

Jagged 1 Regulates The Proliferation and Metastasis of Human MDA-T68 Thyroid Cancer Cells

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Abstract

Objective: The current study evaluated the expression profile and explored the therapeutic implications of Jagged 1 in human thyroid cancer.

Materials and Methods: This experimental study was conducted in 60 paired specimens of papillary thyroid and adjacent normal tissues. Gene expression was determined by quantitative real time polymerase chain reaction (qRT-PCR) and western blotting. Transfection of cancer cells was performed by using Lipofectamine 2000. The cell proliferation of PTC cells was estimated by MTT assay. Clonogenic assay was performed for analysis of colony forming potential of cancer cells. The apoptosis of PTC cells was studied by using AO/EB and Annexin V-FITC/PI staining methods. Flow cytometry was done to analyze the cell cycle phase distribution of cancer cells. Migration and invasion PTC cells were determined respectively with the wound-healing and transwell assays. The impact of Jagged 1 silencing was investigated *in vivo* in a xenografted mice model followed by Immunohistochemistry (IHC) analysis.

Results: We found significant ($P < 0.05$) upregulation of Jagged 1 in human thyroid cancer. Silencing of Jagged 1 caused significant ($P < 0.05$) reduction in proliferation and colony formation of MDA-T68 cells. The inhibitory effects of Jagged 1 silencing were found to be due to the induction of apoptosis. We also found enhancement of Bax and repression of Bcl-2 protein levels in MDA-T68 cells. Wound healing assay indicated significant ($P < 0.05$) inhibition of cell migration of MDA-T68 thyroid cancer cells. Additionally, we found that invasion of the thyroid cancer cells was reduced by 55% upon silencing of Jagged 1. Moreover, Jagged 1 silencing was found to cause inhibition of the Notch intracellular domain (NICD) and Notch target gene, Hes-1 expression. Finally, Jagged 1 silencing inhibited the xenografted tumors *in vivo*.

Conclusion: The findings suggest that Jagged 1 regulates the development of thyroid cancer that may act as a therapeutic target for managing thyroid cancer.

Keywords: Apoptosis, Invasion, Migration, Proliferation, Thyroid Cancer

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Introduction

For more than 30% of the head and neck cancers, thyroid cancer is a prevalent and aggressive cancer with very a high mortality rate (1). Papillary thyroid carcinoma (PTC) is the most prevalent subtype of thyroid cancer (2, 3). Common subtypes of PTC include conventional, follicular and tall cell, while as other uncommon ones are also present. The conventional subtype is having papillary architecture with branching. The follicular subtype when examined grossly looks like follicular neoplasm containing different-sized follicles. Tall Cell subtype is composed of tall cells (with height 2-3 times more than width). Low dietary intake of iodine, increased ionizing radiations and genetic factors significantly increase the risk of thyroid cancer (4). The progression of the disease is slow with no marked clinical symptoms leading to diagnosis of the disease at advanced stages (5). Currently, chemotherapeutic agents like doxorubicin and cisplatin have adverse effects and constant thyroid cancer relapses have been reported (6). This has pressed

the need for efficient chemotherapy and molecular markers/targets for its management.

Notch signalling cascade is dysregulated in different human cancers and involved in their development (7, 8). Notch signalling in humans consists of four Notch receptors (Notch 1-4) and five ligands (Delta 1, 3, and 4 and Jagged 1 and 2) (9). Two proteolytic cleavages are aided by γ -secretase and ADAM metalloprotease during the binding of any Notch ligands to the receptor (10). This results in the discharge of the Notch intracellular domain (NICD) from the cell transmembrane, which subsequently translocates to nucleus (11, 12). The expression of the Notch ligand, Jagged-1, is associated with the growth of different human cancers (13, 14). Nonetheless, the role of Jagged 1 in the development of PTC is yet largely unknown. Given this background, the goal of the current study was to look at Jagged 1's expression pattern and determine how it affects the growth, invasion, and migration of papillary human thyroid cancer.

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Materials and Methods

Tissue samples

In this experimental study, sixty paired tissue specimens of papillary thyroid and adjacent normal tissues were obtained from the Department of General Surgery, Affiliated Haian Hospital of Nantong University, Haian, China. Informed consent was acquired from the patients before collection. The tissue samples were obtained at the time of surgery and subsequently stored in liquid N₂ for further experimentation. The differentiation between the normal and cancerous tissues was carried independently by two pathologists. The research Ethics Committee of Affiliated Haian Hospital of Nantong University, Haian, China approved the study (HH/NU-301H-2019).

Cell lines and culture conditions

The PTC cell lines MDA-T32, TT, K, TPC1, and MDA-T68 were procured from the Shanghai Cell Institute Country Cell Bank. The cell lines were subjected to culturing in DMEM (Gibco, USA) at 37°C in a 5% CO₂ incubator. The DMEM medium was supplemented with fetal bovine serum (10%) (HIMEDIA, USA), penicillin (100 U/mL) (HIMEDIA, USA), and streptomycin (100 U/mL) (HIMEDIA, USA).

Transfection

The Jagged 1 siRNA constructs (si-NC, and si-Jagged 1) were provided by RiboBio, China. The sequence of si-NC was 5'-CCAUCUCCCGGUACAAAUCUGCU-3' and si-Jagged 1 sequence was 5'-GCAAAGAAGCCGUGU-GUAA-3'. After MDA-T68 cells were cultured to 50-60% confluency in 6-well plates, they were subjected to transfection with siRNA using the Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's guidelines.

Quantitative real time polymerase chain reaction

Total RNAs was extracted by Trizol assay (Invitrogen, Carlsbad, CA) and subsequently cDNA synthesized by TaKaRa Reverse Transcription Kit (Shiga, China). Power SYBR Green (TaKaRa) was then used to prepare PCR reaction system. Transcript levels were determined by normalized to 2^{-ΔΔCt} method, actin and GAPDH were utilized as an internal control.

The primer sequences used for expression study were:

Jagged 1:

F- 5'-GAAGCAGAACACGGGCGTT-3'
R- 5'-CAGGTCACGCGGATCTGAT-3'

β-actin:

F- 5'-CCTGGATAGC AACGTAC-3'

R- 5'-CACCTTCTACAATGAGCT-3'

GAPDH:

F- 5'-CAATGACCCCTTCATTGA CC-3'
R- 5'-TGGAAGATGGTGTATGGGATT-3'

Cell viability assay

Transfected MDA-T68 cells were seeded with a density of 4500 cells/well in 96-well plates. Cells were cultured for different intervals of time like 0, 12, 24, 48 and 96 hours. Then 10 μL of MTT (Sigma-Aldrich, USA) was added and the cells were again incubated for 4 hours at 37°C. During this process formazan crystals were formed which were subsequently dissolved by addition of 100 μL of dimethyl sulfoxide (Sigma-Aldrich). Finally, to estimate cell viability, absorbance was measured at 570 nm.

Colony formation assay

In case of colony formation assay, 5000 transfected MDA-T68 cells were added into 6-well plates. Following incubation at 37°C for 14 days, fixation of the colonies was done for 15 minutes in 4% paraformaldehyde (Sigma-Aldrich). Subsequently, staining of the colonies was performed for 10 minutes in 1 mg/mL crystal violet (Sigma-Aldrich). Number of colonies was estimated under a microscope (Olympus, Japan).

Morphological examination

The transfected MDA-T68 cells the density of 5×10⁵/wells subjected to culturing for 24 hours at 37°C. Subsequently, the coverslips with monolayer cells were inverted on the glass slide containing a solution of acridine orange and ethidium bromide (AO/EB) (Sigma-Aldrich). Finally, the cells were examined under a fluorescence microscope (Carl Zeiss, Germany) 200× magnification and images were captured.

Annexin V- FITC/PI dual staining

Annexin V-FITC/PI staining (ThermoFisher Scientific, Germany) was performed to evaluate level of cell apoptosis in transfected MDA-T68 cells. Briefly, transfected MDA-T68 cells at the density of 2×10⁴ cell/well in 6-well plates were cultured for 24 hours at 37°C. Harvesting of treated MDA-T68 cells was immediately followed by two times washing with phosphate-buffered saline (PBS, Sigma-Aldrich, United States). Subsequently the cells were stained using Annexin V-fluorescein isothiocyanate and propidium iodide binding buffer. Finally, the apoptosis percentage was detected using flow cytometry (Beckman Coulter, United States).

Wound healing assay

To determine the effects of Jagged 1 silencing, 1.5×10⁶ MDA-T68 cells/well were cultured in six-

well plates to about 90% confluence. A sterile 200 μ L pipette tip was used to make a scratch on the plates and photographed. Subsequently, the scratched cells were added to the serum-free medium and the photographs were again taken at 24 hours AxioCam single-channel camera (Carl Zeiss, AG).

Invasion assay

Transwell chambers having polycarbonate members (8 μ m pores) were placed in 6-well plates. Coating of the lower compartments was done by using I-type collagen (10 μ g/mL) (Sigma-Aldrich). In the upper chamber 200 mL of MDA-T68 cells (1.4×10^5 cells/mL) and in the lower one 800 μ L media supplemented with fetal bovine serum (20%) was placed and subsequently incubate for 24 hours at 37°C. Thereafter, cells which invaded to lower chambers via the membranes were subjected to staining by crystal violet, examined under inverted microscope, and photographed.

Western blot analysis

The proteins were extracted from MDA-T68 cells and subjected to protein estimation using the Lowry method. The equal quantity of protein samples (60 μ g/well) were loaded in the 8, 10, or 15% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and separated at 50V. The proteins separated in the gel are transferred to the PVDF membrane effectively using the semi-dry method. The membrane is subjected to blocking step using 4% bovine serum albumin (BSA) solution for 2 hours at room temperature and there after incubated with primary antibodies Jagged 1 (Santa Cruz, sc-390177, 1:1000), Hes-1 (sc-13844, Santa Cruz, 1:1000), NICD-1 (ab-52301, Abcam, 1:1000), Bcl-2 (sc-23960, Santa Cruz, 1:1000), Bax (sc-7480, Santa Cruz, 1:1000), and Actin (sc-58673, Santa Cruz, 1:1000) at 4°C for 6 hours. After washing the membrane with 1X TBST buffer the membrane was incubated with HRP-conjugated secondary antibody anti-rabbit IgG HRP (Cell Signaling Technology, 7074s, 1:2000) for 2 hours at 37°C. After finally washing the signals against the secondary antibody which are specific for the primary antibody are developed by using the chemiluminescent substrate (sc-2048, Santa Cruz, Dallas, TX, USA) and photographed using a ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA). The quantification of obtained signals is analysed using Image J software and the results are documented using Gel documentation system. For control purpose, β -actin is used and the final results were normalized to the control protein.

In vivo study

Around 4-6-week-old female BALB/c-nude mice were used for establishment of mice tumorigenic xenografts of thyroid cancer via subcutaneous injections of MDA-T68 cancer cells to induce tumor development. The mice were randomly divided into two groups (n=10) for each group. For analyzing the effects of the Jagged 1 silencing on

mice tumor growth, the mice tumors were given either the intra-tumor injections of si-Jagged 1 or si-NC injections (five in total, each after 3 days). The tumor volumes were monitored after every three days, and the tumor weight was determined at the end of 3 weeks after the mice were sacrificed. The institution's Ethics Committee on Animal Care and Experiments gave its approval to the use of animals in experiments (AKT/20170411).

Immunohistochemistry

Thyroid tissue samples were carefully dissected from scarified animals and fixed with 10% formalin for 2 days at room temperature. The tissues were embedded in wax block and fixed in microtome for thin sectioning (6 μ m size). Following thin sections (5 μ m sizes), the slides were dewaxed and subjected to antigen retrieval in a boiling sodium citrate buffer (0.01 M, pH=6.0) for 20 minutes. The slides were then washed with 1X TBST and prevented non-specific binding of antibody in the slide, the sections were subjected to blocking step using BSA solution (4% in TBST buffer) for 1 hour in 37°C. The primary antibodies like Jagged 1 (ThermoFisher Scientific, PA5-72843, 1:200), Ki67 1 (ThermoFisher Scientific, 701198, 1:200), and cleaved caspas-3 (ThermoFisher Scientific, 700182, 1:200) were diluted with 4% BSA and overlaid with the tissue sections for 6 hours at 4°C. After careful washing for 3 times with 1X TBST, staining was performed out with the help of Histostain-SP kits (Invitrogen) as per the manufacturer's guidelines. As positive control, MTC-M cells (ATCC) cells were used while as nonimmune IgG and incubation with secondary antibody was used as negative control.

Statistical analysis

For achieving statistical significance, the experiments were repeated for at least three times and the results obtained were denoted in mean \pm SD. For comparing two independent groups Mann-Whitney U test, a non-parametric equivalent of the t test was used. The obtained results were considered as statistically reliable when the $P < 0.005$.

Results

Jagged 1 is upregulated in thyroid cancer

Transcript levels of Jagged 1 were estimated in thyroid cancer tissue and normal tissues by quantitative real time polymerase chain reaction (qRT-PCR). We found significant ($P < 0.05$) upregulation of Jagged 1 in human thyroid cancer tissues relative to normal adjacent tissues (Fig.1A). Next, Jagged 1 levels were examined in normal thyrocytes cells and the MDA-T68, MDA-T32, TT, and K1 cell lines. The RT-qPCR revealed Jagged 1 to be considerably upregulated in the all the thyroid cancer cell lines relative to the normal thyrocytes (Fig.1B). Western blot analysis also showed considerable upregulation of Jagged 1 in all the cancer cell lines (Fig.1C). This finding is supported by densitometry analysis (Fig.1D).

Jagged 1 silencing suppresses the growth of thyroid cancer cells

Knowing about the role of Jagged 1 in thyroid cancer, MDA-T68 cells were transfected with si-NC or si-Jagged 1. Next, RT-qPCR analysis was performed to validate the silencing of Jagged 1 (Fig.2A). Transfected MDA-T68 cells were then cultured and subjected to cell viability assessment. We found that silencing of Jagged 1 resulted in significant ($P < 0.05$) decline in the viability of MDA-T68 cells (Fig.2B, C). The inhibition of cell viability was more severe with increase in time. Moreover, colony formation assay indicated that the colony formation was inhibited by around 75% upon MDA-T68 silencing. Above findings indicate that silencing of Jagged 1 leads to the inhibition/repression of thyroid cancer cells.

Jagged 1 silencing induces apoptosis of MDA-T68 cells

Next, AO/EB staining of si-NC and si-Jagged 1 transfected MDA-T68 cells was carried to evaluate the effects of Jagged 1 on apoptotic cell death. We found that silencing of Jagged 1 caused enhancement in red color

cells suggestive of apoptosis (Fig.3A). Annexin V/PI staining showed 3.45% apoptosis in si-NC and 22.88% (early and late apoptotic cells) in si-Jagged 1 (Fig.3B). Jagged 1 silencing also resulted in repression of Bcl-2 and enhancement of Bax further confirming apoptosis in MDA-T68 cells (Fig.3C). This finding is supported by densitometry analysis (Fig.3D). These findings indicate that Jagged 1 silencing promotes apoptosis in thyroid cancer cells.

Jagged 1 silencing suppresses migration and invasion of MDA-T68 cells

We also evaluated the effects of knockdown of Jagged 1 on the migration of the MDA-T68 cells by wound heal assay. We found that the migration of the si-Jagged 1 transfected cells was significantly ($P < 0.05$) suppressed relative to si-NC transfected cells (Fig.4A). The effects of Jagged 1 silencing were also investigated on the invasion of MDA-T68 cells (Fig.4B). It was observed that invasion of the MDA-T68 cells was inhibited by 55%. These findings suggest that Jagged 1 silencing suppresses migration and invasion of MDA-T68 cells.

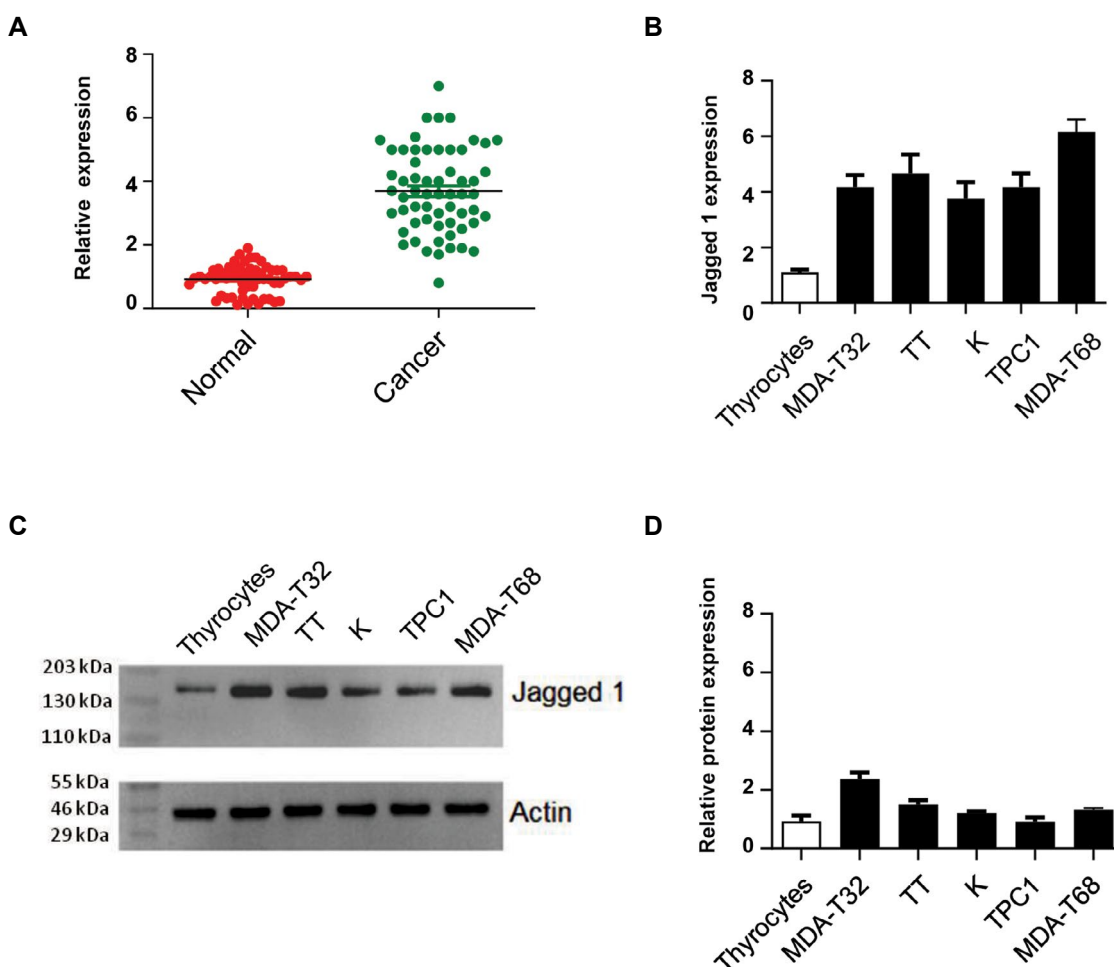


Fig.1: Overexpression of Jagged 1. **A.** Expression of Jagged 1 in human papillary thyroid cancer and normal adjacent tissues as determined by quantitative real time polymerase chain reaction (qRT-PCR). **B.** Expression of Jagged 1 in normal thyrocytes and papillary thyroid cancer cell lines as assessed by RT-qPCR. **C.** Western blots showing expression of Jagged 1 in normal thyrocytes and papillary thyroid cancer cell lines as determined by qRT-PCR. **D.** Densitometry analysis of western blot. All experimentation was done with three replicates.

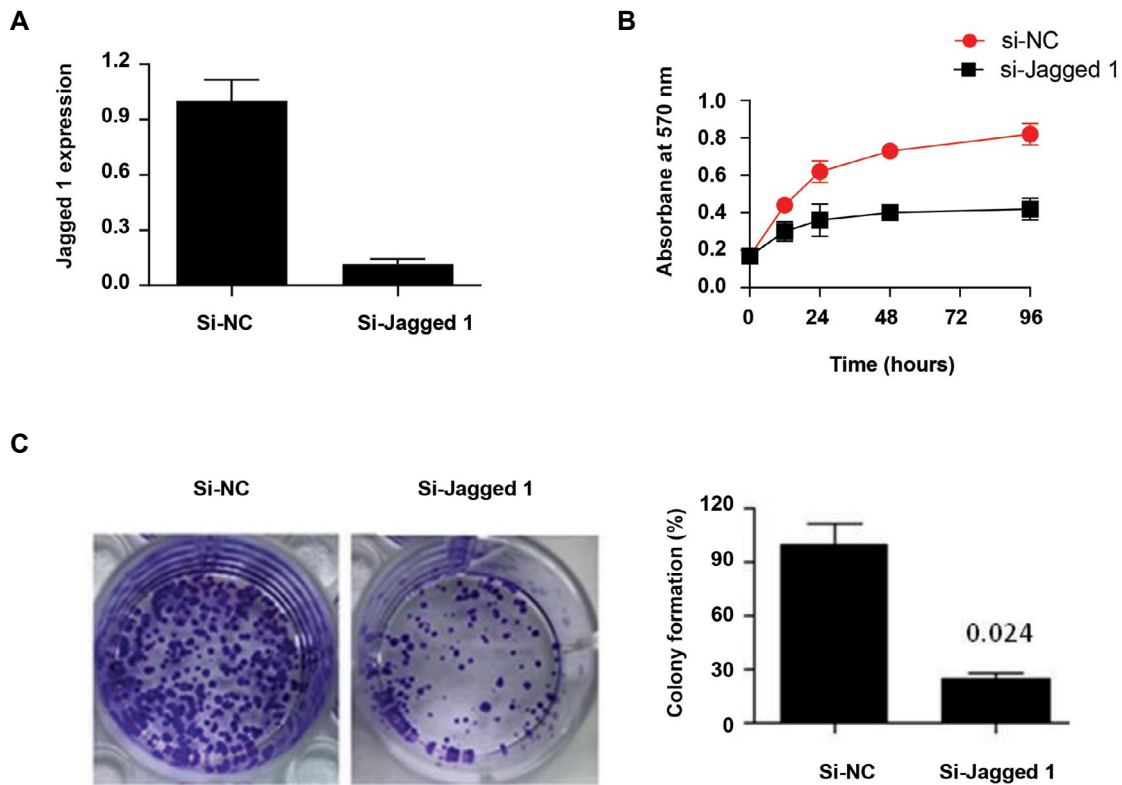


Fig.2: Jagged 1 silencing suppresses colonies of thyroid cancer cells. **A.** Expression of Jagged 1 in si-NC and si-Jagged 1 transfected MDA-T68 cells as evaluated by quantitative real time polymerase chain reaction (qRT-PCR). **B.** Cell viability of si-NC and si-Jagged 1 transfected MDA-T68 cells as determined by MTT assay. **C.** Colony production of Jagged 1 in si-NC and si-Jagged 1 transfected MDA-T68 cells as assessed by RT-qPCR. All experimentation was done with three replicates.

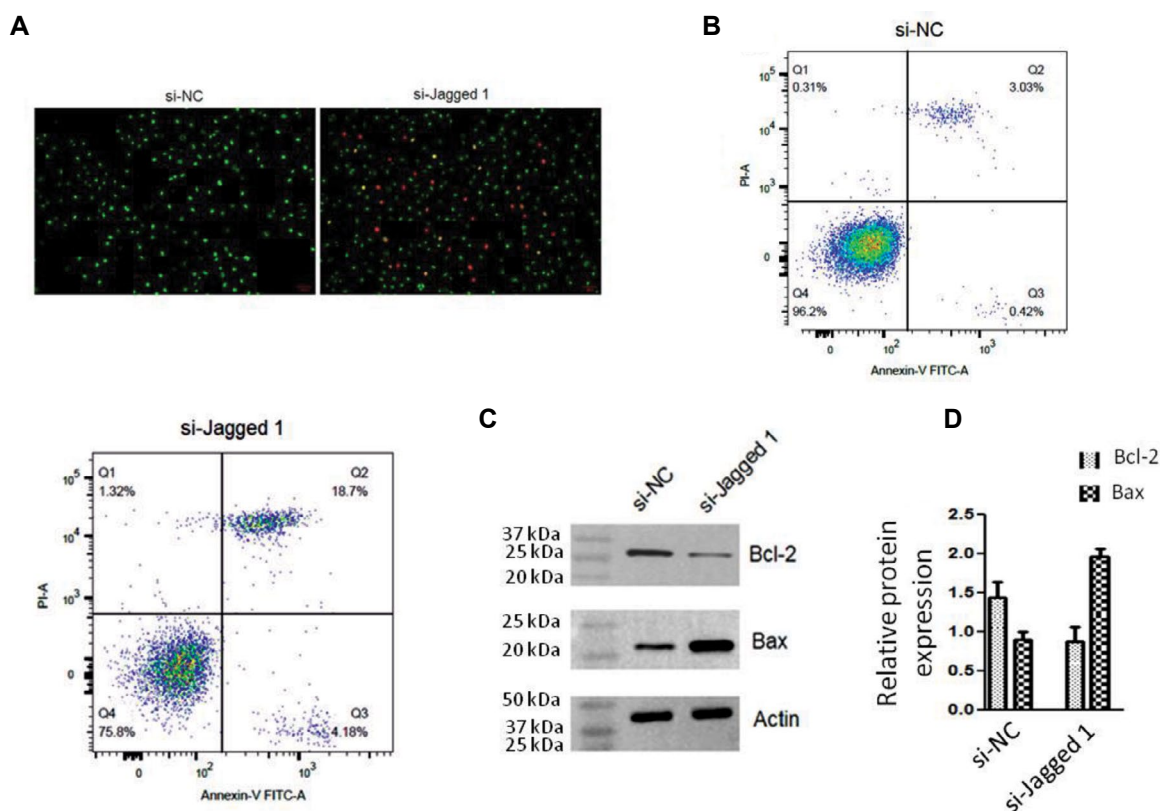


Fig.3: Jagged 1 silencing induces apoptosis. **A.** AO/EB staining of si-NC and si-Jagged 1 transfected MDA-T68 cells showing induction of apoptosis in MDA-T68 cells (red and orange color depicts apoptotic cells) (scale bar: 20 μ m). **B.** Annexin V/PI staining of si-NC and si-Jagged 1 transfected MDA-T68 cells showing percentage of apoptotic cells. **C.** Western blots showing Bax and Bcl-2 proteins in Jagged 1 in si-NC and si-Jagged 1 transfected MDA-T68 cells. **D.** Densitometry analysis of western blot. Experimentation was done with three replicas.

Jagged 1 silencing inhibits Notch signalling in thyroid cancer cells

Next, we evaluated the impact of Jagged 1 silencing on Notch signalling. The results showed that silencing of Jagged 1 in MDA-T68 thyroid cancer cells resulted in a significant decrease of NICD and Hes-1 expression, suggestive of decreased Notch pathway activity (Fig.5).

Jagged 1 silencing inhibits the tumor growth *in vivo*

The impact of Jagged 1 silencing was also

investigated *in vivo* in a xenografted mice model (Fig.6A). IHC analysis showed that expression of Jagged 1 was significantly inhibited in si-Jagged 1 tumors relative to si-NC tumors (Fig.6B). Moreover, the tumor weight and volume of the xenografted si-Jagged 1 tumor was significantly ($P < 0.05$) lower than that of the control (Fig.6C, D). Furthermore, Ki67 protein levels were diminished and that of cleaved caspase-3 were enhanced in si-Jagged 1 tumors (Fig.6E).

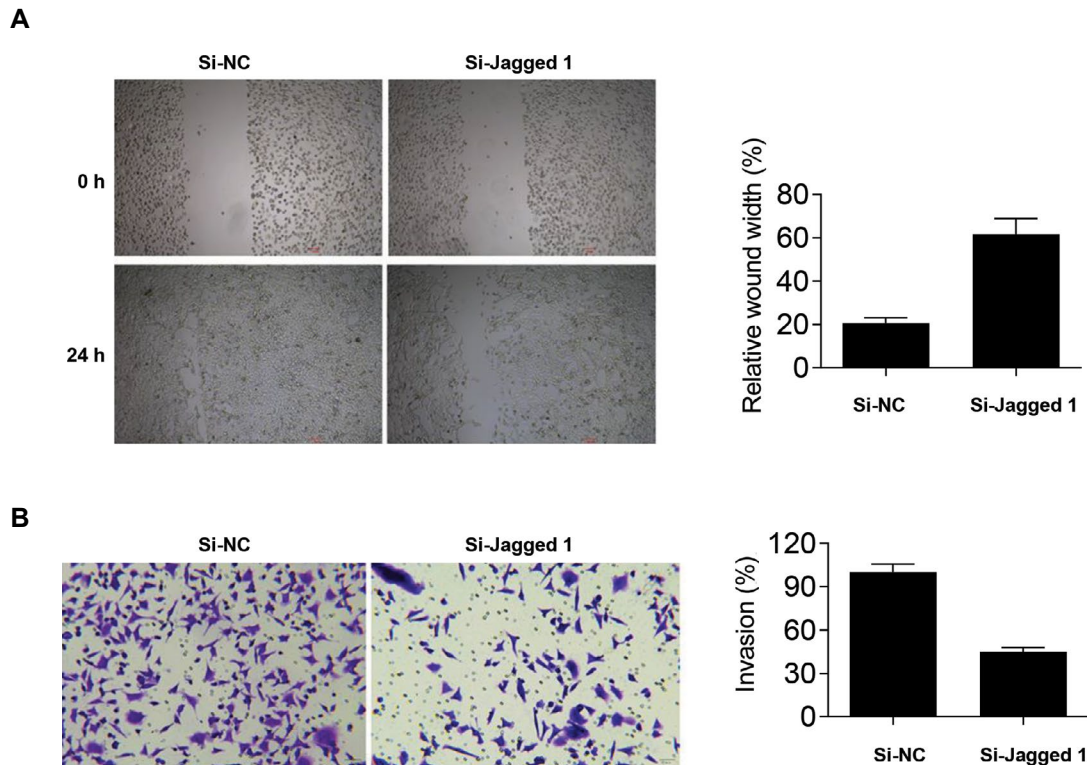


Fig.4: Jagged 1 silencing suppresses the migration and invasion of human thyroid cancer cells. **A.** Wound healing assay revealing cell migration in si-NC and si-Jagged 1 transfected MDA-T68 cells (scale bar: 50 μ m). **B.** Transwell assay revealing cell migration in si-NC and si-Jagged1 transfected MDA-T68 cells (scale bar: 50 μ m). Experimentation was done with three replicas.

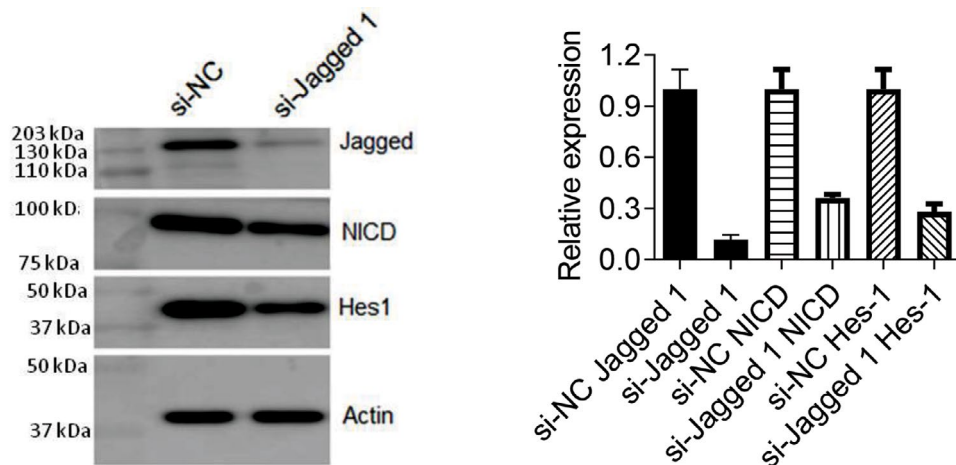


Fig.5: Jagged 1 silencing deactivates the Notch signalling pathway in human thyroid cancer cells. Western blotting showing the expression of NICD and Hes-1 in si-NC and si-Jagged 1 transfected MDA-T68 thyroid cancer cells. Experimentation was done with three replicas.

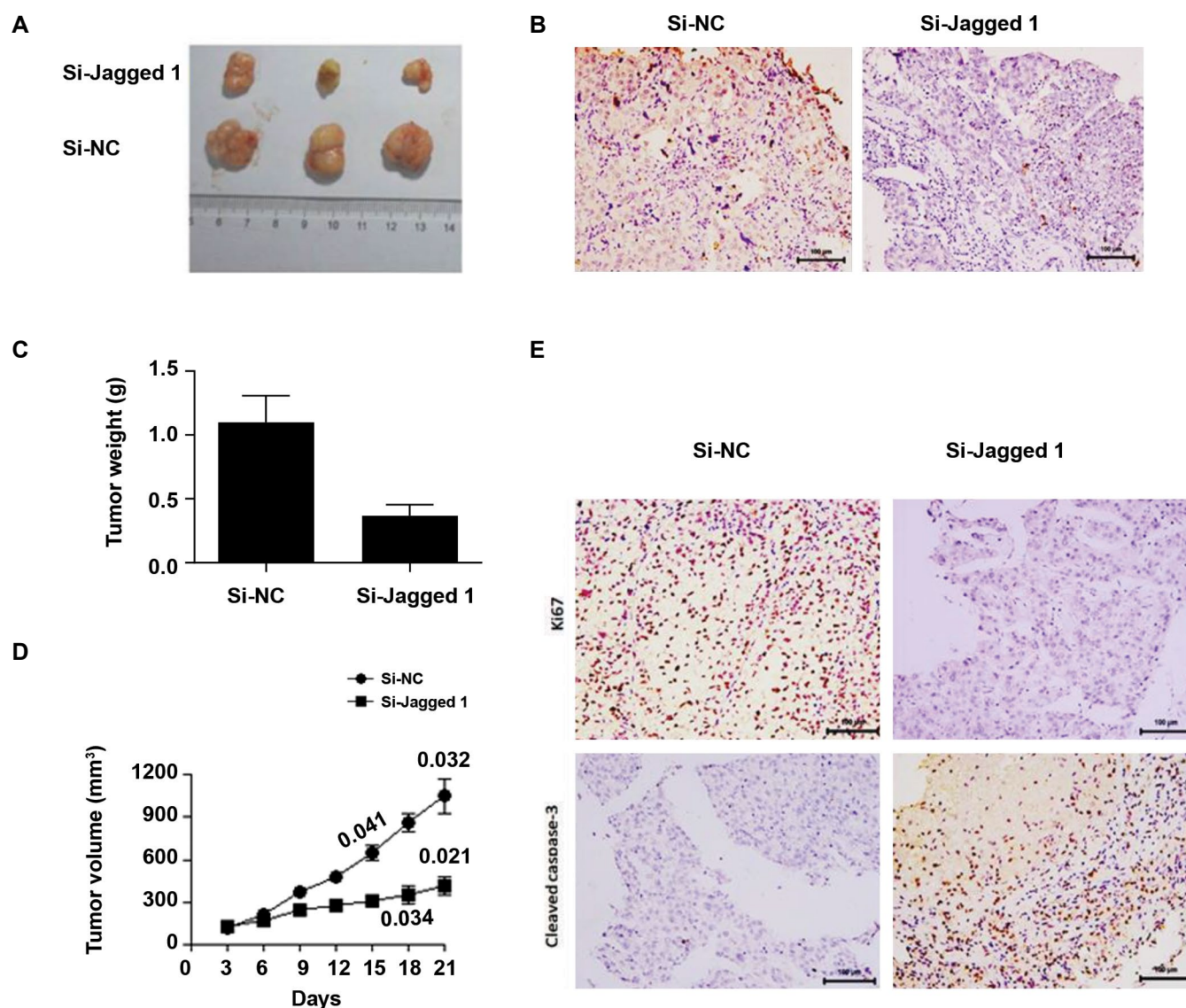


Fig.6: Silencing of Jagged 1 inhibits xenografted tumor growth. **A.** Images of si-NC and si-jagged 1 tumors. **B.** IHC analysis revealing Jagged 1 expression in si-NC and si-Jagged 1 tumors (scale bar: 100 μ m). **C.** Tumor weight of si-NC and si-Jagged tumors. **D.** Tumor volume of si-NC and si-Jagged tumors. **E.** IHC revealing expression of Ki67 and cleaved caspas-3 in si-NC and si-Jagged tumors (scale bar: 100 μ m).

Discussion

Papillary thyroid cancer (PTC) is the most prevalent thyroid cancer with alarmingly increasing incidence globally (15). It has been reported to cause tremendous human mortality leading to increased demand for the development of reliable molecular markers and therapeutic targets for its management (16). Herein we investigated the role and therapeutic implications of Jagged 1 in thyroid cancer. Over the years, research on Notch signalling pathway has indicated its involvement in developing different cancer types (17). Furthermore, Jagged 1 regulate the Notch pathway and is involved in developing different tumor types (18).

Sethi et al. (19), reported that Jagged 1 promotes the metastasis of breast cancers. Similarly, Jagged 1 is critical for the proliferation of glioma cells (20). Herein, we evaluated the expression of Jagged 1 in human PTC

tissues and cell lines that found it to be significantly upregulated. This confirms the earlier studies wherein Jagged 1 is significantly upregulated in head and neck cancers (21). In another study, Gao et al. (22), reported the enhanced expression of Jagged 1 in human colon adenocarcinoma. The silencing of Jagged 1 suppressed the viability and colony formation of the human PTC cells via stimulation of the programmed cell death. Apoptosis is a vital process enabling the removal of cancer cells. Different proteins have been found to act as biomarkers for apoptosis, like Bax and Bcl-2 (23). Herein, we found that Jagged 1 silencing enhanced Bax and reduced Bcl-2 expression further validating the stimulation of apoptosis. Among the imperative process, cell migration and invasion are essential for cancer metastasis (24). Herein, we found that Jagged 1 silencing caused suppression of migration and invasion of the MDA-T68 cancer cells. The Notch signalling pathway has been shown to be

significantly upregulated in cancer cells (25). It has been shown that Notch ligand binding triggers the cleavage of NICD, rapidly translocating to the nucleus wherein it binds to DNA-binding proteins, eventually triggering the activation of target genes (7). Thus, signalling cascade regulates cell proliferation, differentiation, and apoptosis (26).

In the present study, we observed that silencing of Jagged 1 is associated with the downregulation of NICD and Hes-1, which triggers cell death. Although Jagged 1 may exhibit therapeutic implications, some limitations of the present study need more research endeavours. For instance, the effect of chemotherapeutic drugs should be assessed on the expression of Jagged 1 or lead molecules which can prevent the binding of Jagged 1 to Notch need to be identified. Moreover, the effects of Jagged 1 silencing must also be assessed in normal thyroid cells. Although it has been reported that knockout of Jagged-1 is embryonic lethal due principally to vascular defects (27, 28).

Conclusion

Collectively, Jagged 1 is significantly upregulated in PTC cells. Silencing of Jagged 1 suppressed the proliferation of PTC cells via stimulation of apoptosis. Furthermore, Jagged 1 silencing also inhibited the metastasis of the human thyroid cancer cells. Taken together, Jagged 1 may act as a therapeutic target for the treatment of PTC. Nonetheless, more studies are needed to confirm these results.

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Authors' Contributions

J.C., X.W., Y.Z.; Designed the study and wrote the manuscript. J.C., X.W., X.Z., J.Y., Y.Z.; Conducted lab experiments. Y.Z.; Supervised the research project. All authors read and approved the final manuscript.

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