Notch3 is involved in the proliferation of renal cancer cells via regulation of cell cycle progression and HIF-2α

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Abstract. Renal cell carcinoma (RCC) is one of the most common malignant tumors of the urinary system. Although deregulation of the Notch signaling pathway is common in RCC and is involved in the tumorigenic process, the exact role of Notch3 and its underlying molecular mechanism in RCC, particularly in hypoxia, remain unknown. In the present study, RO4929097, a Notch3 inhibitor, was used to alter NICD3 expression. A Cell Counting Kit-8 assay, EdU incorporation assay, colony formation assay, flow cytometry and western blot analysis were used to investigate the effects of altered NICD3 expression on cell proliferation, cell cycle progression and HIF-2α protein expression. The results of western blot analysis showed that RO4929097 dose-dependently decreased the expression of Notch3 intracellular domain (NICD3) in 786-O and ACHN cells, which originate from clear cell RCC (ccRCC). The results of the Cell Counting Kit-8, EdU incorporation and colony formation assays demonstrated that downregulation of NICD3 significantly suppressed cell proliferation in both normoxia and hypoxia. In addition, flow cytometry and western blot analysis demonstrated that hypoxia (2% O2) promoted cell cycle progression in ccRCC cells with the increased expression of G1-S transition-associated proteins, namely cyclin-dependent kinase (CDK)4 and cyclin D1, while downregulation of NICD3 exerted negative effects on cell cycle progression, and the expression levels of CDK4 and cyclin D1. Furthermore, western blot analysis revealed that 2% O2-induced upregulated hypoxia-inducible factor-2α (HIF-2α) expression decreased following downregulation of NICD3 in 786-O and ACHN cells. Following transfection of the vector containing the NICD3 coding sequence, HIF-2α, CDK4, cyclin D1 and proliferating cell nuclear antigen expression, that were inhibited by RO4929097 in hypoxia, were rescued. Collectively, the results of the present study suggest that Notch3 is closely associated with the cell proliferation of ccRCC cells by regulating the cell cycle and HIF-2α.

Introduction

Renal cell carcinoma (RCC) is a complex, multifaceted malignant disease comprising different and specific entities, with over two-thirds of all cases being clear cell RCC (ccRCC) (1). Of all ccRCC cases, 60-80% are considered to be associated with the biallelic inactivation of the Von Hippel Lindau (VHL) gene, which causes aberrant accumulation of hypoxia-inducible factor (HIF) (2). In addition, HIF is a critical component of several proteins that constitute the cellular machinery for adaption to hypoxia, which is the result of rapid cell proliferation and poor vascularization of solid tumors (3). Although previous evidence from genetic and biological studies indicates that hypoxia-associated pathways play key roles in the development and progression of renal cancer (4), the underlying molecular mechanism is complex and remains elusive.

As a highly conserved developmental signaling pathway, deregulated Notch signaling has been reported to be associated with tumorigenesis. The core Notch signaling pathway is composed of five ligands (Jagged-1 and -2 and δ-like-1, -3, and -4) and four receptors (Notch1-4). The binding of a ligand with a Notch receptor results in two proteolytic cleavages of the receptor, which leads to the release of the Notch intracellular domain (NICD). Subsequently, NICD translocates into the nucleus, where it interacts with the DNA-binding factors [CBF1/RBP-J, Su (H), Rbpsuh and Lag-1, CSL] to regulate the expression of downstream genes (5,6). Clinical studies have demonstrated that Notch1 signaling is activated in ccRCC and may be a useful biomarker for prognosis (7,8), whereas treatment with Notch1 inhibitors may inhibit the progression of ccRCC (9). While the Notch1 signaling pathway has been
demonstrated to be involved in the progression of ccRCC, less is known on whether and how Notch regulates the cellular hypoxic response, particularly Notch3, the structure and function of which differs from those of the other three Notch receptors (10). In the present study, RO4929097, a γ-secretase inhibitor that inhibits NICD expression, thereby decreasing the expression of the downstream Notch targets (11), was used to alter NICD3 expression. The present study aimed to investigate the effects of altered NICD3 expression on cell proliferation, cell cycle progression and HIF-2α protein expression, which may prove to be of value in the treatment of RCC.

Materials and methods

Cell lines and cell culture. The human ccRCC cell lines, 786-O and ACHN were purchased from American Type Culture Collection and maintained in RPMI-1640 medium or minimal essential medium (MEM) supplemented with 10% fetal bovine serum (all purchased from HyClone; Cytiva), respectively. 786-O and ACHN cells were treated with or without RO4929097 (cat. no. HY-11102; MCE), and were cultured under normoxic (2% O$_2$, 37°C and 5% CO$_2$) or hypoxic (2% O$_2$, 37°C and 5% CO$_2$) conditions for 48 h, or with 200 µM cobalt chloride (CoCl$_2$) (cat. no. C7300; Beijing Solarbio Science & Technology Co., Ltd.) under 21% O$_2$, 37°C and 5% CO$_2$ condition for 48 h.

Cell Counting Kit-8 (CCK-8) assay. 786-O and ACHN cells were seeded into 96-well plates at a density of 10,000 cells/well and cultured under normoxic or hypoxic conditions at 37°C for 48 h. Then, according to the manufacturer's protocol, the cell proliferation rates of 786-O and ACHN were subsequently analyzed via the CCK-8 assay (cat. no. DJDB4000X; Dojindo Molecular Technologies, Inc.).

EdU assay. EdU incorporation assay was performed using the Cell-Light™ EdU Apollo®488 In Vitro Flow Cytometry kit (cat. no. C10301-3; Guangzhou Ribobio Co., Ltd.), according to the manufacturer's protocol. Briefly, 786-O and ACHN cells were respectively cultured in RPMI-1640 and MEM medium supplemented with 10% EdU for 2 h at 37°C, and washed with cold phosphate buffered saline (PBS) containing 1% bovine serum albumin (Beijing Solarbio Science & Technology Co., Ltd.) for three times. Cells were resuspended in 500 µl of 1X Apollo reaction buffer and subsequently incubated at room temperature for 30 min. 786-O and ACHN cells were re-washed twice with PBS containing 0.5% Triton X-100, stained with 1X Hoechst33342 reaction buffer for 5 min at room temperature, re-washed twice with PBS containing 0.5% Triton X-100, and subsequently added to 500 µl PBS. Cells were observed under an inverted immunofluorescence microscope at x10 magnification [IX70/SPOT RT-KE (color); Olympus Corporation/Diagnostic Instruments Inc.] and named as p3xFLAG-CMV-14 NICD. For the transfection experiments, according to the manufacturer's instructions, 786-O and ACHN cells (2x10$^4$ cells/well) were seeded in 6-well plates overnight, then transfected with p3xFLAG-CMV-14 vector as the control or p3xFLAG-CMV-14-NICD vector (2 µg/ml) using Lipofectamine 3000 (cat. no. L3000015; Thermo Fisher Scientific, Inc.). After 24 h post-transfection, RO4929097 was added to cell culture wells. Subsequently, the cells were cultured under hypoxic conditions for 48 h, and the cells were harvested for further analysis.

Western blotting. 786-O and ACHN cells were lysed using RIPA lysis buffer (cat. no. P0013C; Beyotime Institute of Biotechnology). Cell extracts were collected via centrifugation and protein concentration was measured by BCA method. Subsequently, 50 µg protein per lane was separated by SDS-PAGE on a 10% gel and transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk for 2 h at room temperature, and then incubated with the following primary antibodies: Rabbit polyclonal anti-HIF-2α (1:500; cat. no. NB100-122; Novus Biologicals, LLC), rabbit polyclonal anti-Notch3 (1:500; cat. no. ab23426; Abcam), mouse monoclonal anti-cyclinD1 (1:500; cat. no. 60186-1-Ig; ProteinTech Group, Inc.), rabbit polyclonal anti-CDK4 (1:1,000; cat. no. YT5198; http://www.imunoway.com/), mouse monoclonal anti-cyclinD1 (1:500; cat. no. 60186-1-Ig; ProteinTech Group, Inc.), rabbit polyclonal anti-CDK4 (1:1,000; cat. no. YT5198; http://www.imunoway.com/) and named as p3xFLAG-CMV-14 NICD.
rabbit polyclonal anti-PCNA (1:1,000; cat. no. ab152112; Abcam) and mouse monoclonal anti-β-actin (1:1,000; cat. no. AF0003; Beyotime Institute of Biotechnology) secondary antibodies at room temperature for 2 h. Protein bands were visualized using the chemiluminescent reagent SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). Densitometry values were normalized to levels of β-actin.

Quantitation analysis for western blot were performed using ImageJ software (version 1.52; National Institute of Health).

Statistical analysis. Statistical analysis was performed using (version 6.0; GraphPad Software, Inc.). All experiments were performed in triplicate and data are presented as the mean ± standard deviation. One-way analysis of variance, followed by Tukey's post hoc test was used to compare differences between multiple groups. GraphPad Prism 6.0 was used to create the histograms. P<0.05 was considered to indicate a statistically significant difference.

Results

RO4929097 dose-dependently inhibits NICD3 expression and decreases ccRCC cell proliferation in normoxia and hypoxia.

Aberrant cell proliferation is the main biological characteristic of cancer (12), and is affected by the tumor microenvironment, including the O2 concentration around cancer cells (13). In the present study, cells were treated with or without RO4929097 for 48 h. Western blot analysis demonstrated that NICD3 expression decreased with increasing concentration of RO4929097 in 786-O and ACHN cells (Fig. 1A). Moreover, after the treatment with 20 µM RO4929097 for 48 h, not many dead cells were obviously observed via invert-microscope, suggesting that the direct cytotoxicity of RO4929097 to cells was low. The CCK-8 assay was performed to determine whether inhibition of Notch3 decreased cell proliferation. As presented in Fig. 1B, a significant increase in proliferation was induced by hypoxia in the control groups of 786-O cells (P<0.01) but not ACHN cells, and the increases weren't significant in the treatment groups of two cell lines. However, the proliferation of 786-O and ACHN cells was significantly inhibited by RO4929097 under normoxic and hypoxic conditions (P<0.05 in 786-O in normoxia; P<0.01 in 786-O in hypoxia; P<0.01 in ACHN in normoxia or hypoxia). EdU incorporation assay was performed to validate the effect of RO4929097 or hypoxia on cell proliferation. The results demonstrated that the number of EdU-positive cells significantly increased in response to hypoxia in the control groups and the treatment groups of two cell lines (all P<0.01), whereas the number of EdU-positive cells significantly decreased following RO4929097 treatment in normoxia and hypoxia (all P<0.05) (Fig. 1C).

The colony formation assay is considered the gold standard for analyzing cell proliferation potential in cellular systems in vitro (14,15). In the present study, 786-O and ACHN cells were cultured under normoxic or hypoxic conditions for 10 days. At the end of the culture period, the cells derived from a single cell were separable and did not form a dense clone. Thus, the sum of the IOD of cells stained with methylene blue in each well was measured, rather than the number of colonies. The results demonstrated that under normoxic conditions, the sum of the IOD significantly decreased following RO4929097 treatment in 786-O cells (P<0.01), but not in ACHN cells. Furthermore, under hypoxic conditions, the sum of the IOD significantly increased in both 786-O and ACHN cells (P<0.05 in 786-O and P<0.01 in ACHN), the effects of which were reversed following RO4929097 treatment (P<0.05 in 786-O and P<0.01 in ACHN) (Fig. 1D). Collectively, these results suggest that hypoxia promotes the proliferation of ccRCC cells in vitro, and inhibiting NICD3 expression may repress the proliferation of ccRCC cells under both normoxic and hypoxic conditions.

Downregulation of NICD3 inhibits RCC cell cycle progression via CDK4 and cyclin D1, under both normoxic and hypoxic conditions.

Cell replication and division are tightly regulated by the cell cycle, and dysregulation of the cell cycle leads to aberrant cell proliferation in cancer (16). Cell cycle distribution was assessed to determine the molecular mechanism underlying the regulatory effect of Notch3 on the proliferation of ccRCC cells. As presented in Fig. 2A and B, hypoxia decreased the percentage of cells in the G1 phase compared with that in normoxia in 786-O (P<0.01) but not ACHN, and RO4929097 treatment significantly increased the percentage of cells in the G1 phase, under both normoxic (all P<0.05) and hypoxic conditions (all P<0.01). Furthermore, the increased range of the percentage of cells in the G1 phase induced by RO4929097 in hypoxia was higher compared with that in normoxia.

The biological properties modulated by hypoxic signaling and Notch signaling pathways are due to the initial expression levels of a set of downstream genes, such as cyclin D1, CDK4, P21 and so on (21,27). For example, hypoxic signaling and Notch signaling regulated the migration and invasion of breast cancer via their regulation to the targets, snail and slug (21). Thus, the expression levels of cyclin D1 and CDK4, which regulate G1-S transition (22,23), were detected via western blotting. Western blot analysis demonstrated that hypoxia upregulated the expression of cyclin D1 and CDK4 of the control groups (all P<0.01). After treatment RO4929097, their expressions were downregulated in no matter normoxia (all P<0.05) or hypoxia (all P<0.01) (Fig. 2C). Given that an increasing number of cells in the G1 phase indicates low proliferation (24,25), consistent with those of cell cycle analysis, the results suggested that downregulation of NICD3 may decrease cell proliferation by regulating the cell cycle.

NICD3 overexpression rescues the expression of HIF-2α, CDK4, cyclin D1 and PCNA, which are inhibited by RO4929097 in hypoxia. A set of cellular responses, such as proliferation, drug resistance and metastasis, caused by low oxygen levels are mediated by HIFs (3,4,13). HIF-α is an oxygen-dependent transcriptional activator, and three α subunits (HIF-1α, HIF-2α and HIF-3α) have been identified (4). Several lines of investigation indicate that HIF-2α, but not HIF-1α, is upregulated in ccRCC, which promotes ccRCC procession (26,27). In addition, HIF-1α is not expressed in 786-O cells, which
lack HIF-1α mRNA (28). Previous studies have reported that HIFs interact with types of Notch in cancer, such as in breast cancer and prostate cancer (29,30), thus the effect of hypoxia on Notch3 expression and the effect of NICD3 on HIF-2α...
expression were assessed in the present study. First, the vector containing NICD3 coding sequence was constructed. The results demonstrated that NICD3 expression significantly increased in 786-O and ACHN cells following transfection, by 47.0 and 168.1%, respectively (Fig. 3A). To determine the effect of hypoxia on Notch3, the expression levels of the full length of Notch3 (Notch3FL), NICD3 with transmembrane domain (TD+NICD3) and NICD3 were detected via western blotting. As the anti-Notch3 antibody used in this present study could bind with Human NOTCH3 aa 2300 to the C-terminus (C terminal), which Notch3FL, TD+NICD3 and NICD3 all contain, the bands of different molecular weights, 220kDa, more than 90kDa and 90kDa, relatively represented Notch3FL, TD+NICD3 and NICD3. The results demonstrated that the expression levels of Notch3FL, TD+NICD3 and NICD3 did not change in 786-O and ACHN cells following exposure to
The effects of CoCl₂ and 2% O₂ on HIF-2α expression were subsequently assessed. As presented in Fig. 3C, 2% O₂, but not CoCl₂, increased HIF-2α expression in 786-O cells (P<0.01), whereas HIF-2α expression increased in ACNH cells following treatment with both CoCl₂ (P<0.05) and 2% O₂ (P<0.01), respectively. These results may be due to the inactivation of VHL in 786-O cells (2,28). As presented in Fig. 3D, HIF-2α expression was significantly decreased by RO4929097 under 2% O₂ (all P<0.01), but not 21% O₂ for 48 h, the effects of which were reversed following overexpression of NICD3 overexpression. Collectively, these results suggest that NICD3 affects HIF-2α expression; however, hypoxia does not affect Notch3 expression.

To further investigate the association between hypoxia and Notch3 signaling, the effects of altered NICD3 expression on the cell cycle and proliferation-associated protein expression in hypoxia were assessed in the present study. Western blot analysis demonstrated that compared with the control groups, the protein levels of CDK4, cyclin D1 and PCNA decreased in RO4929097-treated 786-O and ACHN cells cultured under 2% O₂ (all P<0.05), whereas NICD3 overexpression rescued the protein levels, suggesting that the hypoxia-induced protein expression was regulated by NICD3 (Fig. 3E). Taken together, the results of the present study suggest that regulation of NICD3 to these proteins is associated with its regulation to HIF-2α.
Discussion

Notch family members are single-pass transmembrane proteins that function both as cell surface receptors and nuclear transcriptional regulators. The latter function is predominantly mediated by NICD. Upon Notch binding to their ligands, the extracellular subunit, NEC, dissociates from the transmembrane subunit (5). NEC is subsequently cleaved by a disintegrin, metallopeptase and γ-secretase, whereas the NICD is released into the cytoplasm and translocates into the nucleus to regulate the transcription of Notch target genes, which subsequently regulates proliferation and differentiation (6,31). In the present study, RO4929097 dose-dependently inhibited NICD3 expression in 787-O and ACHN cells, suggesting that RO4929097 downregulates Notch3 signaling.

Hypoxia and activation of HIFs have also been described in the major RCC subtypes, and are closely associated with ccRCC development and progression (4). In normoxia, HIF-α molecules are subjected to a regulatory process involving the enzymatic hypoxylation of conserved prolyl and asparaginyl residues, thus leading to VHL protein-mediated ubiquitination and proteasomal degradation (2). The HIF-specific prolyl hydroxylases have an iron-binding core, which can be replaced by CoCl$_2$, resulting in the inhibition of HIF-α degradation; thus, CoCl$_2$ is used to mimic chemical hypoxia effects (32). However, to the best of our knowledge, it has not yet been demonstrated that the function of cells in response to hypoxia can completely be recapitulated by CoCl$_2$. In the present study, CoCl$_2$ did not affect HIF-2α protein expression in 786-O cells, which lack VHL (28). However, 2% O$_2$ increased HIF-2α protein expression in 786-O and ACHN cells. These findings were consistent with the results that demonstrated that 2% O$_2$ promoted cell proliferation and cell cycle progression in 786-O and ACHN cells, suggesting that culturing cells in 2% O$_2$ for 48 h did induce the response of 786-O and ACHN cells to hypoxia in the present study. In D324 medulloblastoma cells, CoCl$_2$ does not significantly alter HIF-2α expression (33). Previous studies have demonstrated that hypoxia increases HIF-2α protein expression in 786-O cells (34), and also increases cyclin D1 expression, which is a target gene of HIF-2α (17). The results of the present study demonstrated that although the CoCl$_2$ experimental group exhibited different results from 2% O$_2$, 2% O$_2$ did affect ccRCC through its regulation of HIF-2α expression.

Multiple signaling pathways, including the Notch signaling pathway, have been reported to affect the response to hypoxia and interact with HIFs at several levels (29,35-37). In the present study, although hypoxia did not affect Notch3 expression, NICD3 was demonstrated to regulate HIF-2α protein expression, suggesting an association between Notch3 and HIF-2α in ccRCCs. Furthermore, downregulation of NICD3 was demonstrated to decrease the proliferation of 787-O and ACHN cells under both normoxic and hypoxic conditions, suggesting the involvement of Notch3 in the progression of ccRCC in normoxia, and the response of ccRCC cells to hypoxia.

High cell proliferation is characterized by rapid cell division and G$_1$-S transition (16,24,25). A previous study demonstrated that Notch3 affects cell cycle progression (38). In esophageal squamous cell carcinoma, inhibiting Notch3 induces G$_1$ phase arrest and inhibits cell proliferation (39). In Notch-dependent T-cell lymphomas, cyclin D3 and CDK4 are highly expressed, and γ-secretase inhibitor induces G$_1$ arrest in these cell lines (18). HIFs have also been reported to affect cell cycle progression through transcriptional targets, such as cyclin D1 and CDK4, p21 and p27 (2,19,20). In the present study, RO4929097 treatment increased the number of cells in the G$_1$ phase by downregulating the expression of cyclin D1 and CDK4 in normoxia and hypoxia, and the increased range of the percentage of cells in the G$_1$ phase induced by RO4929097 in hypoxia was higher compared with that in normoxia. Notably, overexpression of NICD3 reversed the RO4929097-induced downregulated expression of cyclin D1 and CDK4 in hypoxia. Taken together, these results suggest that Notch3 promotes cell cycle progression of ccRCCs by regulating cyclin D1 and CDK4 expression, and acts synergistically with HIFs to regulate cyclin D1 and CDK4 expression in hypoxia.

In conclusion, the results of the present study demonstrated that Notch3 is closely associated with the cell proliferation of ccRCC cells through its regulatory effects on the cell cycle, and by acting synergistically with HIF-2α in hypoxia. These findings provide novel insights into targeting Notch3 as a promising therapeutic option in RCC. However, the exact molecular mechanisms underlying the synergistic effect of Notch3 and HIF-2α on ccRCC tumorigenesis and progression were not investigated in the present study, and further investigations are required.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

QH, CL and BW conceived and designed the present study. QH, YF, FH, BL, JX, WS, DH and HK performed the experiments, and QH drafted the initial manuscript, analyzed and interpreted the data, and performed statistical analysis. CL and BW revised the manuscript for intellectual content. All authors contributed to data interpretation, and QH, CL and BW conceived and designed the present study.

Ethics approval and consent to participate

Not applicable.
Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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