CTNNB1 Knockdown Inhibits Cell Proliferation and Aldosterone Secretion Through Inhibiting Wnt/β-Catenin Signaling in H295R Cells

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Abstract
Aldosterone-producing adenomas (APA) is one of the causative factors of primary aldosteronism. Previous studies have suggested that there are somatic CTNNB1 mutations in APA, but the specific mechanism of CTNNB1 mutation in APA tumorigenesis and aldosterone secretion remains unclear. In the present study, human adrenocortical carcinoma cell line H295 R was used to establish stable CTNNB1 knockdown cell lines. Cell proliferation and aldosterone secretion of H295 R cells in response to angiotensin II (Agn II) were analyzed. We found that CTNNB1 knockdown reduced β-catenin expression and inhibited proliferation of H295 R cells. CTNNB1 knockdown inhibited Wnt/β-catenin signaling pathway and downregulated expression of downstream genes including axin 2, lymphoid enhancer binding factor 1 (LEF1), and cyclin D1. In addition, CTNNB1 knockdown decreased responses of H295 R cells to Agn II and decreased aldosterone secretion. Our findings suggest that CTNNB1 knockdown can inhibit H295 R cell proliferation and decrease aldosterone secretion in the responses of H295 R cells to Ang II through inhibiting Wnt/β-catenin signaling pathway, indicating that targeting Wnt/β-catenin signaling pathway may be an important approach to decrease aldosterone secretion in the treatment of aldoster-producing adenomas.

Keywords
aldosterone-producing adenomas, CTNNB, Wnt/β-catenin signaling pathway, aldosterone secretion, H295 R cell

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Introduction
Primary aldosteronism (PA), which is characterized by hyperaldosteronism, affects 20% of patients with refractory hypertension.1 The excessive production of aldosterone leads to hypertension, low plasma renin activity, varying degrees of hypokalemia, and metabolic alkalosis.2 Now, PA is being recognized as the most common cause of secondary hypertension with a prevalence of 5-10% in hypertensive individuals and up to 20% in therapy-resistant hypertension.3,4 The pathogenesis of PA is caused by adrenal aldosterone-producing adenomas (APA) or bilateral hyperplasia in 95% of patients.5 Clinical therapy of PA relies on surgical resection and/or treatment with mineralocorticoids antagonists to minimize hypertension.6,7 However, the medication time is still inconclusive and serious side effects are still not resolved. Therefore, existing treatment strategies are limited, which warrants exploring the molecular mechanisms of PA to improve therapeutic outcomes.

Wnt/β-catenin signaling pathway has extensive biological effects and is relatively conservative in evolution, which has important effects on ontogeny, cell differentiation, apoptosis and necrosis.8 Previous studies indicate that the abnormal activated

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aldosterone in the responses of H295 R cells to Ang II.

nin signaling pathway is a vital role in mediating the secretion of carcinoma cell line H295 R. Our study suggests that Wnt/b-catenin signaling to induce aldosterone secretion. 18 The specific mechanism of Wnt/b-catenin in the adrenal cortex present with increased aldosterone production and even a risk of adrenocortical tumors.15,16 These researches suggest that Wnt/b-catenin signaling pathway triggers off APA tumorigenesis and may be involved in aldosterone secretion.

APA patients with CTNNB1 mutation are older and have more obvious hypertension symptoms. After adrenalectomy, CTNNB1 mutation carriers have a higher likelihood of residual hypertension than other APA patients.17 Akerstrom et al reported that APA tissue containing the CTNNB1 mutant gene has a high expression of CYP11B2.13 As the last rate-limiting enzyme for aldosterone synthesis, CYP11B2 expression is up-regulated by angiotonin II (Ang II) through activating calcium signaling to induce aldosterone secretion.18 The specific mechanism of CTNNB1 mutation in APA-induced hyperaldosteronism remains unclear.

In order to explore the effect of Wnt/b-catenin signaling pathway on the development of APA and to better understand the role of CTNNB1 gene in APA cell proliferation and aldosterone secretion, we knocked down CTNNB1 gene expression in vitro and investigated the effect of Wnt/b-catenin signaling pathway on cell proliferation and aldosterone secretion of human adrenocortical carcinoma cell line H295 R. Our study suggests that Wnt/b-catenin signaling pathway is a vital role in mediating the secretion of aldosterone in the responses of H295 R cells to Ang II.

Materials and Methods

Cell Culture

Human adrenocortical carcinoma H295 R cell line was cultivated in Dulbecco’s Modified Eagle Medium (DMEM)/Ham’s F12 medium complemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 50 U/mL penicillin and 100 mg/mL streptomycin, at 37°C with 5% CO₂. All media and supplements in this study were purchased from Invitrogen (Carlsbad, CA, USA).

CTNNB1 Knockdown

CTNNB1 was knocked down in H295 R cells with siRNA (RiboBio Co., Ltd., Guangzhou, China) in accordance with the manufacturer’s instructions. There were 3 target sequences: si-CTNNB1_001, 5’-TGTTGCTTGCTCAACAA-3’; si-CTNNB1_002, 5’-GCTTGGAATGAGACTGCTG-3’; si-CTNNB1_003, 5’-AGCTGATATTGAGACAG-3’. The corresponding negative control sequence for the target gene was purchased from RiboBio Co., Ltd. To stably and efficiently knockdown CTNNB1 in H295 R cells, siRNA targeting the si-CTNNB1_003 coding sequence 5’-AGCTGATATTGAGACAG-3’ was designed and inserted into a pGMLV-SC5RNAi lentiviral vector (Genomeditech Co., Ltd, Shanghai, China), and an interference-free siRNA was used as a negative control. Quantitative reverse transcription PCR (qRT-PCR) and Western blot analysis were used to determine the knockdown efficiencies.

Total RNA Extraction and qRT-PCR

Total RNA extraction kit (Solarbio, Beijing, China) was used to extract total RNA from H295 R cells, and was in accordance with the manufacturer’s instructions. were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to determine the RNA concentrations. The iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA, USA) was used to reverse-transcribe 1 microgram of total RNA to synthesize cDNA. CFX96 real-time system (Bio-Rad) and SYBR Green Supermix (Bio-Rad) were used to perform PCR and the procedures were performed according to the manufacturer’s instructions. The sequences of the primers used in this study are listed in Table 1.

Western Blot Analysis

The prepared protein samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 12% gel. Subsequently, in Tris buffered saline containing 5% skim milk and 0.1% Tween-20, the separated protein samples were transferred to the nitrocellulose membranes after blocking at room
temperature for 1 h. The nitrocellulose membranes were placed in a suitable concentration of primary antibody and incubated for 18 h at 4 °C. Then, membranes were placed in an incubator and stained with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Finally, membranes were visualized with enhanced chemiluminescence (SuperSignal, Pierce, Rockford, IL) or ECL Plus (Amersham Pharmacia Biotech, Buckinghamshire, UK) substrates in accordance with the manufacturers’ instructions.

**Cell Viability Assays**

Cell viability was assessed by cell counting kit-8 (CCK-8) and colony formation assays. Briefly, cells were inoculated in a 6-well plate (Corning, Corning, NY, USA) at 500 cells per well after being treated with or without CTNNB1 knockdown. The cells were cultured for 10 days, and the medium was changed at least every 3 days depending on the growth of the cells. The colonies were washed with phosphate-buffered saline (PBS), fixed in methanol, and stained with crystal violet. The number of colonies was counted under a microscope. CCK-8 assays were performed in accordance with the manufacturer’s instructions.

**Quantification of Aldosterone Using ELISA Kit**

After incubation of H295 R cells with the respective control medium, aldosterone in the supernatant of the cells was measured using a commercial human aldosterone ELISA Kit (ALD ELISA kit) in accordance with the manufacturer’s protocol (AmyJet Scientific, Wuhan, China).

**Statistical Analysis**

Each experiment was performed at least 3 times independently. The GraphPad Prism 7.0 statistical program (GraphPad, San Diego, CA, USA) was used for data analysis. The results were presented as the mean ± standard deviation (SD) unless otherwise indicated. The significance of the difference between the 2 groups was analyzed by 2-tailed Student’s t-tests. The otherwise indicated. The significance of the difference between the groups was analyzed by chi-square test. P < 0.05 was considered statistically significant.

**Results**

**CTNNB1 Knockdown Reduced β-Catenin Expression and Inhibited Proliferation of H295 R Cells**

Stable CTNNB1 knockdown in H295 R cell lines were established to actively explore the function of endogenous CTNNB1. Based on qRT-PCR and western blot assays, si-h-CTNNB1_003 significantly downregulated the mRNA level and protein expression level of β-catenin (Figure 1A). We utilized this lentivirus-mediated siRNA to induce CTNNB1 knockdown in H295 R cells (Figure 1B). CCK-8 assays showed that CTNNB1 knockdown markedly reduced cell viability, compared with the control group (Figure 1C). Similarly, colony formation assays showed that cell proliferation was significantly decreased with CTNNB1 knockdown in H295 R cells (Figure 1D).

**Wnt/β-Catenin Signaling Pathway Was Inhibited by CTNNB1 Knockdown**

To explore the effects of CTNNB1 knockdown on Wnt/β-catenin signaling pathway in H295 R cells, we detected the downstream target genes of Wnt/β-catenin signaling pathway including AXIN2 and lymphoid enhancer binding factor 1 (LEF1). Compared with the control group, the mRNA expression levels of AXIN2 (Figure 2A) and LEF1 (Figure 2B) in CTNNB1 knockdown group were significantly decreased. Western blot analysis revealed that the protein expression levels of AXIN2 and LEF1 were downregulated in CTNNB1 knockdown group (Figure 2C). After adding angiotensin II (Ang II) to the cells for 6 hours, the results were not affected (Figure 1B and Figure 2A-C). These results suggest that CTNNB1 knockdown inhibited Wnt/β-catenin signaling pathway.

**CTNNB1 Knockdown Reduced Expression of Cycling D1**

Since cycling D1 is known to play an important role in cell proliferation, qRT-PCR and Western blot analysis were used to detect expression of cycling D1 in H295 R cells. In CTNNB1 knockdown group, the mRNA levels of cycling D1 were significantly decreased (Figure 3A) and the protein expression levels of cycling D1 were also downregulated (Figure 3B), compared to the control group. In addition, the expression levels of cycling D1 were not affected by adding Ang II. These results suggest that CTNNB1 knockdown inhibits cell proliferation through reducing expression of cycling D1.

**CTNNB1 Knockdown Decreased Aldosterone Secretion in H295 R Cells**

Cytochrome P450 family 11 subfamily B member 2 (CYP11B2) is one of the rate-limiting enzymes for aldosterone synthesis and is also the terminal enzyme. In order to explore the effect of CTNNB1 on aldosterone secretion, we detected the basal and Ang II-treated CYP11B2 expression levels. CTNNB1 knockdown inhibited the mRNA and protein expression levels of CYP11B2 (Figure 4A and C), but not the expression levels of CYP11B1 (Figure 4B and C). In addition, Ang II significantly increased the expression levels of CYP11B2 in H295 R cells, which was reversed by CTNNB1 knockdown (Figure 4A and C). On the contrary, Ang II also increased the expression levels of CYP11B1, which was not influenced by CTNNB1 knockdown (Figure 4B and C). Moreover, aldosterone ELISA Kit was used to detect the concentration of aldosterone in the supernatant of H295 R cells. We found that Ang II-induced upregulation of aldosterone secretion was reversed by CTNNB1.
knockdown (Figure 4D). These results suggest that CTNNB1 knockdown reduces the responses of H295 R cells to Ang II and decreases secretion of aldosterone.

**Discussion**

Hypertension is one of the most common cardiovascular diseases in China. In recent years, with the clinical application of plasma aldosterone/renin activity ratio (ARR) and the extensive application of computed tomography, magnetic resonance imaging and other imaging technologies, the detection rate of PA in hypertensive population has been significantly improved. PA has become the common cause of intractable hypertension. In addition, the tissues in the heart, brain and kidneys in PA patients were more seriously damaged than those in PH patients, which results in more grievous consequences. Therefore, exploring novel therapeutic targets is an urgent and promising undertaking for the clinical treatment of PA.

Aldosterone production is stimulated by Ang II or extracellular K⁺ and is mediated mainly by Ca²⁺ influx into
adrenal glomerulosa cells through calcium signaling pathway. During the past years, significant progress has been made in the knowledge of the genetic basis of APA development. The mutation site of APA is related to the specific biochemical and clinical features of PA. Over 50% of APA patients have been identified to have multiple gene mutation sites, such as \textit{KCNJ5}, \textit{CACNA1D}, \textit{ATP1A1}, and \textit{ATP2B3} mutations, which belong to cell membrane ion channel protein encoding gene. These mutations may increase intracellular calcium concentrations through various ways, opening voltage-dependent calcium channels, thereby activating calcium signaling and aldosterone secretion. However, there are no obvious association between these mutations and adrenal tumorigenesis.

Most majorities of APA patients have been detected to possess activation of Wnt/\(\beta\)-catenin signal pathway. In adrenal tumors, the main cause for activation of Wnt/\(\beta\)-catenin signal pathway is \textit{CTNNB1} mutation. Patients with \textit{CTNNB1} mutation have larger adenomas size, but not higher level of aldosterone, compared to the patients with other mutations. H295 R cells have been shown to be an Ang II-responsive steroid-producing adrenocortical cell line. Gaujoux et al has reported that silencing \textit{CTNNB1} can inhibit cell proliferation and stimulate apoptosis of H295 R through decreasing Wnt/\(\beta\)-catenin-LEF/TCF dependent transcription, but the relationship between Wnt/\(\beta\)-catenin signaling and Ang II-induced aldosterone secretion is unclear. In the present study, the secretion of aldosterone and the expression of rate-limiting enzyme gene \textit{CYP11B1} and \textit{CYP11B2} were increased by Ang II, and the silencing \textit{CTNNB1} inhibited aldosterone secretion and the expression of \textit{CYP11B2} but not \textit{CYP11B1} in H295 R cells. Furthermore, the expression of \textit{CYP11B2} and aldosterone secretion of H295 R cells in response to Ang II were both decreased by \textit{CTNNB1} knockdown, but \textit{CTNNB1} knockdown had no effect on expression of \textit{CYP11B1}. These results suggested that silencing \textit{CTNNB1} genes reduced aldosterone secretion and responsiveness to Ang II of H295 R cells by inhibiting the expression of \textit{CYP11B2}.

In addition, the relationship between \textit{CTNNB1} knockdown and H295 R cell proliferation in response to Ang II was also
investigated. Our results showed that CTNNB1 knockdown decreased expression of β-catenin and inhibited proliferation of H295 R cells. Moreover, the downstream target genes of Wnt/β-catenin signaling pathway, AXIN2 and LEF1, were downregulated by CTNNB1 knockdown in H295 R cells, which were consistent with Gaujoux et al.14 However, there was no difference in the Wnt/β-catenin signaling pathway of H295 R cells after Ang II treatment. These results indicate that the activation of Wnt/β-catenin signal pathway promoted APA tumorigenesis, which was independent of Ang II.
Uncontrolled cell proliferation is the most important hallmark of cancer cells. Abnormal progression of cell cycle is the leading reason for dysregulation of cell proliferation. Cell cycle is regulated by a coordination of several cyclins and cell cycle inhibitors.26 Different cyclins are involved in different cell cycle stages. Cyclin D1 is a key protein that drives G1/S transition of the cell cycle.27 In our study, expression of cyclin D1 was investigated in H295 R cells. The protein and mRNA levels of cyclin D1 were significantly reduced by CTNNB1 knockdown. These results suggest that CTNNB1 knockdown downregulates cyclin D1 expression to inhibit H295 R cell proliferation.

In conclusion, our findings suggest that CTNNB1 knockdown can inhibit H295 R cell proliferation and decrease aldosterone secretion in the responses of H295 R cells to Ang II through inhibiting Wnt/β-catenin signaling pathway, indicating that targeting Wnt/β-catenin signaling pathway may be an important approach to decrease aldosterone secretion in the treatment of aldosterone-producing adrenomas.

Authors' Contributions
P.L. and T.Z conducted the study design; P.L. and L.W carried out experiments, data analysis and wrote the manuscript; S.Y, J.L, S.Q and Z.W provided the technical or material support. All authors read and approved the final manuscript.

Tingting Zhou, MM Pengwei Luo, MM are authors contributed equally to this work.

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

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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