Targeting Notch1 inhibits invasion and growth of ovarian cancer cell through regulation of miR-124/flotillin-1 pathway

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Abstract: Background and aims: Notch-1 signaling is active in ovarian cancer, and transfection of active Notch-1 into ovarian cancer cells promotes tumorigenesis in vivo. In contrast, targeting Notch-1 inhibits growth and invasion of ovarian cancer cells in vitro and in vivo. However, the associated molecular mechanisms have not been thoroughly defined. Herein, we investigated the mechanisms of Notch-1 inhibition on proliferation, invasion and metastasis of ovarian cancer cells. Methods: Notch-1 expression was inhibited by small interfering RNA targeting Notch-1 (Notch-1 siRNA). The effect of Notch-1 inhibition on growth, apoptosis and invasion of ovarian cancer A2780 cells in vitro was detected using the methyl thiazol tetrazolium (MTT) assay, colony formation assay, Annexin V/PI double staining followed by flow cytometry, and Matrigel transwell and wound healing assays. The effect of Notch-1 on miR-124 and flotillin-1 (FLOT1) expression in A2780 cells was detected by quantitative real-time PCR (qRT-PCR) and western blot assay. A subcutaneously implanted tumor model using A2780 cells in nude mice was used to assess the effects of Notch-1 inhibition on tumorigenesis and lung metastasis development. Results: Targeting Notch-1 by Notch-1 siRNA transfection induced apoptosis and inhibited growth and invasion of A2780 cells in vitro. Furthermore, targeting Notch-1 inhibited tumorigenesis and lung metastasis of A2780 cells in nude mice. Notch-1 inhibition increased miR-124 expression and decreased miR-124-dependent FLOT1 expression. Targeting miR-124 reversed the effects of Notch-1 inhibition on A2780 cells in vitro. In addition, targeting FLOT1 rescued the effects of miR-124 inhibition on A2780 cells in vitro. Conclusions: Therapies targeting the Notch-1 signaling pathway may be more effective in preventing primary tumor formation and inhibiting organ metastasis of ovarian cancer cells. The ability of this therapy to decrease tumorigenesis and metastasis may be related to miR-124/FLOT1 signaling.

Keywords: Ovarian cancer, Notch-1, miR-124, Flotillin-1, metastasis, apoptosis

Introduction

Notch signaling is a highly conserved pathway that is activated by ligands [1]. When the Notch receptor is activated, the receptor-ligand then triggers a second Notch extracellular domain cleavage by the metalloproteinase ADAM, which in turn downregulates ligand activity [2]. To date, only four Notch genes have been identified in mammals (Notch-1, Notch-2, Notch-3 and Notch-4) [1]. All of the Notch receptors are very similar in structure, although there are some subtle differences in their cytoplasmic and extracellular domains [3].

Increasing evidence has shown that Notch signaling plays an important role in cell invasion, apoptosis and proliferation, which are all involved in development of functional organs [4-6]. Notch-1 is the most studied gene among the Notch family. Many studies have demonstrated that the Notch-1 gene is abnormally activated in several human malignancies [7-12]. Activation of Notch-1 might predict poor survival and more aggressive tumor behavior in patients with malignancies [13-16].

Active Notch-1 is known to stimulate proliferation and survival in ovarian cancer cells [17]. However, the mechanisms of how Notch-1 functions remain unclear. MicroRNAs (miRs) are small non-coding RNAs that regulate gene expression post-transcriptionally. miRs mediate fundamental cellular processes, such as prolif-
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eration, differentiation and apoptosis, and are actively involved in carcinogenesis [18]. miR-124 was recently reported to be expressed at low levels in ovarian cancer cells [19, 20]. Overexpression of miR-124 could inhibit invasion and migration of ovarian cancer cells in vitro [19]. In gastric cancer (GC) cells, overexpression of Notch-1 repressed miR-124 expression, followed by increased growth and invasion of GC cells in vitro [21].

Flotillin-1 (FLOT1) is a plasma membrane lipid raft-localizing protein involved in internalizing membrane-localizing proteins into the cytosol by endocytosis. FLOT1 was recently shown to be overexpressed in various types of human cancers, and high FLOT1 expression was correlated with advanced tumor stage and poor patient survival [22, 23]. FLOT1 levels determine cancer proliferation, migration and invasion [24]. In breast cancer cells, FLOT-1 was found to be a target of miR-124 [25]. However, it is unclear whether FLOT1 is responsible for malignant transformation of ovarian cancer.

In this study, we assessed the effect of Notch-1 inhibition on growth and invasion of ovarian cancer cells in vitro and explored related molecular mechanisms. Our findings demonstrate that targeting Notch-1 induced apoptosis and inhibited growth and invasion of ovarian cancer cells in vitro by activating miR-124/FLOT1 signaling.

Materials and methods

Cell line and culture

Ovarian cancer A2780 cells were purchased from American Type Culture Collection (ATCC, Shanghai, China). Cells were maintained in RPMI 1640 (Life Technologies) and supplemented with 10% (v/v) fetal bovine serum, 3 mmol/L L-glutamine and 100 units/mL penicillin/streptomycin.

Notch-1 siRNA transfection

Notch-1 or FLOT1 siRNA and control siRNA were purchased from Santa Cruz Biotechnology (Shanghai, China). A2780 cells were transfected with Notch-1 or FLOT1 siRNAs and corresponding control siRNA for 48 h using Lipofectamine 2000 according to the manufacturer's instructions. At 24 h after Notch-1 or control siRNA transfection, cells were split into 96-well plates and subjected to G418 (1 mg/ml) selection for 2 weeks. The transcriptional silencing Notch-1 protein was screened using western blot assay. All transfection experiments were performed at least three times.

Synthesis oligo-nucleotide and cell transfection

A miR-124 inhibitor (anti-miR-124) and negative control (NO) were synthesized at Ribobio Inc. (Guangzhou, China) based on the miR-124 sequence in the miRBase database (MIMAT-0000422). A2780 cells were transfected with oligonucleotides at working concentrations of 20 µM using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instruction.

MTT assay

A2780 cell viability was assessed using the methyl thiazol tetrazolium (MTT) assay. Briefly, cells were plated in 96-well plates containing 10% FBS. After transfection with Notch-1 siRNA or FLOT1 siRNA or/and anti-miR-124 for 24, 48, or 72 h, cells from each group were collected and plated in 96-well plates at a density of 1.0 × 10^4 cells/well. Absorbance was measured at 570 nm. Each assay was performed in triplicate. Cell growth (mean absorbance ± standard deviation) was plotted versus time.

Colony formation assay

Stable Notch-1 or control siRNA-transfected A2780 cells or untreated A2780 cells were seeded at a density of 300 cells/ml on 35-mm dishes. Colonies were allowed to grow for 14 days. The medium was discarded and each well was carefully washed twice with phosphate buffered saline (PBS). Cells were fixed in methanol for 15 min and stained with crystal violet for 20 min, and positive colony formation (more than 50 cells/colony) was counted.

Flow cytometry

Apoptosis induction was quantified by annexin V/PI double staining followed by flow cytometry. Annexin V/PI double staining was performed using an apoptosis detection kit (Biovision, Mountain View, CA) following the manufacturer's instructions. Briefly, after transfection, cells were gently detached by brief trypsinization and washed with ice cold PBS. After another wash with binding buffer, cells were suspended in 300 µL binding buffer containing
annexin V and propidium iodide and incubated for 5 min at room temperature. Early apoptotic cells were identified as annexin V positive/Pl negative cells, while late apoptotic/necrotic cells were identified as annexin V positive/Pl positive cells using a BD LSR II cell analyzer.

Wound healing assay

A2780 cells in different groups were seeded onto 6-well plates. When cultures reached 100% confluence, a scratch was made across the cell monolayer. Cells were gently washed with PBS, and new media containing hydroxyurea 30 uM (Sigma, Shanghai, China) was added to block cell division. Cells were then incubated for 24 h and photographed using an inverted tissue culture microscope at 100 × magnification.

Invasion assay

The upper chamber of each transwell was coated with Matrigel (BD Biosciences, MA, USA). A2780 cells (2 × 10^4) were seeded in upper chambers in DMEM and incubated in 24-well plates with 10% FBS supplemented in DMEM. After 24 h, cells remaining on the upper surface of the membrane were removed with a cotton swab. Cells that invaded through the Matrigel precoated membrane filter were fixed. Cells were counted at 100 × magnification in 10 random fields of view under a microscope. Three independent experiments were performed in each case.

Western blotting

Cells were lysed on ice in lysis buffer, clarified by centrifugation at 6,000 × g for 5 min in a microcentrifuge at 4 °C, separated by SDS-PAGE, and then transferred to Hybond-P (Amersham, Hangzhou, China) membranes. Antibodies used for western blot were anti-Notch-1, FLOT1 and goat anti-β-actin (Santa Cruz, Shanghai, China). Secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit serum (BD Biosciences) and rabbit anti-goat serum (DAKO). Chemiluminescence was detected using ECL reagents.

qRT-PCR analysis of mRNA and miR-124

Quantitative real-time PCR (qRT-PCR) was performed on cDNA using the Taqman Fast System and reagents (Applied Biosystems, Foster City, CA) per the manufacturer’s instructions. Total RNA (1 μg) was reverse-transcribed using random primers and MultiScribe RT (High-Capacity cDNA Archive Kit) for mRNA analysis and miScript Reverse Transcription Kit for miRNA analysis. PCR was performed with the resulting reverse transcription products using specific oligonucleotide primers. Primer sequences for miR-124 were 5'-TAAGGACGCGGTGAATGCC-3' and 5'-TGCGGGTGCTGCTTCGGCAGC-3', and primer sequences for Notch-1 were 5'-GGGTCACCAGTTGAAATG-3' and 5'-GTGATTGTTCCGCACCAT-3'. Primer sequences for FLOT1 were 5'-GGCAGAAATTCTCAGAACAG-3' and 5'-GGTGCAATAGTCTGGTCTCAT-3'. All reactions were performed in triplicate. Results were analyzed using the 2^ΔΔCt or 2^-ΔCt method.

In vivo A2780 xenograft assay

All animal experiments were performed in accordance with Institutional Animal Care and Use Committee procedures and guidelines of the People’s Hospital of Weifang in Weifang, China. Female nude mice (4-6 weeks old) were maintained in pathogen-free conditions. Untreated A2780 cells (5 × 10^6) or stable Notch-1 shRNA-transfected A2780 cells were subcutaneously injected into the right flank of nude mice. Tumor size was measured with calipers, and tumor volume was determined using the formula: volume = 0.5 × width^2 × length. Six weeks after tumor inoculation, the mice were sacrificed and subcutaneous tumors were excised with the attached muscle layers. Tumor tissue was stained by hematoxylin and eosin (H&E). Lungs were collected to determine the number of metastatic nodes. Incidence of metastasis was determined by the presence of macroscopic lesions on the surface of the lung. mRNA and miR-124 were detected by qRT-PCR.

TUNEL assay

Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) was performed with an in situ cell death detection kit (Roche). Cell apoptosis was quantified by determining the percentage of positively stained cells for all of the nuclei in 20 randomly chosen fields/section at 200 × magnification. Slides from apoptosis studies were quantified in a blind manner by two independent reviewers two different times.
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**Statistics**

Statistical differences were analyzed using unpaired 2-tailed Student’s t test. P values less than 0.05 were considered significant.

**Results**

**Effect of Notch-1 siRNA transfection on Notch-1 expression in A2780 cells**

A2780 cells were transfected with Notch-1 or control siRNA for 24-72 h. Following transfection, Notch-1 expression levels in the A2780 cells were evaluated by qRT-PCR and western blot assay. As shown in Figure 1A, the number of Notch-1 transcripts was reduced by more than 60 to 90% in A2780 cells after Notch-1 siRNA transfection. We then studied whether reduction of Notch-1 transcripts by Notch-1 siRNA transfection were reflected at the protein level by western blot assay. As estimated by densitometry, inhibition of Notch-1 expression was significantly increased by Notch-1 siRNA transfection (Figure 1B). However, Notch-1 transcripts and Notch-1 protein expression were not affected by control siRNA transfection in the A2780 cells (Figure 1A, 1B).

**Targeting Notch-1 decreases miR-124-dependent FLOT1 expression in A2780 cells**

Following Notch-1 siRNA transfection in A2780 cells, we evaluated miR-124 levels using a qRT-PCR assay. As shown in Figure 1C, the number of miR-124 transcripts increased by more than 10- to 15-fold, demonstrating that knockdown of Notch-1 induced miR-124 expression. In addition, targeting Notch-1 also inhibited FLOT1 expression in A2780 cells (Figure 1C, 1D)
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expression in a time-dependent manner by western blot (Figure 1B) and qRT-PCR assay (Figure 1D). Peak FLOT1 mRNA and protein levels were detected at 72 h following Notch-1 siRNA transfection, demonstrating that targeting Notch-1 could inhibit FLOT1 expression. However, when Notch-1 siRNA/A2780 cells were treated with anti-miR-124 for 24 h to inhibit miR-124 expression, FLOT1 mRNA (Figure 1E) and protein expression were increased (data not shown), demonstrating that targeting Notch-1 inhibited FLOT1 expression through a miR-124-dependent pathway.

**Figure 2.** Targeting Notch-1 decreases proliferation of ovarian cancer cells through miR-124/FLOT1 signaling in vitro. A. Proliferation of A2780 cells transfected with Notch-1 siRNA, anti-miR-124 or/and FLOT1 siRNA was measured by an MTT assay (*P*<0.05, **P*<0.01; Student's t-test). B. Growth of A2780 cells stably transfected with Notch-1 siRNA was measured by colony formation assays (*P*<0.01; Student's t-test). C. Cell apoptosis was examined by flow cytometry (*P*<0.05, **P*<0.01; *P*<0.05; Student's t-test).

**Targeting Notch-1 induces apoptosis and inhibits growth of A2780 cells by miR-124/FLOT1 signaling**

To assess the role of Notch-1 in regulating cell viability, we also observed significant inhibition of anchorage independent growth by Notch-1 siRNA transfection in A2780 cells (Figure 2B).

To examine the mechanisms of inhibition of cell viability, cell apoptosis was evaluated in A2780 cells. Flow cytometry analysis shows (Figure 2C) that targeting Notch-1 induced apoptosis/cell death in a time-dependent manner.

Next, we investigated the mechanisms underlying Notch-1 inhibition-induced apoptosis in A2780 cells. A2780 cells were treated with anti-miR-124 for 24 h, then transfected with Notch-1 or control siRNA for 24-72 h. Flow cytometry showed that treatment with anti-miR-124 reversed Notch-1 inhibition-induced apoptosis of A2780 cells (Figure 2C). However, when anti-miR-124-treated Notch-1 siRNA/A2780 cells were transfected with FLOT1 siRNA, Notch-1 siRNA-induced apoptosis of A2780 cells was rescued (Figure 2C). Similarly, treatment with anti-miR-124 was able to reverse Notch-1 inhibition-induced proliferation.
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Targeting Notch-1 on A2780 cells (Figure 2A). However, when anti-miR-124-treated Notch-1 siRNA/A2780 cells were transfected with FLOT1 siRNA, Notch-1 siRNA-induced growth of A2780 cells was rescued (Figure 2A).

**Figure 3.** Targeting Notch-1 suppresses migration and invasion of A2780 cells *in vitro*. A. A2780 cells transfected with Notch-1 siRNA, anti-miR-124, or/and FLOT1 siRNA were analyzed by wound-healing assay. B. Invasion assays were performed on Matrigel-coated polyethylene terephthalate membrane inserts (""P<0.01; Student’s t-test).}

of A2780 cells (Figure 2A). However, when anti-miR-124-treated Notch-1 siRNA/A2780 cells were transfected with FLOT1 siRNA, Notch-1 siRNA-induced growth of A2780 cells was rescued (Figure 2A).

**Targeting Notch-1 inhibits migration and invasion of A2780 cells by miR-124/FLOT1 signaling**

We first detected the role of Notch-1 inhibition by Notch-1 siRNA transfection on migration of A2780 cells using a wound healing assay. Compared to untreated or control siRNA-transfected A2780 cells, cells transfected with Notch-1 siRNA for 48 h exhibited a decreased migration rate (""P<0.01, Figure 2A).}

Next, we detected the role of Notch-1 inhibition on invasion of A2780 cells using the BD Biocoat growth factor-reduced Matrigel invasion chamber assay. As shown in Figure 3B, transfection of Notch-1 siRNA for 48 h significantly inhibited invasion of A2780 cells compared to untreated or control siRNA-transfected A2780 cells.

These experiments indicate that targeting Notch-1 inhibits migration and invasion of A2780 cells *in vitro*.

Next, we investigated the mechanisms underlying Notch-1 siRNA-induced inhibition of cell invasion and migration. A2780 cells were transfected with Notch-1 or control siRNA for 24 h, then treated with anti-miR-124 for 24 h. As shown in Figure 3A, wound repair was only observed in anti-miR-124/Notch-1 siRNA-treated A2780 cells. However, when anti-miR-124-treated Notch-1 siRNA/A2780 cells were transfected with FLOT1 siRNA, Notch-1 siRNA-induced wound repair was rescued in the
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A2780 cells (Figure 3B). These results were confirmed using a transwell chamber assay.

Targeting Notch-1 inhibits growth and lung metastasis of A2780 cells in vivo

Given that targeting Notch-1 inhibits growth, invasion and migration of A2780 cells in vitro, we next determined whether targeting Notch-1 could exert a therapeutic effect in vivo. To assess the in vivo antitumor efficacy of Notch-1 inhibition, we subcutaneously implanted Notch-1 shRNA/A2780 cells or shRNA/A2780 cells or untreated A2780 cells into the right flank of female 4- to 6-week-old BALB/c nude mice. The results showed that the tumor volume of Notch-1 shRNA/A2780 groups was markedly suppressed compared to shRNA/A2780 or untreated A2780 cells groups (P<0.05) (Figure 4A). The number of macroscopic lung metastatic nodes in the Notch-1 shRNA transfected groups was 6.4±1.2, which was significantly decreased compared to the control shRNA-transfected (23±6.4) and untreated groups (25±7.8) (P<0.05). A TUNEL assay was performed to detect cell apoptosis. The average number of apoptotic cells from Notch-1 shRNA-transfected groups was 10.4±1.8, which was significantly increased compared to control shRNA-transfected (1.7±0.2) or untreated groups (1.46±0.3) (P<0.05; Figure 4B).

The qRT-PCR assay revealed that Notch-1 mRNA and FLOT1 mRNA expression levels were markedly decreased and that miR-124 levels were significantly increased in the Notch-1 shRNA-transfected groups compared to the untreated or control shRNA-transfected groups (Figure 4C). Western blot assay confirmed the results of the qRT-PCR assay (Figure 4D).

Discussion

Notch-1 signaling has been found to be overexpressed in ovarian cancer cells [26, 27]. Furthermore, transfection of active Notch-1 promoted proliferation and colony formation in ovarian cancer cells [27], suggesting that Notch-1 signaling plays a role in ovarian cancer growth. The Notch pathway could be a target in developing therapies for ovarian cancer. In the present study, we demonstrated that targeting Notch-1 by siRNA inhibited growth, invasion and metastasis in ovarian cancer cells in vitro and vivo.

Figure 4. Targeting Notch-1 inhibits xenografted tumors from A2780 cells in mice. A. Stable Notch-1 shRNA-transfected A2780 cells were subcutaneously implanted into the right flank of female 4- to 6-week-old BALB/c nude mice for 6 weeks. A significant reduction in tumor volume was observed in Notch-1 shRNA-transfected tumors, *P<0.05. B. Representative fields showing tumor cell apoptosis by TUNEL assay in A2780 tumors from mice, *P<0.05. C. Relative expression of Notch-1, miR-124 and FLOT1 mRNA in tumor tissues as determined by qRT-PCR. D. Relative expression of Notch-1 and FLOT1 proteins in tumor tissues as determined by western blot assay.
Several studies have revealed the functional significance of miR-124 signaling [19, 20]. In prostate cancer cells, miR-124 shed light on anti-migration and anti-invasion mechanisms [28]. In NPC cell lines, ectopic miR-124 expression dramatically inhibited cell proliferation, colony formation, migration and invasion in vitro, as well as tumor growth and metastasis in vivo [29]. The suppressive capability suggested that miR-124 functioned as tumor-suppressive microRNA in cancer cells. Jiang et al. demonstrated that blocking Notch-1 signaling could upregulate miR-124 expression [21]. However, Gallardo et al. found that miR-124 expression upregulated Notch-1 expression [30]. In this study, we found that targeting Notch-1 by siRNA inhibited growth and induced apoptosis of A2780 cells, followed by upregulation of miR-124 expression. However, when A2780 cells were transfected with anti-miR-124 to inhibit miR-124 expression, the increase in Notch-1 siRNA-induced growth inhibition and apoptosis was reversed, suggesting that the anti-tumor activity of Notch-1 inhibition is correlated with increased miR-124 expression. Further study found that targeting Notch-1 inhibited invasion and migration of A2780 cells. However, targeting miR-124 can reverse the anti-metastatic activity of Notch-1 inhibition in A2780 cells, suggesting this anti-metastatic activity is correlated with increased miR-124 expression.

FLOT1s are highly conserved proteins that localize in specific cholesterol-rich microdomains in cellular membranes. FLOT1 has been documented in the literature to regulate cellular processes, particularly cancer growth, proliferation, migration, metastasis and tumorigenesis. A previous study found that miR-124 could directly target and downregulate FLOT1, resulting in inhibition of invasion and growth of breast cancers [25]. In the present study, we found that targeting Notch-1 in A2780 cells upregulated miR-124 and downregulated FLOT1 expression. However, targeting miR-124 expression reversed Notch-1 inhibition-induced downregulation of FLOT1 expression. We therefore suggested that Notch-1 inhibited FLOT1 expression via a miR-124-dependent pathway. We also found that invasive and migration rates of A2780 cells in Notch-1 siRNA transfectants were significantly decreased compared with Notch-1 siRNA/anti-miR-124 groups (P<0.01). Furthermore, FLOT1 siRNA transfectants counteracted the increase in FLOT1 expression, resulting in decreased invasive and migratory ability of A2780 cells. Our results also show that targeting Notch-1 by siRNA inhibited cell growth and induced cell apoptosis, whereas anti-miR-124 could reverse the effects. However, FLOT1 siRNA transfectants rescued the effects of anti-miR-124 on cell growth and apoptosis. In vivo, we also found that targeting Notch-1 inhibited tumor growth and lung metastasis, followed by miR-124 upregulation and FLOT1 downregulation. We therefore suggest that targeting Notch-1 inhibits invasion and migration and induces apoptosis of A2780 cells through Notch-1-miR-124-FLOT1 signaling.

Conclusion

In this study, we present experimental evidence that strongly supports the antitumor effects of targeting Notch-1 in ovarian cancer cells in vitro and in vivo. Targeting Notch-1 could potentially upregulate miR-124 expression and downregulate FLOT1 expression, resulting in inhibition of cell growth, invasion and metastasis and induction of apoptosis of ovarian cancer cells. This study suggests that Notch-1 may be an effective target for treating ovarian cancer.

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Disclosure of conflict of interest

None.

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