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Article

Ginsenoside Rh4 Suppresses Notch3 and PI3K/Akt Pathway to Inhibit Growth and Metastasis of Gastric Cancer Cells

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Abstract

Background: Rh4 (a compound derived from ginsenoside) has excellent anti-tumor property. It has been documented that an abnormally elevated level of Notch3 expression is linked to poor prognosis of gastric cancer (GC). Currently, the function of Rh4 in GC malignant progression is still unclear, and the manner in which Notch3 regulates GC progression remains undefined.

Methods: The level of Notch3 in GC cells was determined utilizing qRT-PCR and western blot. The relationship between Notch3 level and patient overall survival was forecasted by Kaplan-Meier Plotter database. The impacts of Notch3 and Rh4 on the biological characteristics of GC cells were examined through CCK-8, clone formation, scratch healing, and transwell assay. Using western blot, PI3K/Akt pathway protein levels were measured. Finally, a mouse subcutaneous transplantation tumor model was utilized to explore the influence of Rh4 treatment *in vivo*.

Results: The level of Notch3 was notably elevated in GC. The patient's overall survival exhibiting high Notch3 levels was considerably lower than those with low expression. Silencing of Notch3 reduced GC cell viability and inhibited malignant progression. Rh4 exhibited a dose-dependent decrease in GC cell viability. In addition, Rh4 significantly suppressed Notch3 levels in GC cells, with over-expression of Notch3 attenuating the inhibitory influence of Rh4 on GC malignancy. Notch3 over-expression activated the PI3K/Akt pathway, while Rh4 effectively blocked this pathway by targeting Notch3, but PI3K agonists reversed this effect. Rh4 treatment reduced Notch3 expression and hindered tumor growth *in vivo* by inhibiting the PI3K/Akt pathway.

Conclusion: Notch3 expression was found to be heightened in GC, but Rh4 effectively suppressed PI3K/Akt pathway by targeting Notch3, and subsequently inhibiting cell proliferation, migration, and invasion.

Highlights

Expression of Notch3 is significantly elevated in GC and is linked to a negative prognosis.

Silencing Notch3 reduced GC cell viability and inhibited malignant progression.

Ginsenoside Rh4 reduced GC cell viability and inhibited migration and invasion, while over-expression of Notch3 attenuated this effect.

Inhibition of the PI3K/Akt pathway by Rh4 is achieved through the reduction of Notch3 expression, ultimately hindering GC malignant progression.

The growth of tumors *in vivo* is inhibited by Ginsenoside Rh4.

1. Introduction

Gastric cancer (GC) ranks among the prevalent form of cancers affecting the digestive tract, with over 1 million new cases of GC and around 800,000 deaths reported each year globally [1-3]. Risk factors for GC including poor dietary habits, *Helicobacter pylori* and EBV infections, smoking, and alcohol consumption [4]. Classified according to anatomical site and histologic type, more than 95% of GC are Stomach adenocarcinoma (STAD) [5]. The primary method for treating GC is through radical surgery, however, GC has an insidious onset and rapid progression, and the majority of patients are usually already in the mid-to-late stages when diagnosed, resulting in missed the best time for surgery and leading to an unfavorable prognosis [6,7]. According to reports, patients diagnosed with GC have a five-year survival rate of 32%, whereas the rate for patients with distant metastases is only 6% [8]. Therefore, further exploration of the pathogenesis of GC, searching for novel and efficient drugs will have an important impact on the early detection and management of GC patients.

Ginseng is a traditional medicinal plant, mainly categorized as Koryo ginseng, American ginseng, and Panax ginseng, popular and widely used in East Asia [9-11]. Ginsenoside is the main active ingredient in ginseng, the diverse pharmacological and therapeutic properties of ginsenoside have garnered significant interest in its use for treating various conditions like immunomodulatory functions, diabetes, inflammation, cancer and aging [12-14]. Rh4 (a compound derived from ginsenoside) is a unique triol saponin converted from the original ginsenoside triol group of saponins, with better anti-tumor, anti-diabetic and anti-inflammatory effects than ordinary ginsenosides [15-17]. For example, Zhang *et al* found that Rh4 could block the lung adenocarcinoma cell cycle, inhibit cell proliferation, and suppress lung adenocarcinoma metastasis through hindering the JAK2/STAT3 pathway [18]. Currently, Rh4 is understudied in GC progression.

Notch signaling pathway is highly conserved, and it mediates intercellular information exchange and is essential for cell proliferation, apoptosis, differentiation, and epithelial mesenchymal transition [19-21]. Notch3 included in the four Notch receptor proteins expressed in mammals, and previous studies have verified a correlation between abnormally elevated Notch3 expression and various cancer types [19-22]. For example, dysregulation of Notch signaling plays a pivotal role in the initiation and progression of cancers such as breast carcinomas [23], liver cancer [24], colorectal cancer [25], and GC [26]. Cui *et al.* indicated that Notch3 is crucial for immune tolerance of GC and has potential as a predictive marker [27]. Additionally, recent studies have shown that Notch3 could activate the phosphoinositol-3 kinase (PI3K)/AKT pathway, thereby promotes the malignant progression of osteosarcoma and bladder cancer [28,29]. Notably, suppressing PI3K/AKT pathway caused a decline in PD-L1 level in GC cells and improved T-cell anti-tumor immunity, thereby inhibiting GC progression [30]. Furthermore, the functional relationship between Notch and mTOR in regulating cancer cell proliferation [31] remains underexplored, describing the importance of further investigation into these interconnected signaling pathways in GC [19-22,31].

Nevertheless, the specific function of Notch3 in GC malignant progression remains uncertain and requires further study. Therefore, our study intends to explore the regulation of Rh4 on Notch3 expression and its effects on GC malignant biological behaviors. The influence of Rh4 on GC progression *in vivo* was determined by subcutaneous transplantation tumor, and the specific mechanism of regulating GC malignant progression was explored, for the purpose of providing new biomarkers of GC.

2. Material and Methods

2.1 Cell Culture and Transfection

Human GC cells (N87, AGS, and HGC-27) and gastric mucosal cells (GES-1) were sourced from American type culture collection (ATCC). After that, the cells were grown in a mixture of RPMI 1640 (12633020), 10% fetal bovine serum (FBS, A5670701) and 1% penicillin/streptomycin (15140122), and the above three materials were obtained from Gibco (Grand Island, NY, USA). The temperature was set to 37°C, the fluid change interval was 3 days, and the passaging ratio was 1:3.

2.2 Cell Transfection

Notch3 over-expression plasmid (OE-Notch3), Notch3 silencing plasmid (si-Notch3), or negative control (OE-NC and si-NC) were obtained from RiboBio Co., Ltd. (Guangzhou, Guangdong, China). Referring to the instructions of Lipofectamine 3000 (L3000001, Invitrogen, Austin, TX, USA), the above testing plasmids and control plasmids were transfected into GC cells, respectively. Afterward, the cells were subjected to 48-hour incubation in an incubator before doing the subsequent testings.

2.3 CCK-8 Assay

GC cells in the log-phase were collected and seed into 96-well cell culture plates (2.0×10^4 cells/well), and after the cells were wall-approximated, the original medium in the cell culture wells was discarded and substituted with medium containing Rh4 (G860635, Macklin Inc., Shanghai, China). After 24 h of action, introduced 10% CCK-8 reagent (C0038, Beyotime, Shanghai, China) into every well and mixed well. After incubation for 2 h in the dark, the cell viability was determined by assessing the OD_{450} value by a microplate reader.

2.4 Clone Formation Assay

500 cells were seed into each well of a 6-well plate and cultured in a cell culture incubator for 14 d [32,33]. Every 2~3 days, the culture medium was replaced with the new. The culture was terminated when clonal cell clusters were visible to the naked eye. The culture solution was aspirated and rinsed twice with PBS. 4%

paraformaldehyde (P0099, Beyotime) was added and fixed for 20 min. The fixative was discarded, exposed to crystal violet (C0775, Sigma-Aldrich, St. Louis, MO, USA) for 15 min. Photographs were taken using a fluorescent inverted microscope (DM IL LED, Leica, Heidelberg, Germany), image processing with Image J software, and the clone formation rate was calculated.

2.5 Scratch-Wound Assay

The GC cells were taken, trypsin-digested, collected, and cell suspension (1 mL, 1×10^6 cell/mL) was seed into 6-well plates with the horizontal lines drawn in advance. When the cells had completely attached to the wall and grew to more than 80% density, a uniform straight line was drawn perpendicularly to the bottom of the well plate with a 200 μL sterile pipette tip. The suspended cells were cleaned using PBS and resupplied with fresh medium. Microscopy images were taken at 0 h and 24 h, and the scratch widths were calculated using Image J, respectively, to calculate the migration rate of cells.

2.6 Transwell Assay

Matrigel (HY-K6002, MedChemExpress) was melted in a refrigerator at 4°C overnight, and subsequently mixed with serum-free RPMI-1640 medium. Transwell chambers (Corning, Tewksbury, MA, USA) were put into 24-well plates, the above dilution gel (100 μL) was pipetted and spread on the bottom of each chamber and placed in the incubator overnight. The following day, the remaining liquid in the chambers was cleaned and filled with serum-free RPMI-1640 medium. AGS and HGC-27 cell suspension (200 μL) was seed into the upper section, the medium was put into the lower section, and then incubated for 36 h. The cell suspension inside the chambers was discarded, and 4% paraformaldehyde for fixation, then stained with 0.1% crystal violet. The field of view was randomly selected and captured using microscope to count the amount of invasive cells.

The transwell chamber basement membrane was hydrated in 1640 medium without serum in advance. Cell suspension was put in the upper section, and medium was put in the lower section, and then incubated for 24 h. Then treated with 4% paraformaldehyde, after that, treated with 0.1% crystal violet aqueous. After washing, the number of migrated cells was ascertained.

2.7 *In vivo* Experiment

Balb/c nude mice were obtained from Vitalriver (Beijing, China) and housed at a constant temperature environment at 22°C, 55% to 60% humidity. Mice were injected subcutaneously with 200 μL of HGC-27 cell suspension (2×10^6 cells/mice), randomized into 3 groups, and administered starting on day 7 after inoculation (recorded as day 1). The low-dose group (L-Rh4, n=3) was injected intraperitoneally with 50 mg/kg of Rh4, the high-dose group (H-Rh4, n=3) was injected with 100 mg/kg of Rh4, and the control (n=3) was injected intraperitoneally with an equal amount of substrate solution once a day for 21 days. The size of subcutaneous tumors in nude mice was measured by vernier calipers on 1, 6, 11, 16, and 21 d, and mice were anesthetized and executed on the 21st day, tumors were excised and weighed. The Institutional Animal Ethics Committee approved all the experiments involving animals.

2.8 Immunohistochemistry

To preserve the tumor tissues, 4% paraformaldehyde was used, followed by dehydration and embedding in paraffin, and the paraffin blocks were divided into pieces of 4~5 μm. DAB solution (P0203, Beyotime) was used to stain the sections, the reaction was terminated by distilled water. Mayer hematoxylin (C0107, Beyotime) was used as a counterstain, and neutral tree glue was employed for sealing. Ki-67 staining was performed carefully according to the Ki-67 kit (E607238, Sangon Biotech, Shanghai, China) instructions.

2.9 RT-qPCR

To extract total RNA, cells and tumor tissues were treated with Trizol reagent (15596018CN, Invitrogen). After that, cDNA was obtained through reverse transcription with the inclusion of AMV reverse transcriptase (2621, Takara, Tokyo, Japan). Then, TB Green FAST qPCR kit (CN830S, Takara) was applied for PCR amplification. β-actin serving as an internal control.

These were the primer sequences:

Notch3:

F: 5'-TTCCCCTCTCACCTCGGAAG-3'
R: 5'-TTTCCCTGCGTGTTTCTTGC-3'

β-actin:

F: 5'-TCCTATGGGAGAACGGCAGA-3'
R: 5'-TCCTTTGTCCCCTGAGCTTG-3'

2.10 Western Blotting

RIPA lysis buffer (P0013B, Beyotime) was utilized for lysing cells or tissues to extract proteins, and the BCA kit (P0012, Beyotime) was for assessing protein concentrations. After gel electrophoresis was performed, the samples were moved to PVDF membranes (Invitrogen) and closed for 1 h. Following rinsing the membrane, it was incubated overnight with Notch3 primary antibody (ab23426, 1:100, Abcam, Cambridge, MA, USA), PI3K primary antibody (PA5-29220, 1:1500, Invitrogen), p-PI3K primary antibody (PA5-17387, 1:1000, Invitrogen), p-Akt primary antibody (44-621G, 1:1000, Invitrogen) or Akt primary antibody (44-609G, 1:1000, Invitrogen) at 4°C. On the following day, after being rinsed thrice, the membrane was cultured with secondary antibody IgG (31460, 1:10000, Invitrogen) for 1.5 h. The ultra high sensitivity ECL reagent (HY-K1005, MedChemExpress) was evenly dripped onto the membrane. β-actin (MA1-140, 1:5000, Invitrogen) served as the internal reference, and the grayscale value was obtained after image processing with Image J software.

2.11 Statistical Analysis

A minimum of 3 repetitions in each experiment, with the result being reported as the average value ± standard deviation. SPSS26.0 software was employed to process and analyze the data statistically. A one-way analysis of variance was utilized to conduct several comparisons among the different groups. In cases of independent samples, Student's *t*-test was applied if normally distributed, if not, non-parametric tests were used. Prism software (Graphpad 9.0) was utilized for plotting. * and # denote that there was a significant difference.

3. Results

3.1 Expression of Notch3 in GC

Notch3 expression in the TCGA dataset was examined through GEPIA2 database (http://gepia2.cancer-pku.cn/#index), revealing a notable rise in Notch3 level in GC tissues (Figure 1A). We examined the level of Notch3 in different cells through RT-qPCR and western blot, and the findings demonstrated that GC cells exhibited notably higher expression of Notch3 in comparison to GES-1 cells (Figure 1B-1D). Among them, Notch3 expression was found to be highest in AGS and HGC-27 cells, leading us to choose these cells for further biological characterization experiments. In addition, the connection between Notch3 expression and patient overall survival was assessed through the Kaplan-Meier plotter (http://kmplot.com/analysis/), it was found that patients with elevated Notch3 level experienced a notably reduced overall survival in comparison to those with lower levels (Figure 1E). This suggested that elevated levels of Notch3 expression are linked to a negative prognosis.

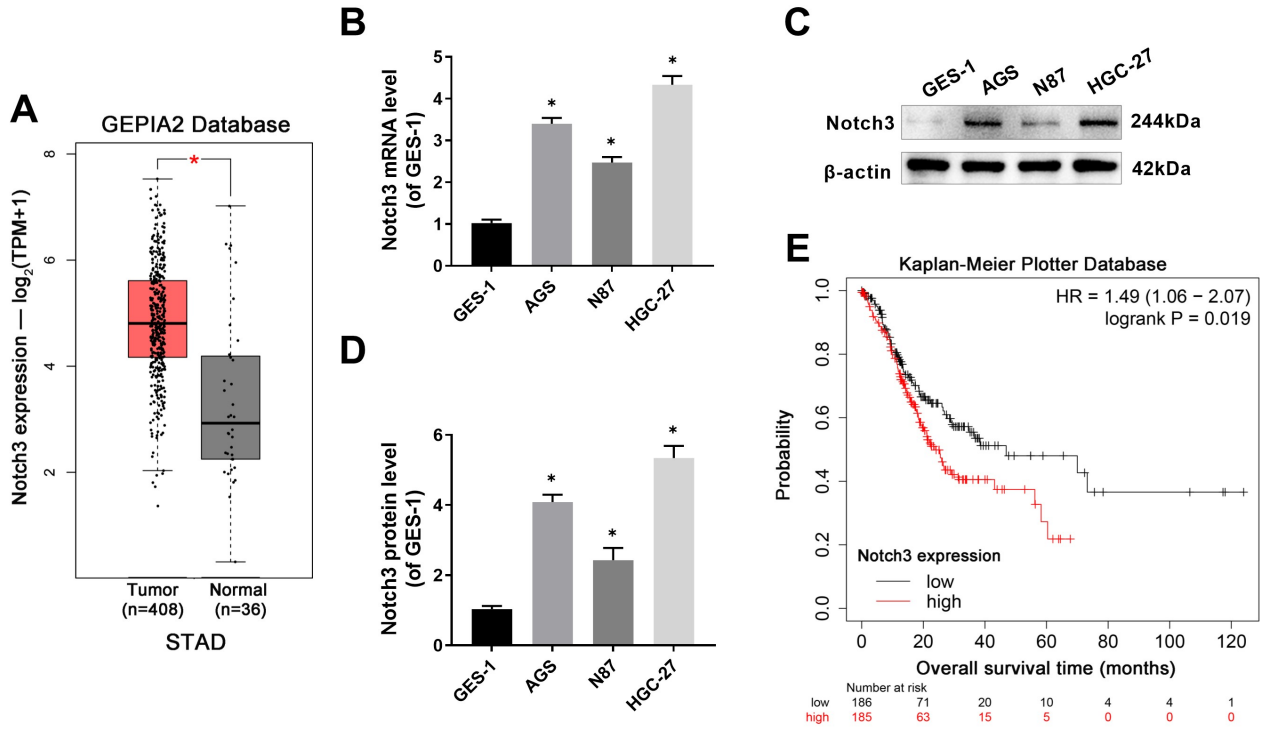

Figure 1. Expression of Notch3 in GC. (A) GEPIA2 database shows Notch3 expression in TCGA gastric cancer dataset. (B-D) Notch3 expression was assessed by qRT-PCR and western blot. (E) Kaplan-Meier Plotter Database shows effect of Notch3 expression levels on OS. *P<0.05.

3.2 Silencing Notch3 Inhibits GC Malignant Progression

We performed transfection of si-Notch3 in AGS and HGC-27 cells and tested its silencing effectiveness, the findings verified a notable decrease in both Notch3 mRNA and protein levels, which could be used for subsequent functional experiments (Figures 2A-2C). Silencing Notch3 led to a considerable decline in the viability of GC cells, as detected by the CCK-8 assay (Figure 2D). Clone formation assay results revealed a notable decrease in clone formation ability of cells following the silencing Notch3 (Figure 2E-2F). The cell scratch assay established that silencing Notch3 reduced the migration capability (Figure 2G-2H). In addition, Transwell assay results similarly indicated that silencing Notch3 markedly reduced the invasion and migration abilities of AGS and HGC-27 cells (Figure 2I-2L). These findings indicated that silencing Notch3 inhibited the malignant biological behavior of GC cells.

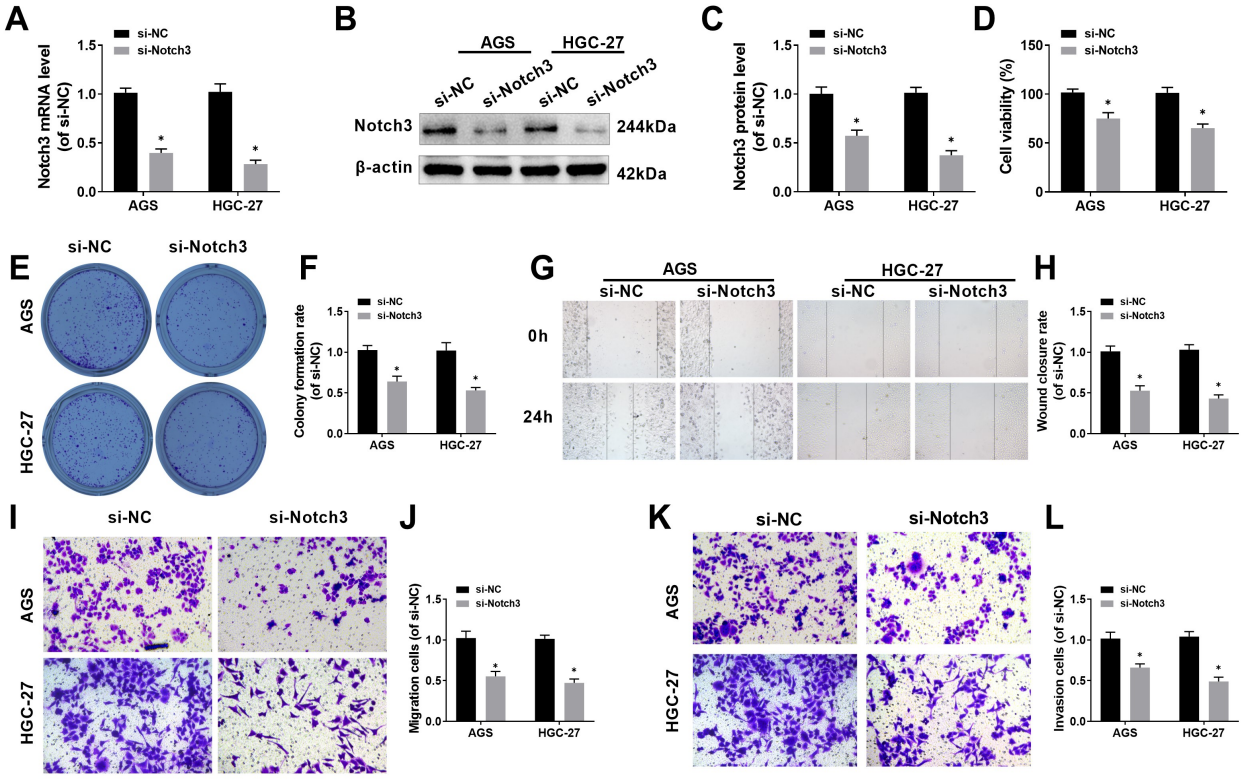

Figure 2. Silencing of Notch3 inhibits the malignant progression of GC. (A) Examining Notch3 silencing efficiency through qRT-PCR. (B-C) Examining Notch3 level through western blot. (D) After transfected si-NC or si-Notch3, GC cell viability was assessed by CCK-8. (E-F) Clone formation assay was performed to evaluate cell proliferation. (G-H) The wound closure rate was scrutinized using scratch-wound assay (10×, bar=200 μm). (I-L) Transwell assay examined cell invasion and migration, with the amount of invasive and migrated cells was counted (20×, bar=100 μm). *P<0.05

3.3 Rh4 Inhibits GC Malignant Progression and Notch3 Over-Expression Partially Attenuated the Inhibition of Rh4

We used the Coremine Medical database (https://coremine.com/medical/) to analyze herbal medicines associated with the Notch3 gene and found that Panax ginseng may be a key herbal medicine for intervening in the Notch3 target (Figure S1A). Figure S1B demonstrates the molecular structure of Rh4. After transfected OE-Notch3 into GC cells, there was a substantial increase in Notch3 level (Figure S1C-S1E). We utilized the CCK-8 assay to examine the impact of Rh4 on cell viability to screen the appropriate treatment concentration. The findings indicated a gradual decline in AGS and HGC-27 cell viability with increasing doses of Rh4 (20, 40, 60, or 80 μM) (Figure 3A). In subsequent experiments, we chose to treat cells with 20 μM Rh4. Interestingly, Rh4 significantly reduced Notch3 mRNA and protein expression (Figure 3B-3D). According to the CCK-8 assay, Rh4 had a significant impact on reducing cell viability in GC cells. However, when Notch3 was over-expressed, it partially reversed the inhibition of Rh4 (Figure 3E). Additionally, Rh4 greatly diminished the clone formation capacity of GC cells, while the over-expression of Notch3 lessened the impact of Rh4 (Figure 3F-3G). The scratch healing rate of GC cells was notably reduced by Rh4 treatment, which was attenuated by over-expression of Notch3 (Figure 3H-3I). Additionally, Rh4 treatment notably declined the invasion and migration capacities of GC cells, while over-expression of Notch3 attenuated the effect of Rh4 (Figure 3J-3M). The above findings implied that Rh4 suppressed malignant progression of GC and Notch3 expression, and Notch3 overexpression partially attenuated the suppressive impact of Rh4, suggesting that Rh4 acts by downregulating Notch3.

3.4 Rh4 Suppresses the PI3K/Akt Pathway by Regulating Notch3

In order to delve deeper into the molecular mechanisms how Rh4 hinders the malignant progression of GC, we analyzed the influence of Rh4 on the PI3K/Akt pathway. We examined the proteins involved in the PI3K/Akt pathway, revealing a notable rise in the phosphorylation level of PI3K and Akt in GC cells after over-expression of Notch3, while Rh4 attenuated this effect (Figure 4A-4D), which suggested that Rh4 may hinder the PI3K/Akt pathway by regulating Notch3.

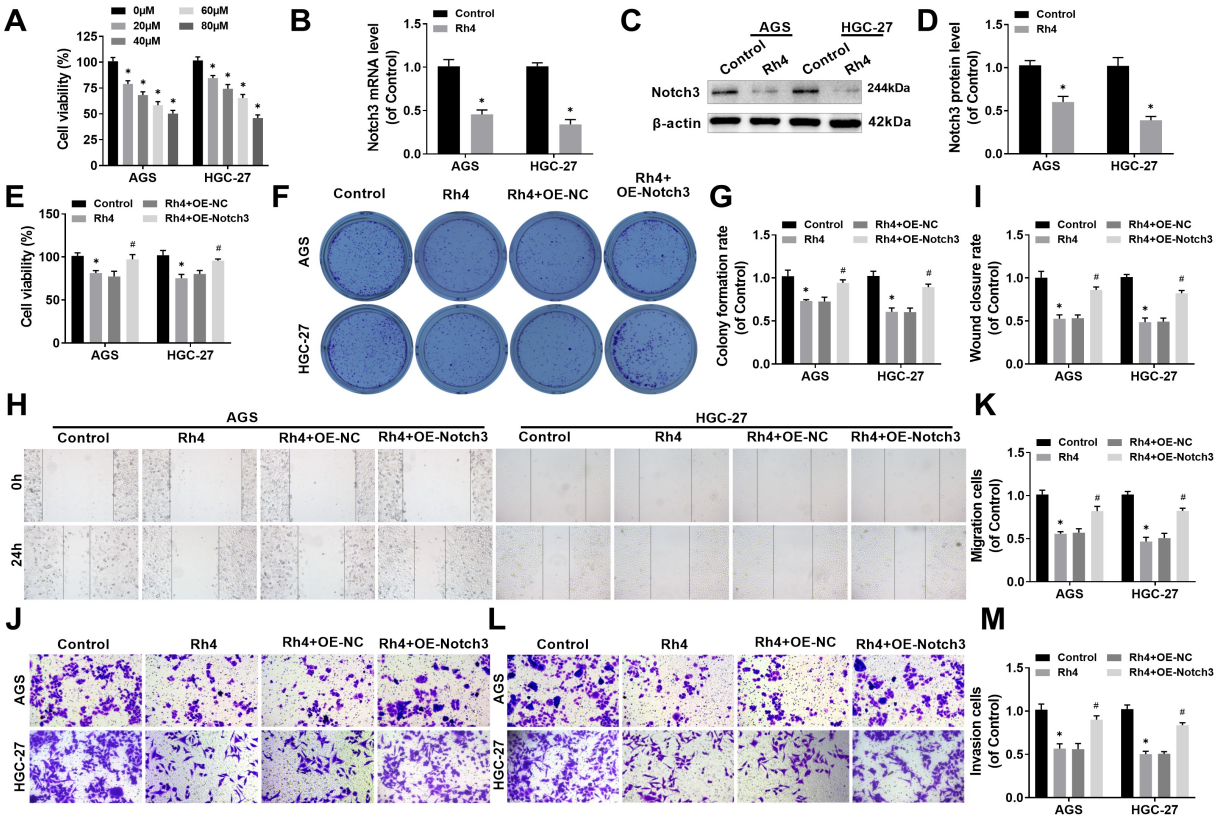

Figure 3. Rh4 inhibits GC malignant progression and Notch3 over-expression partially attenuated the inhibition of Rh4. (A) The influences of Rh4 on cell viability were identified utilizing CCK-8 assay. (B-D) The influences of Rh4 on Notch3 expression were examined through qRT-PCR and western blot. (E) Viability of GC cells after RH4 treatment and overexpression Notch3 was monitored through CCK-8 assay. (F-G) Clone formation assay was performed to evaluate cell proliferation. (H-I) The wound closure rate was scrutinized utilizing scratch-wound assay (10×, bar=200 μm). (J-M) The amount of migration and invasion cells were calculated through transwell assay (20×, bar=100 μm). *P<0.05 vs Control, #P<0.05 vs Rh4+OE-NC

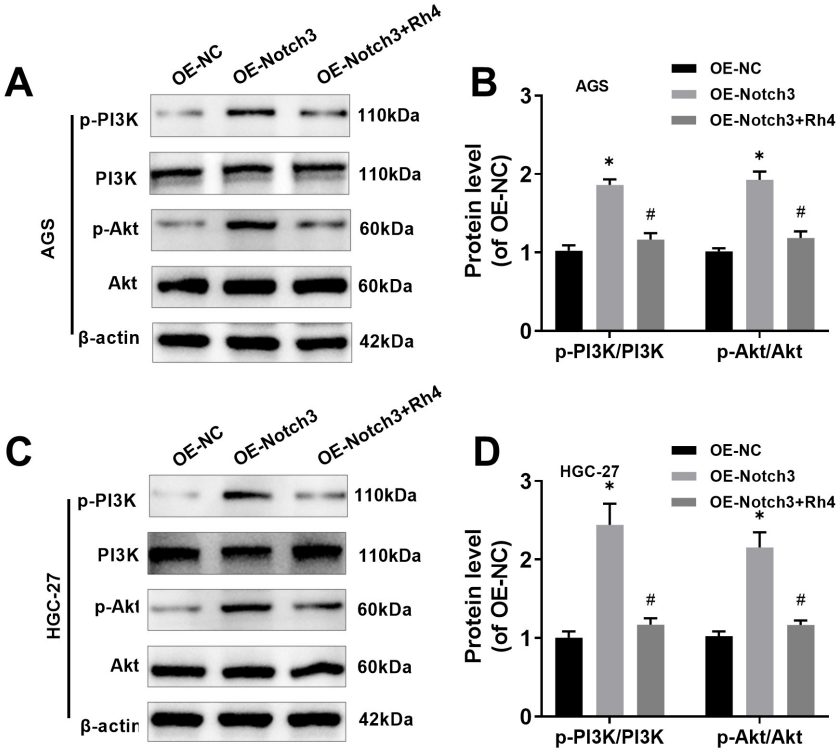

Figure 4. Rh4 hinders the PI3K/AKT pathway by regulating Notch3. (A-D) Assessing PI3K/AKT pathway-related protein levels through western blot. *P<0.05 vs OE-NC, #P<0.05 vs OE-Notch3.

3.5 Rh4 Suppresses GC Malignant Progression Through Hindering the PI3K/AKT Pathway

To further determine whether Rh4 inhibited the GC malignant progression through inhibiting the PI3K/Akt pathway, we intervened in Rh4-treated AGS and HGC-27 cells using 740 Y-P (30 μM), a PI3K agonist, and assayed PI3K/Akt pathway-related protein levels. The findings demonstrated that Rh4 notably decreased the PI3K and Akt phosphorylation levels, while 740 Y-P attenuated this effect (Figure 5A-5C). Not only that, 740 Y-P partially declined the inhibition of Rh4 on cell viability and clone formation (Figures 5D-5F). Scratch assay and transwell results showed that Rh4 notably reduced cell migration and invasion ability, while 740 Y-P attenuated the inhibitory effect of Rh4 (Figures 5G-5L). The findings above indicated that Rh4 effectively inhibited GC malignant progression through suppressing the PI3K/AKT pathway.

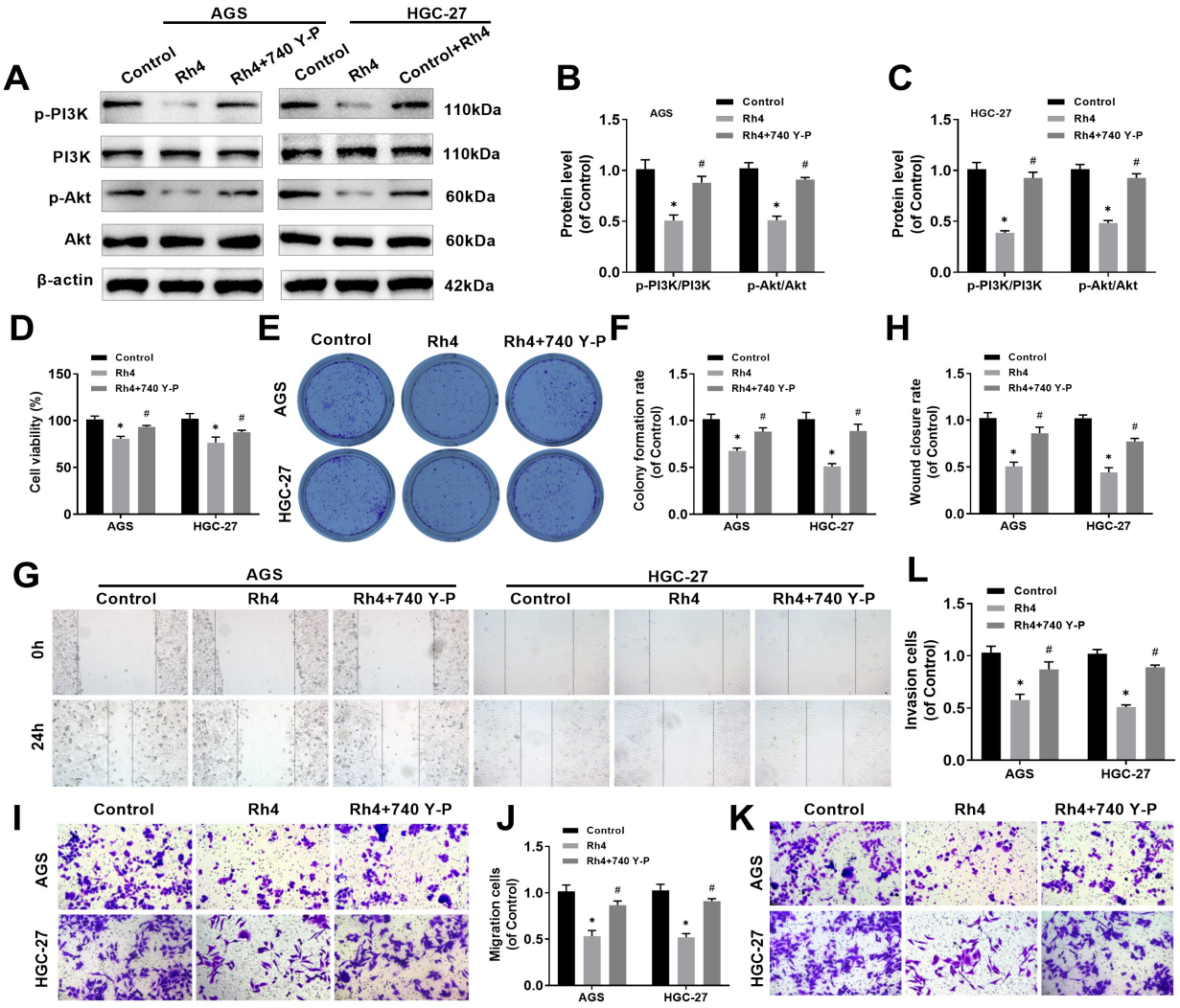

Figure 5. Rh4 inhibits GC malignant progression through hindering the PI3K/AKT pathway. (A-C) Examining PI3K/AKT pathway-related protein levels by western blot. (D) Viability of GC cells after RH4 and 740-YP treatment was monitored through CCK-8. (E-F) Clone formation assay was performed to evaluate cell proliferation. (G-H) The wound closure rate was scrutinized using scratch-wound assay (10×, bar=200 μm). (I-L) The amount of migration and invasion cells were identified by transwell assay (20×, bar=100 μm). **P*<0.05 vs Control, #*P*<0.05 vs Rh4

3.6 Rh4 Inhibits Tumor Growth *in vivo* by Regulating Notch3 and PI3K/AKT Pathways

Rh4 injection caused a noticeable declined in both the volume and weight of tumors, and the inhibitory effect of H-Rh4 (100 mg/kg) was markedly higher than that of L-Rh4 (50 mg/kg) (Figure 6A-6C). The impact of Rh4 on the Ki-67 positive cells was determined utilizing immunohistochemistry. The findings revealed a notable decrease in the Ki-67 positive cells in GC tissues after Rh4 injection, and the influence of Rh4 in suppressing the growth of GC cells *in vivo* improved with higher dosages (Figure 6D-6E). Notably, following Rh4 injection, the levels of Notch3 protein, PI3K and Akt phosphorylation were markedly decreased in GC tissues, further confirming that Rh4 inhibits tumor growth by regulating Notch3 and PI3K/AKT pathway (Figure 6F-6G).

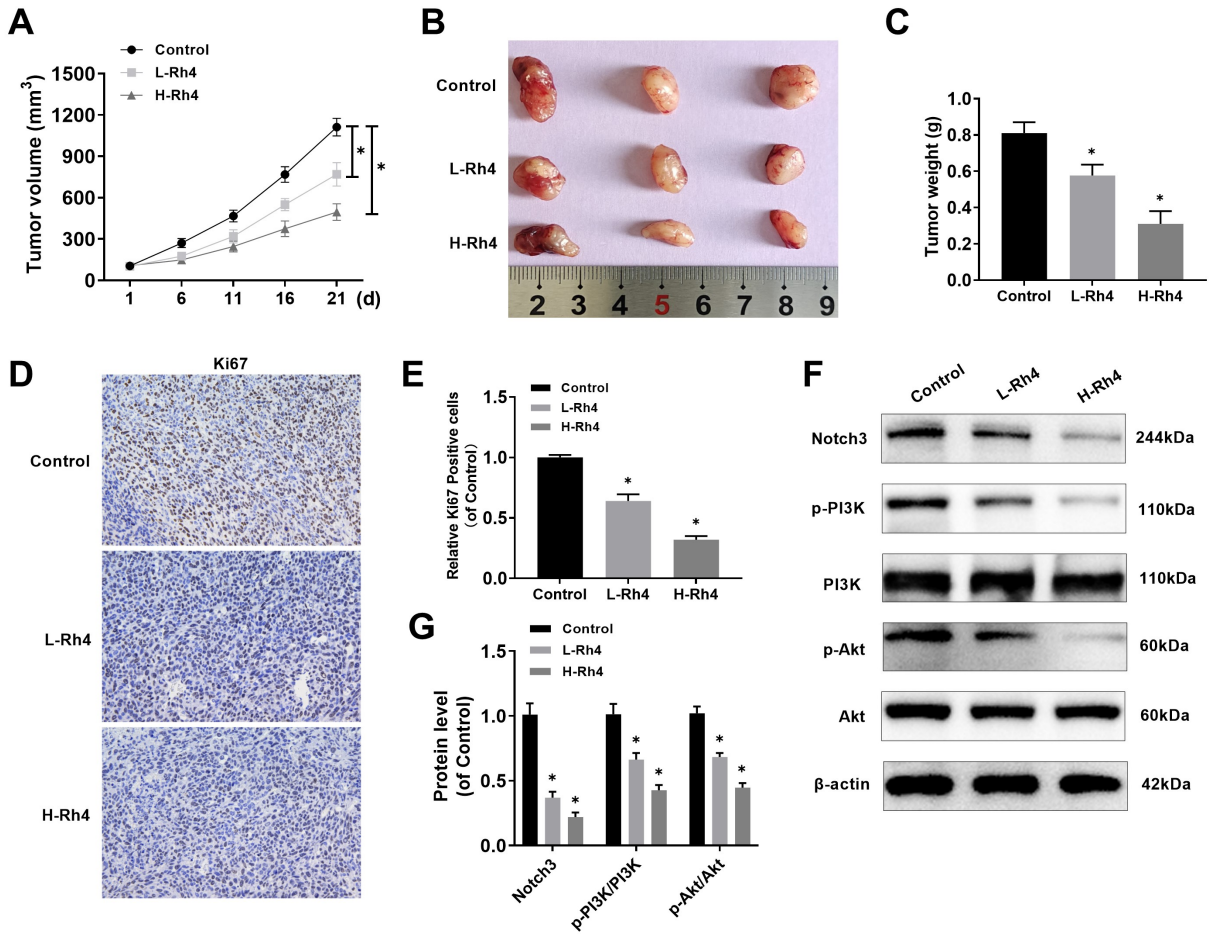

Figure 6. Rh4 inhibits tumor growth in vivo by regulating Notch3 and PI3K/AKT pathways. (A) The changes in tumor tissue volume. (B-C) The weight of tumor tissue (21st d). (D-E) Analyzing Ki-67 expression in GC cells via immunohistochemistry (40×, bar=50 μm). (F-G) Examining Notch3 and PI3K/AKT pathway-related protein level through western blot. *P<0.05 vs Control

4. Discussion

The pathogenesis of GC is very complex, influenced by multiple factors and genes, and its etiology is unclear and rapidly evolving, leading to a low rate of early diagnosis and survival of GC [34,35]. Therefore, in-depth elucidation of the specific mechanisms of GC development, identifying reliable biomarkers for early detection, and discovering potent drugs for treatment are critical factors in enhancing the diagnosis and survival rates of GC.

There is growing evidence that Notch3 is present in various tissues, like vascular, smooth muscle, central nervous and immune system [36,37]. Not only that, Notch3 is implicated in the development of various types of malignant tumors, like colorectal [38], prostate [39], ovarian [40], and pancreatic cancer [41]. Wei *et al.* found that among four Notch receptors, the expression of Notch3 was found to be elevated in various GC datasets and was correlated with unfavorable clinical results [42]. Our research revealed a marked elevation in Notch3 level in GC. Notably, patients exhibiting elevated Notch3 levels had a markedly reduced overall survival rate in comparison to those with low levels, indicating that Notch3 could be a valuable prognostic marker for GC. In addition, silencing Notch3 inhibited the viability, clone formation, migration, and invasive abilities of GC cells, further suggesting that Notch3 is crucial in regulating GC malignant progression.

According to the previous reports, the ability of Notch family receptors to regulate transcription of downstream target genes is well-established. This occurs through binding of activated Notch receptors to CSL (CBF1/Su(H)/Lag-1) elements within the promoter regions of target genes, initiating transcriptional activation [43,44]. However, the precise mechanisms by which Notch signaling modulates cancer progression in certain malignancies, such as gastric cancer, remain poorly understood. A previous report elucidated that Notch3 directly binds to CSL elements within the promoter of SPP1 gene, thereby enhancing SPP1 transcription. This, in turn, activates PI3K/Akt signaling, promoting the malignant progression of BLCA. SPP1, a multifunctional glycoprotein, plays a critical role in tumor cell adhesion, migration, and invasion, and its expression is linked to various stages of cancer progression [45]. Furthermore, in pancreatic cancer, for example, Notch3 has been shown to increase the activity of PI3K/Akt pathway in response to gemcitabine treatment, a common chemotherapeutic agent. This effect is reversible by silencing Notch3 with specific siRNAs, highlighting Notch3's regulatory role in

modulating chemotherapy response and tumor progression [46,47]. By searching the Coremine Medical database for traditional Chinese medicines related to Notch3 gene, we found that Panax ginseng may be a key traditional Chinese medicine to intervene in Notch3. Rh4 is a crucial active compound of Panax ginseng, and several studies have confirmed the favorable anticancer effects of Rh4. According to Wu *et al.*, Rh4 hindered the proliferation capacity of colorectal cancer cells and induced ferroptosis via the autophagy pathway [48]. Jiang *et al.* discovered that Rh4 could suppress the SIX1-TGF-β/Smad2/3 axis, resulting in reduced GC cell growth and metastasis [49]. Our results showed that Rh4 could notably reduce the viability of GC cells, aligning with the report of Jiang *et al.* Interestingly, Rh4 could markedly down-regulate Notch3 in GC cells and mouse GC tissues, whereas over-expression of Notch3 attenuated the inhibition of Rh4 on GC malignant progression, hinting at the role of Rh4 in inhibiting cancer through the suppression of Notch3 expression.

Aberrant activation of the PI3K/Akt pathway is vital in regulating cell proliferation, metastasis, metabolism, oxidative stress and apoptosis [50,51]. When a growth factor interacts with a receptor tyrosine kinase, PI3K is commonly activated, which in turn triggers the recruitment and phosphorylation of Akt at the plasma membrane [52]. Wang *et al.* found that ApoC-II promotes GC malignant progression through the PI3K/Akt/mTOR pathway, resulting in peritoneal metastasis [53]. Shan *et al.* showed that Eriodictyol inhibits clone formation and enhances apoptosis in GC cells by hindering PI3K/Akt pathway [54]. Our research revealed that over-expression of Notch3 activated the PI3K/AKT pathway, while Rh4 attenuated the influence of Notch3, and that the suppression of Rh4 on PI3K/AKT pathway was attenuated by PI3K agonist intervention. Not only that, PI3K agonists reduced the inhibition of Rh4 on GC malignant progression, suggesting that Rh4 may suppress GC malignant progression through hindering the PI3K/AKT pathway. Notably, the subcutaneous graft tumor model and immunohistochemical assay results also indicated that Rh4 inhibited tumor growth in mice *in vivo* through PI3K/AKT pathway.

5. Conclusion

Our findings revealed that Rh4 was able to reduce the GC viability, suppressed Notch3 expression in both GC tissues and cells, which in turn inhibited migration and invasion. Additionally, Rh4 was able to hinder tumor growth by regulating Notch3 and PI3K/AKT pathway *in vivo*. Importantly, we identified a new mechanism in which Rh4 hinders the PI3K/AKT pathway by suppressing Notch3 levels. In conclusion, Rh4 shows promising prospects in the clinical management of GC and deserves further study in the future.

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Author's Contributions

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Ethics Approval

This study involved animal experimental models. Hence, a full ethical approval was obtained from the Institutional Ethical committee of Raghavendra Institute of Pharmaceutical Education and Research (RIPER) India.

Consent for Publication

Not applicable

Conflict of Interest

The authors declare no conflict of interest

Data Availability

Not applicable

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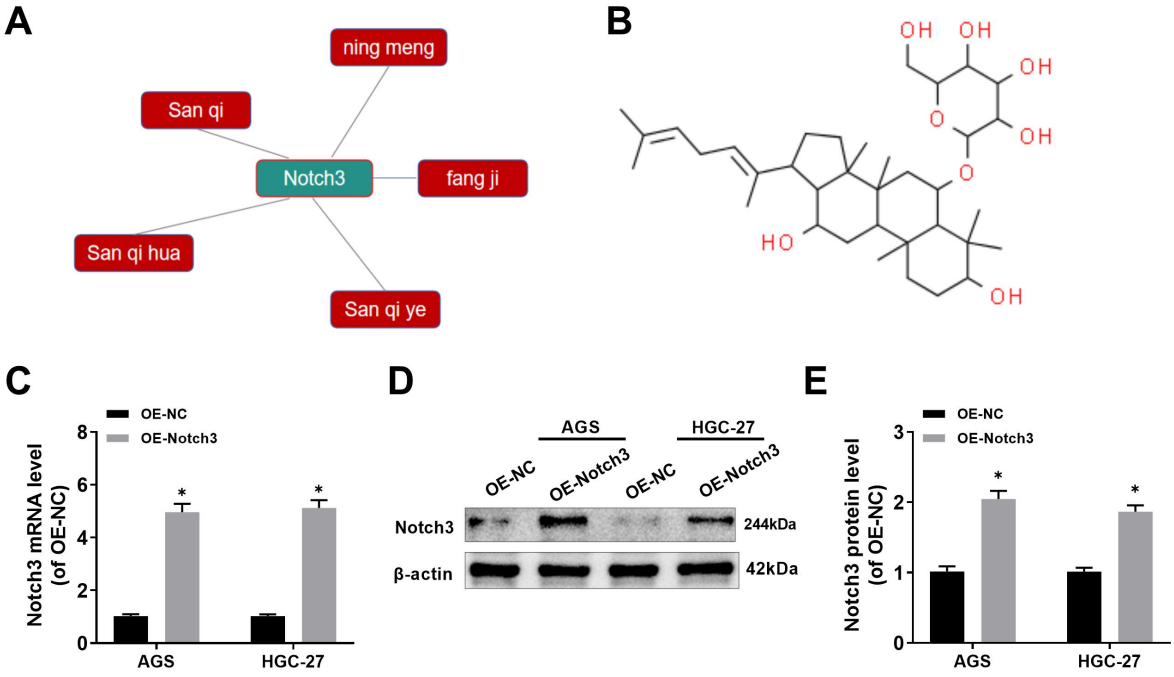

Supplementary Figure S1: (A) Search for herbal medicines related to Notch3 gene via Coremine Medical Database. (B) Molecular structure of Rh4. (C) Examining Notch3 over-expression efficiency by qRT-PCR. (D-E) Assessing Notch3 level by western blot. **P*<0.05

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