Numb (Numb-L; P<0.05;

Fig. 3B). By contrast, overexpression of Numb-S exhibited a significant inhibitory effect on cell viability (P<0.01; Fig. 3B). Thus, these data suggest that increased expression of Numb-L promotes cell viability, whereas expression of Numb-S inhibits cell viability. It is therefore possible that mechanisms that promote increased inclusion of Numb exon 12 may contribute to the viability of cervical carcinoma cells.

Effect of Numb isoforms on Notch signaling. Previous studies have revealed that Numb is a negative regulator of the conserved Notch signaling pathway, and that it is frequently deregulated in cancer (28,29). To determine the contribution of Numb isoforms to the Notch signaling pathway, the mRNA expression levels of Hes1 and Hey2, the Notch targets, were detected in three pairs of cervical carcinoma and corresponding normal tissues, as used in Fig. 2. Both the Hes1 and Hey2 Notch target genes exhibited increased mRNA expression levels in all three tumor tissues relative to that of the matched normal tissues from the patients analyzed earlier in Fig. 2. Moreover, a positive correlation was observed between the degree of increased ratio of Numb exon 12 and the increased levels of Hes1 and Hey2 mRNA expression levels (Fig. 4A). Therefore, these data suggest that the expression levels of Hes1 and Hey2 were significantly increased in cervical cancer samples and the increased ratio of Numb-L relative to that of Numb-S may contribute to the activation of specific Notch targets but not others.

In order to investigate whether Notch target gene expression was affected by the expression of Numb-L and Numb-S isoforms directly, the expression levels of HES1 and HEY2 mRNA were assessed in HeLa cells overexpressing different Numb isoforms, using the same RNA samples as shown in Fig. 3. Overexpression of the Numb-S isoform significantly inhibited the mRNA expression levels of these Notch target genes (P<0.05). In contrast, increased expression of the Numb-L isoform significantly increased Hes1 and Hey2 mRNA expression levels (P<0.05; Fig. 4B). These data indicate that the two isoforms of Numb have opposite effects on Notch signaling activation, and may an important role in the development of cervical cancer.

Discussion

Cervical cancer, which is the second most common gynecological malignant tumor in females worldwide, has a high morbidity in China (30). High-risk HPV infection had been reported as the predominant cause of cervical cancer and precancerous lesions (31). HPV is a small, double-stranded DNA virus, and it can specifically infect squamous epithelial cells in human skin and mucous membranes, causing various benign and malignant lesions (32). HPV infection rate is closely related with cervical cancer incidence. Chronic HPV infection rates are ~20% in countries with high cervical cancer incidence, but are only ~5% in those with low cervical cancer incidence (33). Therefore, HPV detection may be used as a early diagnostic marker of cervical cancer, which is more sensitive than traditional CIN screening.

TCT is widely used in cytological examination due to its high detection rate of cervical cancer (34). In the present study, all cervical cytological sample and slides preparation techniques prior to TCT detection were certified by the Food and Drug Administration. As TCT cytological testing has a high detection rate of cervical cancer, which is reported to be ~100% (35), TCT is recommended for cervical cancer pathological tests to replace traditional cervical smears.

In recently published data, HPV detection combined with cervical TCT dramatically increased the sensitivity of cervical cancer detection and decreased the rate of missed diagnoses (36); therefore, this combined detection method used in CIN screening will be conducive in treatment planning.

Inappropriate activation of Notch signaling has been associated with numerous types of cancer; however, the role of Notch in cervical cancer remains unclear. The results of the present study indicated that Notch overexpression is involved in cervical cancer.

Numb was first identified as a cell fate determinant during Drosophila development (37). It interacts with Notch receptor and functions as a negative regulator of the Notch signaling pathway. Several studies have showed that the alternative splicing of Numb is developmentally regulated, with the Numb isoforms containing exon 12 expressed at early developing stages, which promote viability, and the Numb isoforms lacking exon 12 induced during neurogenesis, which promote differentiation (38,39). The present findings show that alternative splicing of Numb has a key role in regulating the viability of cervical cancer cells. Furthermore, this research indicates the opposite roles that the two NUMN isoforms, NUMB-S and NUMB-L, has in Notch signaling pathway activation. A possible mechanism is that the exon12 of NUMB-L, which encodes a 48 amino acid PRR domain, may be essential in recruiting additional factors via protein-protein interaction to stimulate the expression of Notch targets. These possibilities require further investigation.

The present data also revealed that Hes1 and Hey2 may be associated with the carcinogenesis and progression of cervical carcinoma. Overexpression of Hes1 and Hey2 may be used as markers to predict poor prognosis in patients with early-stage cervical carcinoma.

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