Lentiviral-mediated gene silencing of Notch-4 inhibits in vitro proliferation and perineural invasion of ACC-M cells

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Abstract. Salivary adenoid cystic carcinoma (SACC) is a common type of salivary gland cancer. The poor long-term prognosis for patients with SACC is mainly due to local recurrence, perineural invasion (PNI) and distant metastasis. Notch signaling plays a critical role in determining cell fate such as proliferation, differentiation and apoptosis. Accumulating evidence indicates that aberrant Notch-4 expression has a tumor-promoting function in SACC. In the present study, we used lentiviral-mediated RNA interference (RNAi) targeted against Notch-4 to determine the effects of decreased levels of this protein in the human highly metastatic adenoid cystic carcinoma cell line ACC-M. Furthermore, the proliferative capability as well as the PNI potential of the treated cells were observed in vitro. Our studies demonstrated that RNAi directed against Notch-4 markedly decreased Notch-4 gene expression, resulting in the inhibition of cell proliferation, and G0/G1 to S phase arrest in ACC-M cells. Knockdown of Notch-4 also resulted in a decrease in the in vitro PNI activity in ACC-M cells. To conclude, RNAi targeting against Notch-4 induces the suppression of cell growth and inhibition of PNI in vitro in ACC-M cells. Notch-4 may play an important role in regulating proliferation and PNI activity of SACC.

Introduction

Salivary adenoid cystic carcinoma (SACC) is a common type of salivary gland malignancy, and accounts for 25% of malignant tumors in the major salivary glands (1) and 50% in the minor glands (2). The neoplasm is characterized by heterogeneous phenotypic features and persistently progressive biological behavior. The poor long-term prognosis for patients with adenoid cystic carcinoma is mainly due to local recurrence related to perineural invasion (PNI) and delayed onset of distant metastasis, particularly to the lungs (3,4). PNI, a frequent occurrence in SACC, is difficult to be identified clinically and this often prevents complete surgical resection (5). Vrielinck et al (6) reported the relationship between PNI and poor prognosis. PNI has also been observed frequently in other types of cancer such as melanoma, prostate and pancreatic carcinomas as well as head and neck cancers and is recognized as one of the most important prognostic factors (7-11). Due to their predilection for nerves, these cancers are known as ‘neurotropic cancers’.

Notch signaling is a pathway highly conserved through evolution which regulates various physiological processes, including stem cell maintenance, differentiation, proliferation and apoptosis (12,13). In mammals, key components of the Notch pathway include four transmembrane receptors (Notch-1, Notch-2, Notch-3 and Notch-4) and five ligands (Dll1, Dll3, Dll4 and Jagged-1, -2) (14,15). Direct binding of a ligand from a signaling cell to a Notch receptor on the membrane of the receiving cell initiates two successive proteolytic cleavages by TACE (TNF-α-converting enzyme) and the γ-secretase/presenilin complex, which ultimately results in the release of the intracellular domain (N-IC). N-IC then translocates into the nucleus and directly interacts with the DNA binding protein CBF-1/Su(H)/Lag-1 (CSF) that activates the transcription of target genes including the hairy/enhancer-of-split (HES-1) (16).

Accumulating evidence strongly indicates that aberrant Notch signaling has a tumor-promoting function in many
Table I. Oligonucleotide sequences of Notch-4-specific shRNAs.

<table>
<thead>
<tr>
<th>Name</th>
<th>Base sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>shRNA1-F</td>
<td>5’-CGCGTCCCCCGCAGATATGTAAGGACCAGAATTCTCGTGCTTACATAATCTGCTTTTGGAAAT-3’</td>
</tr>
<tr>
<td>shRNA1-R</td>
<td>5’-CGATTCTCCAAAAAGACGATATGTAAGGACCAGAATTCTCGTGCTTACATAATCTGCTTTTGGAAAT-3’</td>
</tr>
<tr>
<td>shRNA2-F</td>
<td>5’-CGCGTCCCCCTGCGATATGCGAGGAGGATCTCAAGAGAATGCTCTCCGATTATCGAGTTTTTGGAAAT-3’</td>
</tr>
<tr>
<td>shRNA2-R</td>
<td>5’-CGATTCTCCAAAAACTGGCGATAATGCGAGGAGGATCTCAAGAGAATGCTCTCCGATTATCGAGTTTTTGGAAAT-3’</td>
</tr>
<tr>
<td>shRNA3-F</td>
<td>5’-CGCGCTCCCCCAGATATGTAAGGACCAGAATTCTCGTGCTTACATAATCTGCTTTTGGAAAT-3’</td>
</tr>
<tr>
<td>shRNA3-R</td>
<td>5’-CGATTCTCCAAAAAGATATGTAAGGACCAGAATTCTCGTGCTTACATAATCTGCTTTTGGAAAT-3’</td>
</tr>
<tr>
<td>shRNA4-F</td>
<td>5’-CGCGTCCCCCGATGCGAGGAGGAGGATCTCAAGAGAATGCTCTCCGATTATCGAGTTTTTGGAAAT-3’</td>
</tr>
<tr>
<td>shRNA4-R</td>
<td>5’-CGATTCTCCAAAAACTGGCGATAATGCGAGGAGGATCTCAAGAGAATGCTCTCCGATTATCGAGTTTTTGGAAAT-3’</td>
</tr>
<tr>
<td>NC-F</td>
<td>5’-CGCGCTCCCCCTTCTCGGACGTCGTCCTACAGGAGAAGCTGCTCAAGAGATCTGCTTTTGGAAAT-3’</td>
</tr>
<tr>
<td>NC-R</td>
<td>5’-CGATTCTCCAAAAATTTCAGGACGTTACGTTCCTCTCTTGAACGTTGAACGGTACGTTGCGAGAAGGGGA-3’</td>
</tr>
</tbody>
</table>

shRNA1-4 indicates the oligonucleotide sequence of Notch-4, NC, negative control. Underlined sections indicate the forward and reverse target sequence; bold section is the restriction enzyme cutting site, including 5’-ACGCGT-3’ (MluI) and 5’-ATCGAT-3’ (ClaI). shRNA1 and 3 also include the restriction enzyme cutting site of EcoRI (5’-GAATTC-3’, italicized section).

types of tumors, and Notch signaling may be a promising target for cancer treatment. A role for Notch signaling in salivary gland adenocarcinoma cells has been suggested which proposes that 5’-nitro-indirubinoxime (5’-NIO) induces G2/M cell cycle arrest and apoptosis by the down-regulation of Notch-1 signaling (17). Notch-1 cross-talk has also been reported in other major cell growth and apoptotic regulatory pathways through modulating the activity of the transcription factor, for example, nuclear factor (NF)-κB and Wnt/β-catenin signaling (18,19). Notch signaling may contribute to squamous cell carcinogenesis, and it is considered as a candidate marker for squamous cell carcinomas of the head and neck (HNSCC) (20). It was reported that the Notch signaling pathway also contributes to the drug resistance of cancer cells. Inhibition of Notch signaling was found to prevent drug resistance and enhanced chemosensitivity in human myeloma, breast cancer and HNSCC (21-23). In SACC, a recent study suggested that Notch-4 activation contributes to SACC metastasis (24).

In our previous microarray study, Notch-4 was found to play a potential important role in the pathobiology of SACC associated with PNI (25). Thus, we tested our hypothesis on whether knockdown of Notch-4 by short hairpin RNA (shRNA) inhibits the in vitro proliferation and PNI in ACC-M cells.

To examine our hypothesis, we silenced Notch-4 expression in a human highly metastatic SACC cell line, ACC-M (26), by lentiviral vector-mediated RNA interference (RNAi) technology, and evaluated the effect of Notch-4 on cell growth, cell cycle distribution and cell PNI activity in ACC-M cells.

Our data showed that Notch-4 RNAi had antiproliferative activity by modulating G0/G1 and S cell cycle regulators. The knockdown of Notch-4 expression by lentiviral vector-mediated RNAi reduced the PNI activity in vitro in SACC cells. These results suggest that Notch-4 plays an important role in regulating the in vitro growth, proliferation and PNI of ACC-M cells. The suppression of Notch-4 may be a potential therapeutic strategy for SACC.

Materials and methods

Cell lines and cell culture condition. ACC-M and 293T cells were kindly provided by the Department of Oral Biology, School of Stomatology, Fourth Military Medical University, China. The two types of cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% of fetal bovine serum (FBS) (Gibco, Invitrogen), 2.05 mM of L-glutamine, 100 g/ml of penicillin and 100 µg/ml of streptomycin at 37°C in 5% CO2.

Preparation of Notch-4 lentiviral vectors. Lentiviral vector system (pLenOR-THM, pMDLG/pRRE, pRSV-Rev, pMD2.G) was purchased from Innovation Biotechnology Co., Ltd. (Shanghai, China). Referencing siRNA design strategy (27,28), we selected sites of the gene (NM_004557.3) cDNA sequence and determined the specific sequence by BLAST. Four pairs of siRNA and one negative control were designed and synthesized. As shown in Table I, each pair contained a unique 21-nt (shRNA1 and 2) or 19-nt (shRNA3 and 4) double-stranded human Notch-4 sequence that is presented as an inverted complementary repeat and separated by a loop of a 9-nt spacer. DNA oligonucleotides (Table I) targeting Notch-4 were synthesized and inserted into the MluI and ClaI site of the linearized lentiviral-shRNA expression vector according to the manufacturer’s instructions. They were incorporated into a pLenOR-THM expression plasmid. The successful ligation was confirmed by the restrictive cleavage and sequenced for an additional verification (Fig. 1). The recombinant vectors were named pLenOR-Notch-4-shRNA1, 2, 3 and 4.

The recombinant vector was then mixed with virus packaging mix, which including pMDLG/pRRE (HIV-1 gag/pol component), pRSV-Rev (a binding site for the Rev protein which facilitates export of the RNA from the nucleus) and pMD2.G (VSV-G component), packed and transfected by Lipofectamine™ 2000 (Invitrogen) into 293T cells. Viral supernatant was harvested 48 h after transfection, filtered...
through a 0.45-µm cellulose acetate filter and frozen at -70°C. Virus titer was detected by a 96-well plate dilution method and flow cytometry.

**Lentiviral transfection and construction of stable silenced cell lines.** Approximately 2x10⁵ ACC-M cells/well were plated in 6-well plates. Twenty-four hours later, ACC-M cells were transfected with 1x10⁷ specific or negative control lentiviral vectors (multiplicity of infection of 25) containing 500 µl enhancing transfection solution (Innovation Biotechnology Co., Ltd.) and 8 µg/ml Polybrene® (Sigma, St. Louis, MO, USA). At 24 h post-transfection, the medium was replaced by normal medium containing 10% FBS and antibiotics. After 96 h post-transfection, the transfected cells were observed under a fluorescence microscope (Leica). As the lentiviral vector contains a GFP expression cassette, the cell transfection rate was directly observed, which reached 90%.

**Quantitative RT-PCR.** Quantitative RT-PCR (qRT-PCR) for Notch-4 transcripts (shRNA1, 2, 3 and 4) in the ACC-M cell lines was performed. ACC-M cells transfected by zero-loaded lentiviral vector and untreated ACC-M cells were taken as the positive and the negative control separately. Total RNA was extracted from 1x10⁶ cells with TRIzol reagent (Invitrogen). Samples of total RNA (1 µg) were reverse-transcribed into cDNA template supplemented with water to a final volume of 20 µl. The specific primers of Notch-4 (forward, 5'-TCAACACT CCTGGCTCCTTCAACT-3'; reverse, 5'-AGAGGCAC TCATTGTGATCAGCCT-3') were amplified as follows: 94°C for 3 min and 40 cycles at 94°C for 30 sec, followed by 61.1°C for 30 sec, 72°C for 20 sec, then ended with 72°C for 10 min for elongation. Human 18S gene was amplified as the internal control (forward, 5'-CGGCTACCACATCCAAGGAA-3' and reverse, 5'-GCTGGAATTACCGCGGCT-3'). Target genes and the 18S gene were amplified in the same reaction. Each sample was performed in triplicate. Comparative quantification was determined using the 2^(-ΔΔCt) method.

**Western blot analysis.** Cells were washed twice with cold phosphate-buffered saline and lysed on ice in buffer containing protease inhibitors. Equal amounts of protein (20 µg/lane) from the cell lysates were electrophoresed on 10% acrylamide gels. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane. The membrane was incubated for 2 h in PBS plus 0.1% Tween 20 and 5% non-fat skim milk to block non-specific binding. Subsequently, the membrane was incubated for 2 h with an antibody against Notch-4 (R&D Systems, Minneapolis, MN, USA). After washing, proteins were visualized using an ECL detection kit with the appropriate HRP-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were stripped and probed with monoclonal antibodies for GAPDH for loading control as per standard protocols. The experiment was repeated three times to confirm the results.

**Proliferation assay.** The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay was performed to assess the cell proliferation of the transfected cells. Briefly, the cells were plated in 96-well plates at a density of 10⁵ cells/well. Then for 8 days, every 24 h, a batch of cells were stained with 20 µl sterile MTT dye (5 mg/ml; Sigma-Aldrich) at 37°C for 4 h. The culture medium was then removed, and 150 µl of dimethyl sulphoxide (DMSO) was added and thoroughly mixed in for 10 min. Spectrometric absorbance at 490 nm was measured using a microplate reader. Each group consisted of three wells.

**Flow cytometric analysis.** Different cell cycle phases (G₀/G₁, S or G₂/M phase) are characterized by different DNA contents. Fluorescence dye propidium iodide (PI) binds with DNA
strongly at a ratio of 1:1, and hence the DNA contents of cell cycle phases are reflected by varying PI fluorescent intensities. Stable transfected ACC-M cells, 1x10^6, were harvested by trypsinization and fixed in 70% ice-cold (4°C) ethanol for 2 h. Cell pellets were resuspended in 1 mg/ml RNase solution (Sigma-Aldrich) for 30 min at 37°C and subsequently in 0.1 mg/ml PI solution (Sigma-Aldrich) at 4°C for 1 h in the dark. Cell cycle analysis was performed on a flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA).

In vitro perineural invasion assay. The inhibitory effect of RNAi on the PNI ability of ACC-M cells in vitro was demonstrated in modified Boyden chambers. Transwell invasion chambers containing polycarbonate filters (8-µm) (Millipore Corp., Billerica, MA, USA) were coated on the upper surface with Matrigel basement membrane (BD Biosciences, San Diego, CA, USA). Cells (1x10^5) were suspended in DMEM supplemented with 1% fetal bovine serum and added to the upper chamber. The lower chamber contained 600 µl supernatant of 24 h-cultured RSC96 cells (a rat Schwann cell line, purchased from the Cell Bank for Type Culture Collection, Chinese Academy of Sciences) as a chemoattractant to simulate the perineural surrounding environment (29,30). Cells were incubated for 12 h at 37°C in 5% CO_2 incubator. At the end of the incubation, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab. The filters were then fixed in methanol and stained with hematoxylin and eosin. Cells that had invaded the Matrigel and had reached the lower surface of the filter were counted under a light microscope at a magnification of x400. We chose five fields of vision and counted the numbers of the invaded cells on the lower surface of the filter. The assay was performed in triplicate.

Statistical analysis. Results are expressed as means ± standard deviation (SD). Statistical analysis was performed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Data were tested for statistical significance using analysis of variance (ANOVA). Normally distributed, continuous variables were compared using one-way ANOVA. When ANOVA produced a significant difference between groups, multiple comparisons of group means were performed using the Bonferroni procedure with a type I error adjustment. All P-values were two-sided, and significance was defined as P<0.05.

Results

Lentiviral vector-mediated RNAi of Notch-4 causes effective and specific downregulation of Notch-4 expression. The knockdown efficiencies of different Notch-4-specific shRNAs in ACC-M cells were first evaluated using qRT-PCR. Relative Notch-4 mRNA levels in individual stable transfectants were normalized against mRNA levels of an internal control gene, human 18S, performed in the same run. As shown in Fig. 2A, cells transduced with pLenOR-Notch-4-shRNA2, 3 and 4 showed a significantly reduced transcription of Notch-4 mRNA when compared with that of the negative control ACC-M cells and the positive control vector ACC-M Mock, respectively (P<0.01), but there was no significant mRNA transcription reduction in cells transduced with pLenOR-Notch-4-shRNA1. The cells transduced with pLenOR-Notch-4-shRNA2 showed the most significant inhibition of Notch-4 mRNA levels.

The Notch-4 protein is a heterodimer ~210 kDa, and the Notch-4 protein was split by the lysate, so therefore the western blot analysis detected the intracellular and extracellular domain of Notch-4 protein in the cells. Western blot analysis revealed a decreased expression of Notch-4 protein in the ACC-M cells transfected with pLenOR-Notch-4-shRNA1, 2, 4, while cells transduced by pLenOR-Notch-4-shRNA3 showed no notable reduced expression of Notch-4 protein compared with that of the negative control ACC-M cells and the positive control vector ACC-M Mock. These results revealed that the most effective vector was pLenOR-Notch-4-shRNA2. ACC-M, high metastatic potential control used as a negative control; ACC-M Mock, mock transfection control used as the positive control) pLenOR-Notch-4-shRNA1, 2, 3 and 4 represent the four different clones, respectively.
Gene silencing of Notch-4 reduces cell proliferation in vitro. The proliferative activity of tumor cells is important in the invasion/metastasis of tumors. To examine whether the knockdown of Notch-4 expression has any effect on cell growth, an MTT cell proliferation assay was performed. Under the same cell culture conditions, the proliferative activity of the pLenOR-Notch-4-shRNA2-transfected cells, negative and positive control cells was almost similar for the first 24 h. With time-lapse, the cells with Notch-4 gene silencing grew more slowly when compared with the control cells. Among the three groups, Notch-4 RNAi (pLenOR-Notch-4-shRNA2) cells showed decreased cell proliferation, when compared to the negative control (ACC-M) and mock-transfected (ACC-M Mock) cells, supporting the role of Notch-4 in the cell growth of ACC-M cells (P<0.01) (Fig. 3).

We next used flow cytometry (FCM) to study the effect of Notch-4-specific shRNA on the cell cycle distribution in ACC-M cells. The negative control group (ACC-M) and positive control group (ACC-M Mock) resulted in a cell cycle distribution of ~53% and 46% of the cells in the S and G<sub>2</sub>/M phases. In the pLenOR-Notch-4-shRNA2 group, the proliferation index value (PrI) of cycling cells (combined total number of cells in the S and G<sub>2</sub>/M phases) was decreased to ~39%, with a concomitant increase in the number of cells in the G<sub>0</sub>/G<sub>1</sub> phase (Table II) (Fig. 4). Significant decreases in PrI were found in the pLenOR-Notch-4-shRNA2-transfected cells compared with negative and positive control cells (P<0.01). There was no significant difference in PrI between that of the negative and positive control transfectant cells (P>0.05). These findings indicate that knockdown of Notch-4 expression has any effect on cell growth.
may inhibit proliferation of ACC-M cells by modulating G0/G1 and S cell cycle regulators.

**Table II. FCM analysis of the cell cycle of ACC-M cells after Notch-4-specific inhibition.**

<table>
<thead>
<tr>
<th>Cell group</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
<th>Proliferation index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC-M (NC)</td>
<td>46.7±1.45</td>
<td>45.7±0.96</td>
<td>7.6±0.50</td>
<td>53.3±1.45</td>
</tr>
<tr>
<td>ACC-M Mock (PC)</td>
<td>54.2±2.06</td>
<td>40.2±1.27</td>
<td>5.5±0.80</td>
<td>45.8±2.06</td>
</tr>
<tr>
<td>pLenOR-Notch-4-shRNA2</td>
<td>60.8±1.12</td>
<td>37.8±1.16</td>
<td>1.5±0.10</td>
<td>39.2±1.12</td>
</tr>
</tbody>
</table>

*P_{1,3} < 0.01, P_{2,3} < 0.01, P_{1,2} > 0.05. 1, ACC-M (NC); 2, ACC-M Mock (PC); 3, pLenOR-Notch-4-shRNA2.

**Discussion**

The gene encoding the Notch receptor was discovered almost 90 years ago, and gained its name because partial loss of Notch function resulted in notches in the wing margins of *Drosophila* (31). It only became apparent some years later that the Notch signaling pathway has been conserved throughout evolution. The Notch signaling pathway plays a pivotal role in several cell functions, such as cell fate decisions, cell proliferation, differentiation and cell death during development and postnatal life in species as diverse as *Drosophila*, worms and vertebrates (32-36). In the mammalian system, there are four Notch receptors (Notch-1, Notch-2, Notch-3 and Notch-4) and five known ligands (DIll, Dll3, Dll4 and...
Jagged-1, -2) (14,15). Notch is a cellular fate determinant and can induce cell proliferation and/or differentiation, depending on the cellular environment (37). A recent study showed that targeting Notch-1 and Notch-4 may provide a new therapeutic strategy for triple-negative and possibly other breast cancer subtypes (38). In SACC, Notch-4 may play a key role in SACC metastasis, and inhibition of Notch-4 gene expression may have potential therapeutic application in treating metastatic patients (24).

Perineural invasion (PNI), is a typical biological behavior of SACC, which may prevent a complete surgical resection (5). PNI is associated with poor prognosis in SACC patients (6). In our previous study, we established the gene expression profile of SACC associated with PNI by combining the use of laser capture microdissection (LCM) and cDNA microarray. In the profile, Notch-4 was notably overexpressed in the PNI cell group, and this was verified by qRT-PCR (25). Thus, we hypothesized that inhibition of Notch-4 gene expression may reduce in vitro proliferation and PNI in ACC-M cells.

To understand the biological function of Notch-4 in SACC, we examined the effects of a decreased expression of Notch-4 in a human highly metastatic SACC cell line, ACC-M, using a lentiviral-mediated RNAi system. RNAi uses the phenomenon by which double-strand RNA induces potent and specific inhibition of eukaryotic gene expression through the degradation of complementary messenger RNA (mRNA) and is functionally similar to the processes of post-transcriptional gene silencing (39). In the past few years, siRNA and shRNA have been widely used to silence the expression of many target genes, and both methods have had great achievement, but the silencing effect lasts for less than 2 weeks. New systems based on lentiviral vectors have provided new solutions to achieving stable shRNA-mediated knockdown (40,41).

In the present study, we used a lentiviral-mediated RNAi method to obtain effective knockdown of the Notch-4 gene in ACC-M cells and constructed stable silencing clones.

Here, we designed four shRNAs targeted at the Notch-4 gene and successfully transfected them into ACC-M cells. Among the four designed shRNAs, the cells transfected with pLenOR-Notch-4-shRNA2 showed the most significant inhibitory effect as determined by qRT-PCR and western blot analysis. The results indicated that lentiviral-mediated RNAi of Notch-4 silenced the expression of Notch-4 effectively and specifically in ACC-M cells.

We next examined the consequence of ACC-M cells transfected with Notch-4-specific shRNA. The proliferation of the ACC-M cells in which the Notch-4 gene was knocked down was inhibited compared with that of the positive or negative control. In the FC M assay, the Notch-4-knockdown ACC-M cells showed an arrest in G2/M-G1 transition, suggesting growth inhibition of these cells (Table II). Therefore, Notch-4 may be a positive regulator of cell growth to promote a mitogenic signal, which then enhances cell proliferation of ACC-M cells.

In the present study, the silencing of Notch-4 in ACC-M cells inhibited the cell PNI activity in vitro. This result was consistent with the results of the MTT assay and FC M analysis which revealed that silencing of Notch-4 by lentiviral-mediated RNAi inhibited the cell proliferation in vitro by modulating G1/S and G2/M cell cycle regulators. The finding may also be associated with the metastatic ability of Notch-4 in SACC and breast cancer (24,38).

SACC is a common subtype of salivary gland malignancy, and it has an important biological behavior for PNI. It is urgent to develop new therapeutic strategies for SACC. In this report, the knockdown of Notch-4 expression by lentiviral-mediated RNAi successfully inhibited the malignant behaviors of ACC-M cells, particularly PNI ability in vitro, implicating that Notch-4 may be a new candidate target gene for the treatment of SACC.

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