Notch1 suppresses prostate cancer cell invasion via the metastasis-associated 1-KiSS-1 metastasis-suppressor pathway

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Abstract. Notch1 is a type-I transmembrane receptor which has been demonstrated to be involved in proliferation in various organisms. A number of studies have proposed that Notch signaling may be aberrantly activated, thus contributing to development, invasion and metastasis in a variety of human cancers. In the present study, the function and mechanism of Notch1 in human prostate cancer (PCa) LNCaP cells in vitro was investigated. Notch1 and cleaved-Notch1 expression were evaluated in human PCa cell lines, including LNCaP, PC-3 and DU 145, and the human prostate epithelial RWPE-1 cell line. LNCaP cells were transfected with Notch1-targeting short hairpin RNAs (shRNAs) and the level of proliferation, the ability to invade and the expression of genes associated with cancer cell invasion were subsequently investigated. Notch1 was highly expressed in LNCaP, PC-3 and DU 145 cells compared with RWPE-1 cells, while cleaved-Notch1 was expressed in LNCaP, PC-3 and DU 145 cells, and only to a minimal extent in RWPE-1 cells. Knockdown of Notch1 by shRNA in LNCaP cells markedly decreased cell invasion through Matrigel and inhibited cell proliferation 48 h following transfection. Reverse transcription-quantitative polymerase chain reaction analysis indicated that Notch1-knockdown resulted in a significant reduction of metastasis-associated 1 (MTA1) and increase of KiSS-1 metastasis-suppressor (KISS-1), mitogen-activated protein kinase 4 (MKK4) and cluster of differentiation 82 (KAI1). The present data demonstrated that expression of Notch1 was significantly associated with the invasion of prostate cancer. Knockdown of Notch1 decreased the invasive ability of LNCaP cells, which may be caused by downregulating MTA1 and upregulating KISS-1, MKK4 and KAI1. These findings indicated that targeting Notch1 may provide a novel method of suppressing or treating metastasis in prostate cancer.

Introduction

Among all major cancers, prostate cancer (PCa) has one of the worst prognoses; it was ranked 2nd for cancer-associated mortality causes worldwide in 2012 (1). There is evidence to suggest that this may be due to carcinoma in the prostate being more inclined to metastasize (2). The lymph nodes and bone are the destination of metastatic cells in 70-80% cases of prostate cancer-associated mortality (3). However, early stage diagnosis methods currently available for PCa are poor, and the lack of effective therapies for advanced carcinoma results in a high mortality rate among patients.

Notch1 is a type I transmembrane protein, which has a dual function of cell membrane surface receptors and nuclear transcription regulation (4). Previous studies have reported that the Notch signaling pathway may be aberrantly activated, contributing to the development, invasion and metastasis of a wide variety of human cancers, including cervical, lung, colon, head and neck, renal carcinoma, acute myeloid, Hodgkin and large-cell lymphomas and pancreatic cancer (5-7). Notch are a family of transmembrane proteins with epidermal growth factor-like domains. There are four Notch receptors (Notch-1 to -4), as well as six ligands (Jagged1 and 2 and d-like 1, 2, 3 and 4), in mammalian cells (8,9). The signaling pathway is divided into an extracellular and intracellular region, the latter of which may be cleaved when the associated genes are expressed (10-12).

It is established that multiple steps are involved in the progression of metastasis in prostatic tumors, including angiogenesis, migration and intravasation, leading to tumor cell invasion and ultimately, metastasis (13). It has been reported

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that Notch signaling may regulate the metastasis of tumors in multiple tissues and organs (14-17). Therefore, in the present study, Notch1 was knocked down to investigate the function and mechanism of Notch1 in the invasion and metastasis of human PCA LNCaP cells in vitro.

Materials and methods

**Cell culture.** The human prostatic carcinoma LNCaP, PC-3 and DU 145 cell lines and the immortalized human prostatic epithelial RWPE-1 cell line were obtained from the American Type Culture Collection (Manassas, VA, USA). LNCaP, PC-3 and DU 145 cells were maintained in RPMI-1640 medium (Cellgro; Corning Life Sciences, Corning, NY, USA) supplemented with 10% fetal bovine serum (FBS; Biowest USA, Riverside, MO, USA). RWPE-1 cells were maintained in keratinocyte serum-free medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cells were incubated in an environment of 5% CO₂ at 37°C.

**Western blot analysis.** LNCaP, PC-3, DU 145 and RWPE-1 cells were collected and washed twice with cold PBS, and then lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Jiangsu, China) containing the protease inhibitor phenylmethanesulfonyl fluoride, with mild sonication on ice. Cell lysates (20 μg) were used for western blot analysis, subsequent to 11.5% SDS-PAGE, with primary antibodies against Notch1 (cat. no. 36085), cleaved-Notch1 (cat. no. 4147S) and GAPDH (cat. no. 5174), and a secondary HRP-conjugated anti-rabbit IgG (cat. no. 7074S). All antibodies were used at a 1:2,000 dilution and were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Non-specific binding was blocked by incubating with tris-buffed saline with Tween-20 and 5% skimmed milk powder for 60 min at room temperature. The membrane was incubated with the primary antibodies at 4°C overnight, then with the secondary antibody for 2 h at room temperature. The band intensities of the target proteins and GAPDH were quantified using Image Lab 5.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the intensity of each target protein was normalized to the corresponding GAPDH level.

**RNA interference.** In a 6-well tissue culture plate, LNCaP cells at 50-70% confluency in 800 μl of short hairpin RNA (shRNA) plasmid transfection medium (cat. no. sc-108062) was transfected with 200 μl Notch1 shRNA plasmid (cat. sc-36095-SH) or control shRNA plasmid-A/shRNA plasmid transfection reagent complex (cat. no. sc-108060), all from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Following transfection for 24 h at 37°C, RPMI medium was prepared containing twice the usual concentration of FBS (20%) and antibiotics. A total of 1 ml prepared medium was added to each well, and the cells were incubated for an another 24 h at 37°C with 5% CO₂. The medium was then replaced with RPMI with 10% FBS containing 10 μg/ml of puromycin. Every 2-3 days, medium was aspirated out and replaced with freshly prepared RPMI with 10% FBS. The cells were subsequently collected and processed for western blotting, reverse transcription-quantitative polymerase chain reaction (RT-qPCR), cell invasion and proliferation assays.

**RT-qPCR.** RNA was extracted from the cell lines using TRIzol (Thermo Fisher Scientific, Inc.). RNA (1-2 μg) was reverse transcribed to cDNA using the Transcription First Strand cDNA Synthesis kit (Roche Diagnostics, Basel, Switzerland). qPCR analyses were performed using Fast Start Universal SYBR-Green Master (ROX; Roche Diagnostics) on the ABI 7500FAST system (Applied Biosystems; Thermo Fisher Scientific, Inc.) The thermocycling settings were as follows: An initial temperature of 95°C for 5 min; then 35 cycles of 95°C for 15 sec, 60°C for 60 sec, and 72°C for 60 sec; then a final extension step of 10 min at 72°C. qPCR results for target gene expression were normalized using GAPDH as the internal control. Relative target gene expression levels normalized to GAPDH were determined with the formula 2^(-ΔCq), in which ΔCq=Cq_targetgene-Cq(GAPDH). To calculate the fold changes of target gene expression in LNCaP cells transfected with Notch1 shRNA plasmid compared with the control cells, the 2^(-ΔΔCq) method was used (17), in which ΔΔCq=ΔCq_Notch1 shRNA-ΔCq(control cell). The values were calculated. The sequences for primers used in the RT-qPCR assay, as supplied by Invitrogen (Thermo Fisher Scientific, Inc.) are listed in Table I. **Invasion assay.** Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was thawed at 4°C overnight and then diluted 1:4 in serum free-cold cell culture media. Matrigel (50 μl) was added to the upper chambers of a 24-well Transwell plate, and the insert was gently rotated to ensure the entire membrane was coated. Subsequently, the Transwell was incubated at 37°C for 5 h for gelling and to absorb the residual liquid. LNCaP cells transfected with Notch1 shRNA plasmid or control shRNA plasmid-A were then starved for 24 h in serum-free RPMI containing 1% FBS, and cells from the tissue culture flasks were harvested using trypsin/EDTA. The cells were then washed once with serum-free RPMI, and resuspended in serum-free RPMI at a density of 1x10⁶ cells/ml. A total of 100 μl cell suspension was added to the Matrigel, the lower chamber of the Transwell plate was filled with 600 μl cell culture media containing 10% FBS, and the plate was incubated at 37°C for 24 h. Crystal violet dye (as 0.09% crystal violet in 10% ethanol) was then prepared and 500 μl dye was added to the empty wells. The chamber was moved with forceps into the dye well, incubated in the dye for 30 min at room temperature, washed in water for ~5 sec, and the inner surface was then cleaned with a cotton swab to remove the dye that remained inside. Visual/qualitative observations were then made using a microscope at x100 magnification, and the numbers of invading cells were manually counted. All assays were performed in triplicate for 3 independent experiments.

**Cell proliferation assay.** Cell proliferation analysis was performed using the WST-1 assay, a colorimetric assay for the nonradioactive quantification of cell proliferation, cell viability and cytotoxicity (Roche Diagnostics), according to the manufacturer's protocol. Briefly, cells were plated on 96-well plates (5,000 cells/well) and cell viability was then determined.

**Statistical analysis.** Data were expressed as the mean ± standard deviation. Statistical significance of the differences between groups was analyzed by one-way analysis of variance followed by Newman-Keuls multiple comparisons tests.
Results

Notch1 and cleaved-Notch1 are overexpressed in human PCa cells. It was previously reported that Notch signaling may markedly impact prostate development and disease (18). In the present study, western blot analysis was employed to detect Notch1 and cleaved-Notch1 expression in the human prostatic carcinoma cell lines LNCaP, PC-3 and DU 145 and the immortalized human prostatic epithelial RWPE-1 cell line. Notch1 was revealed to be highly expressed in LNCaP, PC-3 and DU 145 cells compared with RWPE-1 cells, while cleaved-Notch1 was expressed in LNCaP, PC-3 and DU 145 cells and only to a minimal extent in RWPE-1 cells (Fig. 1). Notch1-knockdown decreases invasion and proliferation of LNCaP cells. To examine the effects of Notch1-knockdown, LNCaP cells were subjected to cell invasion and proliferation assays. The invasion of Notch1-knockdown cells through the extracellular matrix was reduced compared with non-transfected cells or cells transfected with scrambled shRNA (Fig. 3A). Notch1-knockdown caused a 60% decrease in cell invasion (Fig. 3B), indicating that Notch1 conferred invasive properties to PCa cells. In addition, the proliferation of Notch1-knockdown cells was significantly reduced 48 h post-transfection (Fig. 3C).

Notch1-knockdown changes the expression of genes involved in cell invasion. To define the mechanism of Notch1 in LNCaP cells invasion, RT-qPCR was performed on a number of invasion-associated genes. Notch1-knockdown resulted in a significant decrease in the expression of metastasis-associated 1 (MTA1) and increase of KiSS-1 metastasis-suppressor, MKK4, mitogen-activated protein kinase 4; KAI1, cluster of differentiation 82.

Table I. Sequences for primers used for reverse transcription-quantitative polymerase chain reaction analysis.

| Gene symbol | Sequence |
|-------------|----------|
| Notch1      | Forward 5'-GAGGCGTGCCAGACTATGC-3' Reverse 5'-CTTGACTCCGCTAGCGTG-3' |
| MTA1        | Forward 5'-ACGCAACCCTGTGATCTG-3' Reverse 5'-GGGCGGTTCCACCATTCC-3' |
| KISS-1      | Forward 5'-AGCGAGTGAATCTCGG-3' Reverse 5'-AGGCGGAGGTTCAGTCCC-3' |
| MKK4        | Forward 5'-TGAGAAGGTGACTGCATCG-3' Reverse 5'-ACAAAATTCCGACCGAG-3' |
| KAI1        | Forward 5'-TGTCCTGCAACCTCCTG-3' Reverse 5'-CCATGACCATGTGACTGCC-3' |
| GAPDH       | Forward 5'-GGCTGAGAACGGGAAGCTTTGT CAT-3' Reverse 5'-CAGCCTTCTCCATGGTGAGA-3' |

MTA1, metastasis-associated 1; KISS-1, KiSS-1 metastasis-suppressor, MKK4, mitogen-activated protein kinase 4; KAI1, cluster of differentiation 82.

using SPSS 10.0 for windows (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.
Discussion

The Notch signaling pathway may be aberrantly activated, and contributes to the development, invasion and metastasis of a wide variety of human cancers, including cervical, lung, colon, head and neck, renal carcinoma, acute myeloid, Hodgkin and large-cell lymphomas and pancreatic cancer (19,20). Notch1 has also been reported to be involved in the metastasis of tumors, including osteosarcoma, breast cancer, melanoma and prostate cancer (21-23). In spite of these findings, the involvement of Notch1, in particular aberrantly-activated Notch1 signaling in prostate cancer cell metastasis, requires verification. In the present study, the activity of Notch-1 was investigated by shRNA-knockdown in human PCa LNCaP cells. Notch1 was revealed to be overexpressed in LNCaP,
PC-3 and DU 145 cells compared with RWPE-1 cells, and cleaved-Notch1 was expressed in LNCaP, PC-3 and DU 145 cells, but not RWPE-1 cells. These results were consistent with the findings of Bin Hafeez et al (24), indicating that Notch signaling is involved in prostate cancer development. It was also observed that knockdown of Notch1 by shRNA in LNCaP cells markedly decreased cell invasion through Matrigel, which mimicked the in vivo extracellular matrix, and cell proliferation was also inhibited 48 h post-transfection. The reduction in migration, invasion and proliferation in PCa cells has been demonstrated to be associated with Notch1-knockdown by RNA interference (24-26). The present results verified the involvement of Notch1 in prostate cancer cell metastasis.

The present study revealed that the expression of MTA1 decreased significantly following RNA interference by Notch1 shRNA. MTA1 was initially identified in breast cancer (27), and was classified as a metastasis-associated protein. It exists primarily in the nucleus as a constituent part of the nucleosome remodeling and histone deacetylation complex (28). Subsequently, MTA1 was also detected in the cytoplasm (29).

Xue et al (30) reported that MTA1 exhibits a strong inhibitory activity for the transcription of a variety of tumor suppressor genes. MTA1 is a stress response protein (31). Multiple tumors have been confirmed to be associated with MTA1, including breast cancer and colorectal cancer (32). High expression levels of MTA1 may help cells to migrate to more hospitable areas in order to survive in adverse conditions, including hypoxia (33). An additional study has also indicated that overexpression of MTA1 is consistent with a more advanced tumor stage and increases the rate of metastasis (34). An increase of KISS-1, MKK4 and KAI1 was also observed in Notch1-knockdown cells, indicating that Notch1 may regulate the expression of MTA1, KISS-1, MKK4 and KAI1.

The present data demonstrated the involvement of Notch1 in human PCA invasion and that knockdown of Notch1 inhibited invasion of human PCA cells by downregulating the expression of MTA1 and upregulating the expression of KISS-1, MKK4 and KAI1. These findings indicated that targeting Notch1 may be a novel therapeutic approach for the treatment of prostate cancer metastasis.

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