RESEARCH COMMUNICATION

siRNA-mediated Silencing of Notch-1 Enhances Docetaxel Induced Mitotic Arrest and Apoptosis in Prostate Cancer Cells

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Abstract

Purpose: Notch is an important signaling pathway that regulates cell fate, stem cell maintenance and the initiation of differentiation in many tissues. It has been reported that activation of Notch-1 contributes to tumorigenesis. However, whether Notch signaling might have a role in chemoresistance of prostate cancer is unclear. This study aimed to investigate the effects of Notch-1 silencing on the sensitivity of prostate cancer cells to docetaxel treatment. Methods: siRNA against Notch-1 was transfected into PC-3 prostate cancer cells. Proliferation, apoptosis and cell cycle distribution were examined in the presence or absence of docetaxel by MTT and flow cytometry. Expression of $p21^{waf1/cip1}$ and Akt as well as activation of Akt in PC-3 cells were detected by Western blot and Real-time PCR. Results: Silencing of Notch-1 promoted docetaxel induced cell growth inhibition, apoptosis and cell cycle arrest in PC-3 cells. In addition, these effects were associated with increased $p21^{waf1/cip1}$ expression and decreased Akt expression and activation in PC-3 cells. Conclusion: Notch-1 promotes chemoresistance of prostate cancer and could be a potential therapeutic target.

Keywords: Notch signaling - prostate cancer - siRNA - cell cycle; apoptosis - $p21^{waf1/cip1}$ - Akt

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Introduction

Despite significant advances in the treatment of castration-resistant prostate cancer (CRPC) with the introduction of novel chemotherapies and targeted agents, the overall survival rate remains low due to the development of resistance to standard treatments through the activation of survival-related pathways (Seruga et al., 2011). Many developmentally important signaling pathways including the Notch pathway have been shown to be crucially implicated in tumorigenesis (Ranganathan, et al., 2011). The Notch family of transmembrane proteins in mammals consists of four receptors, Notch 1 through 4, and five ligands, Jagged 1 and 2 and Delta-like ligands (Dlls) 1, 3 and 4 (Artavanis-Tsakonas, et al., 1999). Direct binding of a ligand from a signaling cell to a Notch receptor at the membrane of the receiving cell initiates two successive proteolytic cleavages by TNF-converting enzyme and the γ-secretase/presenilin complex, which ultimately results in the release of the Notch intracellular domain (NICD). NICD translocates into the nucleus, where it forms a complex with the members of the CBF 1/Su (H)/Lag 1 (CSF) transcription factor family (Kopan and Ilagan, 2009). This complex mediates the transcription of target genes such as Hes-1, Hey, cyclin D and $p21^{waf1/cip1}$ to execute the downstream biological effects (Miele, 2006).

Activation of the Notch pathway has been observed in cervical, lung, colon, head and neck, renal, pancreatic and prostate cancers (Miele et al., 2006). Notch signaling contributes to chemoresistance by protecting cells from apoptosis through the activation of targets involved in cell survival, such as phosphoinositide kinase-3 (PI3K)/Akt, Bcl-XL and survivin (Sade et al., 2004; Villaronga et al., 2008). It has been shown that overexpression of Notch-1 increases the resistance of T cells to etoposide (Sade et al., 2004), breast cancers to melphalan and mitoxantrone (Stylianou et al., 2006), and lung cancers to cisplatin and paclitaxel (Mungamuri et al., 2006). However, the relationship between the Notch pathway and the sensitivity of prostate cancer cells to cytotoxic agents has not been fully examined.

Docetaxel is a semisynthetic taxane that binds to β-tubulin and triggers the death of proliferating cells (McGrogan et al., 2008). Clinical trials have shown that combination chemotherapy using docetaxel with other agents improves the survival in prostate cancer patients (Tannock et al., 2004). However, tumor progression occurs after a median of 6.3 months of docetaxel treatment, resulting in treatment failure (Petrylak et al., 2004). Therefore, we speculated that whether Notch signaling is implicated in the resistance of prostate cancer to

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docetaxel. In this study, we used siRNA to silence the Notch-1 receptor in the CRPC cell line PC-3 and found an increased docetaxel chemosensitivity. Our results provide evidence that the down-regulation of Notch-1 inhibited proliferation and induced cell cycle arrest in PC-3 cells and these may be due to the regulation of $p21^{waf1/cip1}$ and Akt through Notch signaling.

Materials and Methods

Cell culture

The PC-3 cell line was originally obtained from the American Type Culture Collection. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and 100 μg/mL kanamycin (Sigma, St. Louis, MO, USA) at 37 °C in a 5% CO_2 humidified incubator.

Reagents

Docetaxel was purchased from Sanofi-Aventis (Dagenham, UK). The Opti-MEM® I serum-free culture medium, Lipofectamine™ 2000 used for transfection and Total RNA Extraction Kit were obtained from Invitrogen (Carlsbad, CA, USA). MTT Cell Proliferation and Cytotoxicity Assay Kit were purchased from KeyGen Biotech (Nanjing, China). Reverse Transcription Kit was purchased from Fermentas (Burlington, Ontario, Canada). Total Protein Extraction Kit was purchased from ProMab (Changsha, China). The rabbit antibodies against Notch-1, Akt, phospho-Akt (p-Akt) and the mouse antibodies against $p21^{waf1/cip1}$ and GAPDH were purchased from Santa Cruz (Santa Cruz, CA, USA).

siRNA transfection

Three different siRNAs targeting specific sequences of Notch-1 (NM_017617) and a negative control scrambled siRNA (not homologous to any gene) were synthesized by GenePharma (Shanghai, China). The Notch-1 siRNA-6150 was selected based on real-time RT-PCR and Western blot results (data not shown); the sequences were as follows: sense 5'-GGGCUAACAAAGAUAUGCATT-3' and antisense 5'-UGCAUAUCUUUGUUAGCCCTT-3'. The PC-3 cells were seeded into 6-well plates ($4\text{-}5 \times 10^4$ cells/well) and cultured in 2 mL of basic culture medium containing 10% FBS until the cells were 70% confluent. The siRNA-Lipofectamine™ 2000 complex was pre-mixed according to the manufacturer's instructions and added to the 6-well plates.

MTT assay

PC-3 cells in the logarithmic phase were seeded into 96-well plates. The cells were transfected with Notch-1 siRNA or scrambled siRNA. Twenty-four hours after transfection, cells were treated with docetaxel (doses ranged from 60-420 nM) or control. Twenty-four hours later, 50 μL of 5 mg/mL MTT solution was added to each well and incubated for 4 h. The reaction was terminated by adding 150 μL DMSO, and the optical density (OD) of each well was measured using a microplate reader (BioRad, Hercules, CA, USA) at a detection wavelength of 570 nm. The experiment was performed in triplicate, and the survival rates were calculated by subtracting the background OD value (complete culture medium without cells) from the OD value from each test well.

Groups

PC-3 cells were divided into 5 groups: (a) control PC-3 cells; (b) scrambled, which indicated scrambled siRNA-transfected PC-3 cells; (c) NS, which indicated Notch-1 siRNA-transfected PC-3 cells; (d) Doce, which indicated PC-3 cells exposed to IC_{30} docetaxel treatment (60 nM); and (e) NS + Doce, which indicated PC-3 cells transfected with Notch-1 siRNA and treated with IC_{30} docetaxel (60 nM).

Detection of apoptosis using flow cytometry

Forty-eight hours after siRNA transfection, cells were trypsinized, centrifuged, and washed with pre-cooled PBS. The cells were then washed with binding buffer containing 10 mmol/L HEPES, 140 mmol/L NaCl and 2.5 mmol/L $CaCl_2$ at pH 7.4 and re-suspended. Each sample (10^5-10^6 cells) was treated with 5 μL Annexin V-FITC and 10 μL propidium iodide (PI), mixed, and incubated at room temperature in the dark for 15 min. Fluorescence was detected using a flow cytometer (FACSAria, BD) within 1 h. The results were averaged, and the percentages of cells undergoing apoptosis were calculated using CellQuest software (Becton Dickinson).

Detection of the cell cycle distribution by flow cytometry

The cells were synchronized by serum deprivation and then released into complete medium containing 10% FBS. Next, 2×10^6 cells were harvested, washed in PBS and then fixed in 70% alcohol overnight at 4 °C. After washing three times in cold PBS, cells were resuspended in 1 mL PBS solution containing 100 μL PI and 1000 μg RNase A for 30 min at 37 °C. DNA content was analyzed with a flow cytometer (FACSAria, BD). The data were analyzed by Modfit software (Verity Software House Inc., Topsham, ME) to determine the proportions of cells in the G_0/G_1, S and G_2/M phases of the cell cycle. The cells with fractional DNA content located on DNA frequency histograms to the left of the G_0/G_1 peak (sub-G_0/G_1 cells) were identified as apoptotic cells.

Real-time RT-PCR

Total RNA was isolated from PC-3 cells using the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA). cDNA synthesis was performed with 2 μg total RNA using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario, Canada). The primers (ProMab) were designed with Primer Express 3.0 software (Applied Biosystems), and the sequences were as follows: Notch-1, forward 5-CCTGTCTGAGGTCAATGAGT-3; reverse 5-GTAGCCACTGGTCATGTCTT-3; Akt, forward 5-GACCATGAACGAGTTTGAGT-3, reverse 5-GAGTACTTCAGGGCTGTGAG-3; $p21^{waf1/cip1}$, forward 5-GATGGAACTTCGACTTTGTC-3, reverse 5-GCACAAGGGTACAAGACAGT-3; GAPDH, forward 5-CAATGACCCCTTCATTGACC-3, reverse 5-GACAAGCTTCCCGTTCTCAG-3. Quantitative PCR was performed using the SYBR Green Master

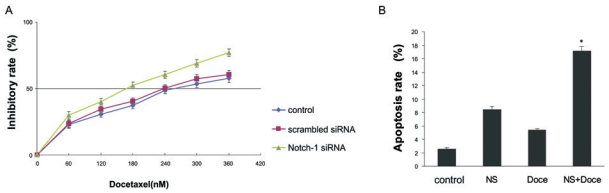

Figure 1. siRNA Mediated Silencing of Notch-1 Enhances Chemosensitivity of PC-3 Cells to Docetaxel. (A) The growth inhibition curve of PC-3 cells subjected to different treatment. (B) Apoptosis of PC-3 cells subjected to different treatment. Early and late apoptotic fractions were calculated as the incidence of apoptotic cell death. The columns indicated the means of at least three experiments. The error bars represented standard deviations. *P<0.05 compared to the control; ΔP<0.05 compared to single treatment

Mix (Fermentas, Burlington, Ontario, Canada) in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The quantification of gene expression was performed using the ΔΔCT calculation with CT as the threshold cycle. The relative levels of target genes, normalized to the sample with the lowest CT, were given as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

Western blot analysis

The cells were lysed in lysis buffer at 4°C for 30 min. The supernatant was taken and the protein concentration was determined using the Bradford protein assay system (Bio-Rad). A total of 20 μg protein was separated by SDS-PAGE and was transferred to polyvinyl difluoride membranes (Millipore, Bedford, MA) by electroblotting. After blocking with 5% non-fat dry milk, the blots were incubated with primary antibodies and developed with alkaline phosphatase-conjugated secondary antibodies (Zymed, San Francisco, CA) using enhanced chemiluminescence (Thermo, Rockford, IL). The membranes were exposed to X-ray film (Kodak, Rochester, NY), which were subsequently digitized and densitometrically analyzed using Gel-Pro Analyzer software version 4.0 (Media Cybernetics, Silver Spring, MD). Protein expression levels were represented as densitometric ratios of the targeted protein to GAPDH.

Statistical analysis

All experiments were performed in triplicate and the data were presented as the means ± standard deviations. The differences between the sample means were compared using analysis of variance. All analyses were performed using SPSS for Windows, version 12.1 (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

Results

Down-regulation of Notch-1 expression enhances the chemosensitivity of PC-3 cells to docetaxel

To investigate whether Notch signaling contributes to the chemoresistance of prostate cancer, the effects of Notch-1 siRNA combined with docetaxel treatment on cell growth were evaluated by MTT assay. The inhibition of cell proliferation by docetaxel and Notch-1 siRNA was significantly higher than that observed in PC-3 cells treated

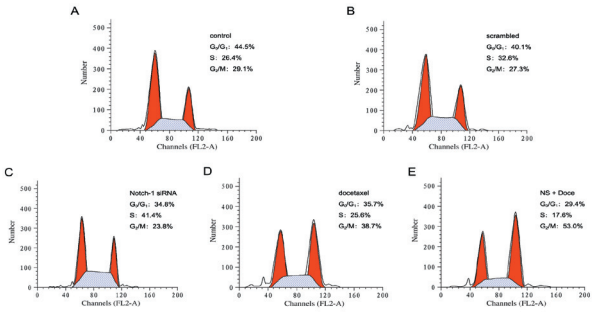

Figure 2. The Effects of Notch-1 siRNA and Docetaxel on Cell Cycle Phase Distribution of PC-3 Cells. X-axis, DNA content; Y-axis, the number of nuclei

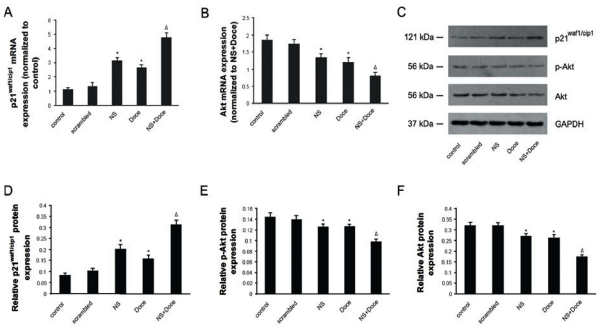

Figure 3. siRNA Mediated Silencing of Notch-1 Regulates the Expression of p21waf1/cip1 and Akt and the Activation of Akt in PC-3 Cells. (A) The expression of Akt mRNA was significantly decreased in the NS+Doce group compared to the NS or Doce groups. (B) The expression of p21waf1/cip1 mRNA was significantly increased in the NS+Doce group compared to the NS or Doce groups. Akt and p-Akt protein expression (C) are significantly decreased, and p21waf1/cip1 protein expression (D) is significantly increased in the NS+Doce group compared to the NS and Doce groups. (E) The protein expression levels of p21waf1/cip1, Akt and p-Akt by Western blot analysis. *P<0.05 compared to the control; ΔP<0.05 compared to single treatment

by Notch-1 siRNA or docetaxel alone. The IC_{50} value of docetaxel in PC-3 cells declined from 264.57±3.12 nM to 149.91±2.80 nM after transfection with Notch-1 siRNA (Figure 1A). Next, we examined the apoptosis of PC-3 cells in different groups by flow cytometry. The results showed that the apoptosis rate was significantly increased in PC-3 cells treated by both Notch-1 siRNA and docetaxel (Figure 1B). These results suggest that the down-regulation of Notch-1 expression enhances the chemosensitivity of PC-3 cells by inducing apoptosis.

Notch-1 siRNA augments the cell cycle arrest induced by docetaxel in PC-3 cells

Docetaxel is known to block cell proliferation at a checkpoint between the G_2 and M phases of the cell cycle. As expected, PC-3 cells exhibited a G_2/M cycle arrest after treatment with 60 nM docetaxel (IC_{30}) for 24 h (Figure 2D). Furthermore, down-regulation of Notch-1 led to a marked arrest in cell cycle progression after 24 h, as characterized by the loss of cells in the G_1 phase and an accumulation of cells in the S phase (Figure 2C). This is in agreement with the reported cell cycle arrest induced by Notch-1 siRNA (Zhang et al., 2006). Notably, compared to docetaxel treatment, the combination treatment with Notch-1 siRNA and docetaxel induced a

significant increase in G_2/M arrest (Figure 2E), suggesting a synergistic effect of Notch-1 depletion and docetaxel in inducing cell cycle arrest.

Down-regulation of Notch-1 induces p21$^{waf1/cip1}$ expression and inhibits Akt activation in PC-3 cells

To elucidate the molecular mechanism by which Notch-1 silencing inhibits cell cycle arrest, we used real-time RT-PCR and Western blot analysis to examine the expression of p21$^{waf1/cip1}$, a well-known cell cycle regulatory factor. In PC-3 cells, docetaxel or Notch-1 siRNA led to a significant increase in p21$^{waf1/cip1}$ expression, consistent with an arrest in the S phase. Furthermore, the combination of Notch-1 siRNA and docetaxel treatment increased the expression of p21$^{waf1/cip1}$ in PC-3 cells to a level much higher than in cells treated by Notch-1 siRNA or docetaxel alone (Figure 3).

The Akt family of proteins integrates a wide array of diverse upstream survival and distress signals to decide cell fate (Reagan-Shaw et al., 2008). To investigate the mechanism by which Notch-1 silencing regulates apoptosis of PC-3 cells, we examined the expression level and activation of Akt. Compared to cells treated by Notch-1 siRNA or docetaxel alone, the expression levels of Akt and the activation of Akt indicated by p-Akt were significantly down-regulated in cells treated with both siRNA and docetaxel (Figure 3). These data suggest that Notch-1 regulates the expression of p21$^{waf1/cip1}$ and the activation of Akt to modulate the proliferation and apoptosis of PC-3 cells.

Discussion

Notch signaling determines cell fate by modulating cell proliferation, differentiation and apoptosis. Notch activation is known to stimulate or inhibit cell proliferation in a cell type-specific and context-dependent manner (Ranganathan et al., 2011). In our study, the proliferation of PC-3 cells was decreased after the down-regulation of Notch-1. Moreover, we found that down-regulation of Notch-1 could enhance the anti-tumor effects of docetaxel by reducing the proliferation and inducing the apoptosis of prostate cancer cells, suggesting that Notch signaling is an effective target for the treatment of prostate cancer. Nefedova et al. (2008) established a mouse model for multiple myeloma and found that the inhibition of the Notch signaling pathway by gamma-secretase inhibitors increased the anti-tumor effects of melphalan and doxorubicin. In addition, it was reported recently that the expression of Notch ligand delta-like ligand 4 (DLL4) was strong in gliomas and the expression level was correlated with glioma angiogenesis (Li et al., 2011). However, the involvement of Notch-1 signaling pathway in the chemosensitivity of prostate cancer has not been previously studied. Our data indicate that chemosensitivity to docetaxel increased in PC-3 cells after siRNA mediated silencing of Notch-1, which supports the notion that Notch-1 is an oncogene in prostate cancer and could be an effective target for prostate cancer treatment.

The down-regulation of Notch-1 by siRNA reduced prostate cancer cell growth, perhaps due to cell cycle arrest. Notably, we found that down-regulation of Notch-1 induces the expression of p21$^{waf1/cip1}$, which binds and inhibits cyclin-dependent kinase/cyclin complexes that regulate the G_1 to S phase transition of the cell cycle (Deng et al., 1995). Furthermore, p21$^{waf1/cip1}$ is an important transcriptional target of p53 and mediates DNA damage-induced cell-cycle arrest in G_1 and G_2 (el-Deiry et al., 1993). Thus, p21$^{waf1/cip1}$ could contribute to S phase arrest following Notch-1 knockdown in PC-3 cells, consistent with the report that p21$^{waf1/cip1}$ plays an important role in controlling prostate cancer growth (Ronchini and Capobianco, 2001).

In addition, we identified G_2/M phase cell cycle arrest in prostate cancer cells when Notch-1 was down-regulated before docetaxel treatment. p21$^{waf1/cip1}$ is also known to promote G_2/M arrest because the introduction of nonfunctional p21$^{waf1/cip1}$ or a p21$^{waf1/cip1}$ antisense oligonucleotide diminished the G_2/M arrest phenotype in various cancer cell lines (Rigberg et al., 1999; De Siervi et al., 2004). It has been proposed that the interaction of p21$^{waf1/cip1}$ with proliferating cell nuclear antigen is critical for inducing G_2 cell cycle arrest (Ando et al., 2001). Therefore, G_2/M cell cycle arrest induced by Notch-1 knockdown in prostate cancer cells is also attributed to the induction of p21$^{waf1/cip1}$.

However, the cell cycle arrest induced by Notch-1 siRNA may not be solely dependent on p21$^{waf1/cip1}$ activation. We observed that down-regulation of Notch-1 inhibited the expression and activation of Akt, suggesting that Akt signaling is involved. Akt has multiple effects on cell cycle regulation, including the capacity to phosphorylate and inactivate two major cell cycle regulators, p21$^{waf1/cip1}$ (Brazil and Hemmings, 2001) and p27^{kip1} (Reed, 2002). We observed that the down-regulation of Notch-1 reduced the expression of the anti-apoptotic protein Bcl-2 and the pro-apoptosis protein Bax in prostate cancer cells (data not shown). In addition, Wang et al. (2011) found that Akt was a downstream target of Notch-1 signaling and a significant reduction in cell viability and increase in apoptosis in prostate cancer cells were correlated with the down-regulation of Notch-1 and Akt. Therefore, it is possible that Akt could contribute to the effects of Notch-1 knockdown on cell cycle arrest and survival. Interestingly, a recent study showed that knockdown of beta-catenin, another important signaling in development, could enhance the chemoresistance of osteosarcoma cells to doxorubicin via the NF-kappaB pathway (Zhang et al., 2011). Therefore, further studies are needed to characterize other downstream pathways or effectors that mediate Notch-1 induced chemoresistance of prostate cancer to docetaxel and other chemotherapeutics.

In summary, we demonstrated that silencing of Notch-1 promoted docetaxel induced cell growth inhibition, apoptosis and cell cycle arrest in PC-3 cells. In addition, these effects were associated with increased p21$^{waf1/cip1}$ expression and decreased Akt expression and activation in PC-3 cells. These results suggest that Notch-1 promotes the chemoresistance of prostate cancer by regulating p21$^{waf1/cip1}$ and Akt and additionally highlight the potential of Notch-1 as a novel therapeutic target for prostate cancer.

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