

## Original Article

# Long non-coding RNA plays a role in aldosterone-producing adenoma through PCP4

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**Abstract:** The molecular mechanisms of primary aldosteronism are unknown. Long non-coding RNA (lncRNA) may play a key role. The objective was to investigate the following: 1) the gene expression in aldosterone-producing adenoma (APA) and normal adrenal cortical (NAC) tissues; and 2) the role of lncRNA in aldosterone synthesis. We analyzed the mRNA and lncRNA profiles of 6 APA and 6 NAC tissues using microarray, and we selected Purkinje cell protein 4 (PCP4) mRNA to be further studied because it had greater gene expression in APA tissues. To find target lncRNAs, we performed Coding-non-coding Gene Co-expression (CNC) network analysis and real-time PCR of expanded samples (APA: 33; NAC: 19). Altered lncRNA and mRNA expression profiles were gained from the microarray. PCP4 was significantly differentially expressed in mRNA profiles (5.11-fold,  $P < 0.05$ ). CNC network analysis showed that 83 lncRNAs were related to PCP4 (Pearson's correlation coefficient (PCC)  $\geq 0.85$ ), and 5 lncRNAs were differentially expressed ( $>1.5$ -fold vs control,  $P < 0.05$ ). Differential expression of 5 out of these 83 lncRNAs was verified by real-time PCR ( $P < 0.05$ ). They were NR\_003023, uc003zpr.2, ENST00000417262, ENST00000503380 and ENST00000512207. PCP4 was over-expressed in APA and was associated with a higher expression of lncRNAs. Thus, the higher expression of PCP4 and lncRNAs could perhaps explain high aldosterone synthesis in human primary aldosteronism. These lncRNAs might be among the important factors controlling aldosterone synthesis.

**Keywords:** Aldosterone-producing adenoma, gene expression, long non-coding RNA, Purkinje cell protein 4, aldosterone synthesis

## Introduction

Primary aldosteronism (PA) is the most common form of secondary hypertension, which affects approximately 8% to 10% of patients with hypertension and as many as 20% of patients with persistent hypertension [1, 2]. Due to autonomous aldosterone production from the adrenal cortex, patients with PA typically present with hypertension, elevated plasma aldosterone levels associated with low plasma renin activity, and varying degrees of hypokalemia and metabolic alkalosis. Because of excess autonomous aldosterone secretion, the risk of serious cardiovascular events and renal or other target organ damage is even higher in PA than in essential hypertension [3, 4]. PA is known to include several subtypes, among which unilateral aldosterone-producing adenoma (APA) accounts for 30% to 60% of all PA patients [5-7].

The mechanism of APA remains unknown. Several studies in the literature have attempted to determine the molecular characteristics of adrenal adenomas using transcriptome analysis to identify the expressed genes that participate in the pathogenesis of the adenoma. Recently, Lifton RP et al identified a mutation of the selectivity filter of the potassium (K<sup>+</sup>) channel (KCNJ5) in some APA patients [8]. Subsequently, mutations in the genes encoding an L-type calcium channel (CACNA1D) and in genes encoding sodium-potassium adenosine triphosphatase (ATP1A1) or calcium adenosine triphosphatase (ATP2B3) were found in other aldosterone-producing adenomas. These findings have provided a working model in which adenoma formation and/or aldosterone production in many cases derives from increased calcium entry [9]. This mechanism might explain a subset of APA patients but not all of them. Some scientists have attempted to find clues

from the studies of the genetic polymorphisms of APA [10, 11]. However, the pathogenesis of PA and nodulation of the adrenal cortex remain poorly understood.

lncRNAs are defined as non-coding RNAs over 200 nucleotides in length. They have been validated to have comprehensive functions in biological processes through various mechanisms [12, 13]. Increasing evidence has indicated that lncRNAs exert significant roles in both normal development and diseases [14, 15]. In particular, the regulatory roles of lncRNAs in the expression, activity and localization of protein-coding genes have attracted greater attention. It has been increasingly recognized that lncRNAs have emerged as an important component in the regulation of gene expression [14, 16].

In this study, we first used microarray to obtain the mRNA and lncRNA profiles of 6 APA tissues and 6 normal adrenal cortical (NAC) tissues. Second, we chose Purkinje cell protein 4 (PCP4) as a study target based on its mRNA profile, which had greater levels of gene expression. Third, we conducted Coding-non-coding Gene Co-expression (CNC) network analysis of PCP4 to find the lncRNAs related to it. Then, we screened the results in differentially expressed lncRNA profiles to search for target lncRNAs. Finally, we performed real-time PCR on expanded samples (APA tissues: 33; NAC tissues: 19) to determine whether these lncRNAs played roles in the mechanism of APA.

### Materials and methods

#### *Subjects*

The study protocol was approved by the Ethics Committee on Human Studies at Zhongshan Hospital, Fudan University, in Shanghai. From Jan. 2011 to May 2014, we collected 39 APA tissues and 25 NAC tissues. The APA pathology specimens used in this study were obtained from patients who underwent adrenalectomy. Ipsilateral normal adrenal glands from 19 cases undergoing nephrectomy for renal tumors served as the control group. Before the removal of adrenal glands, all of the APA patients in this study were diagnosed with APA on the basis of endocrinal tests, computed tomography (CT) scans of adrenal glands and/or adrenal vein sampling (AVS). All of the adenoma cases were histopathologically diagnosed as adrenocortical adenoma.

#### *RNA extraction and RNA quality control*

The tissue samples were homogenized in TRIzol reagent (Invitrogen, CA, USA). The total RNA was isolated according to the manufacturer's protocol (Invitrogen). RNA quantity and quality were measured by NanoDrop ND-1000, and RNA integrity was assessed using standard denaturing agarose gel electrophoresis.

#### *Microarray and computational analysis*

RNA purified from total RNA after removal of rRNA (mRNA-ONLY™ Eukaryotic mRNA Isolation Kit, Epicentre) was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias, utilizing a random priming method. Then, the labeled cRNAs were hybridized onto the Human lncRNA Array, version 2.0 (8 × 60K, Arraystar). The array contains 33,045 lncRNAs and 30,215 coding transcripts. The lncRNAs were carefully collected from the most authoritative databases, such as RefSeq, UCSC Knowngenes, and Ensembl, and from the related literature. The Arraystar lncRNA Array Protocol was as follows. Step 1 was to prepare the RNA sample, kit and reagents: TRIzolH Reagent (Invitrogen Life Technologies), Biopulverizer (Biospec), and Mini-Bead-Beater-16 (Biospec). Step 2 was total RNA Clean-up and RNA QC. Step 3 was to prepare the labeling reaction. Step 4 was to purify the labeled/amplified RNA and labeled cRNA QC. Step 5 was hybridization. Step 6 was the microarray wash. Step 7 was scanning. Step 8 was the extraction of data using Agilent Feature Extraction software. The arrays were scanned by the Agilent Scanner G2505B, and the acquired array images were analyzed by Agilent Feature Extraction software (version 10.7.3.1). Quantile normalization and subsequent data processing were performed using the GeneSpring GX software package (Agilent Technologies), version 11.5.1. The microarray work was performed by KangChen Bio-tech, Shanghai, P. R. China.

#### *Construction of the coding-non-coding gene co-expression network*

The network construction procedures include preprocessing the data, screening the data, calculating the Pearson's correlation coefficient (PCC), using the R value to calculate the PCC between lncRNA coding genes, screening by PCC and drawing the CNC network using

## lncRNA plays a role in APA through PCP4

**Table 1.** List of primers

LncRNA primer	Sequence	Product length (bp)	Application
GAPDH total F	GGGAAACTGTGGCGTGAT	299	qPCR
GAPDH total R	GAGTGGGTGTCGCTGTTGA		
NR_003023 total F	CCTGGGTCCTGGGTGTTGT	215	qPCR
NR_003023 total R	CTTTTCATTTCCGGGTCGGC		
ENST00000417715 total F	GCTGAAGCACCTGATGTTTGG	188	qPCR
ENST00000417715 total R	GCTTATGGGCATTTTGGGAA		
uc003zpr.2 total F	AAGTCGGTACCACATCGTCC	165	qPCR
uc003zpr.2 total R	TCTGCTTCTATGCTTGCTCCA		
ENST00000417262 total F	TCTCTGCCTTGCTCGTGC	217	qPCR
ENST00000417262 total R	CAAGCGTTTGTGGGTTTCA		
CR605514 total F	AGGTGAAGCATCAACATGGAAC	115	qPCR
CR605514 total R	GACAATGAAAAAGCAGGAACGA		
ENST00000503380 total F	CCCAGCAGTCCATCCACAAT	162	qPCR
ENST00000503380 total R	GCGAACAGGAACAGGAAAAACA		
ENST00000512207 total F	TTTGGGGCCAGTTCTTGTG	104	qPCR
ENST00000512207 total R	TGGTGGTGAGGATGTCGGT		

Cytoscape. Thus, the CNC network showed the lncRNAs related to mRNA.

### *RT-PCR and statistical methods*

Total RNA was first isolated using TRIzol reagent (Invitrogen Life Technologies) and then was reverse transcribed using the SuperScript™ III Reverse Transcriptase Kit (Invitrogen), according to the manufacturer's recommendations. Target lncRNAs were measured by real-time PCR using an Applied Biosystems ViiA 7 Real-time PCR System. The expression values of individual lncRNAs were normalized to GAPDH, and they are shown relative to control samples as indicated. The primers used in this study are presented in **Table 1**. The lncRNA expression differences between the APA and control groups were analyzed using Student's t test with SPSS software (version 20.0; SPSS Inc.). A value of  $P < 0.05$  was regarded as statistically significant.

## Results

### *Quality control of microarray data*

The box plot is a convenient method for visualizing the distribution of a dataset in lncRNA and mRNA profiles. After normalization, the distributions of log<sub>2</sub>-ratios among the tested samples were nearly the same. The scatter plot was used for assessing the lncRNA and mRNA expression variations between the APA and NA

arrays. Hierarchical clustering is one of the most widely used clustering methods for analyzing lncRNA and mRNA expression data. Cluster analysis arranges samples into groups based on their expression levels, allowing us to hypothesize about the relationships among samples.

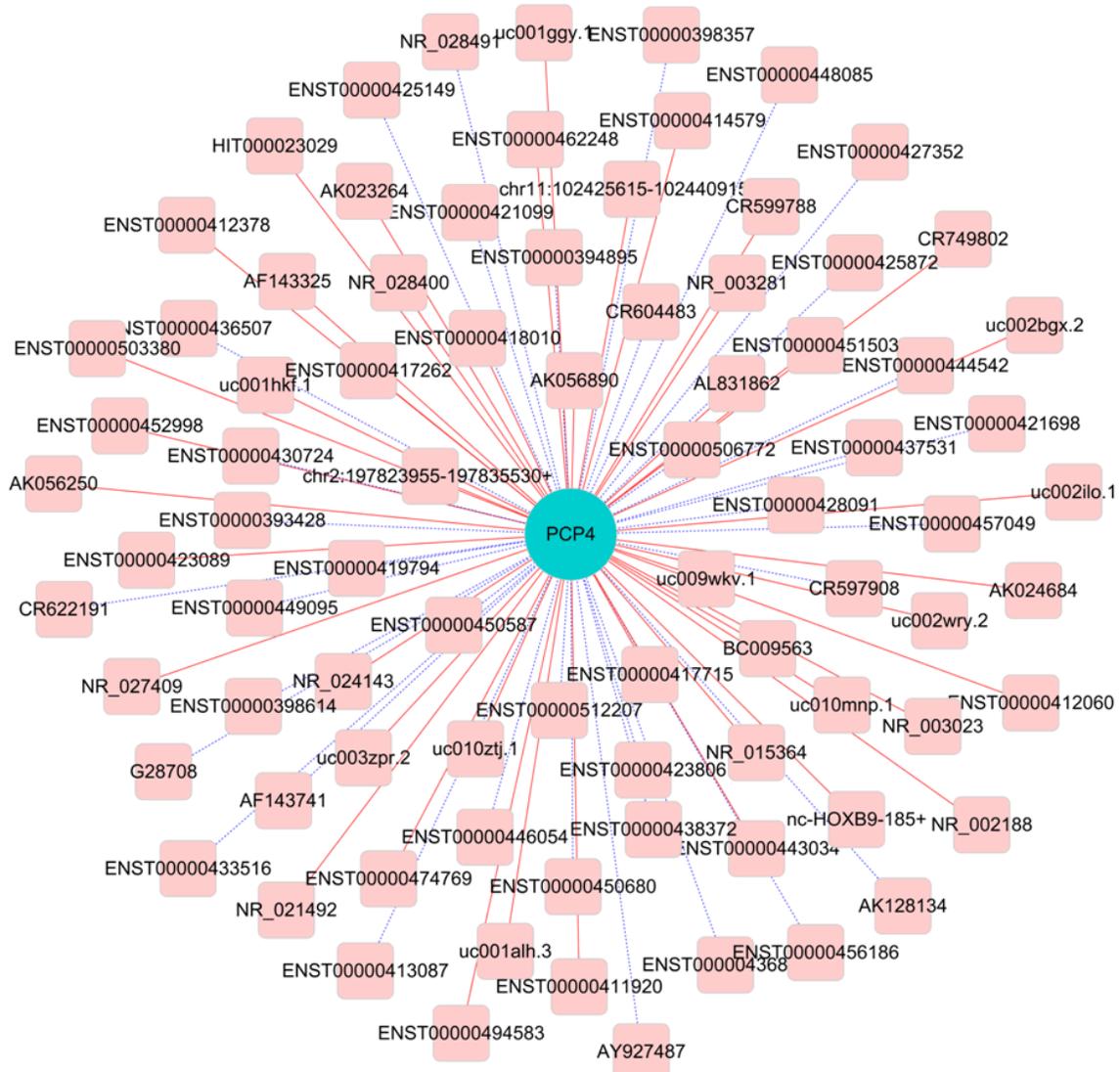
### *Altered lncRNA expression profiles*

We compared the lncRNAs expression levels between the 6 APA tissues and NAC tissues. To identify differentially expressed lncRNAs with statistical significance, we performed volcano plot filtering between the two groups (fold change  $\geq 1.5$ ,  $P < 0.05$ ). In total, we detected 1809 lncRNAs differentially expressed between the APA and normal control groups, among which 1181 lncRNAs were up-regulated, and 628 were down-regulated. The majority of differentially expressed lncRNAs in the APA group were from intergenic regions (~60 anti-sense to protein-coding loci ~20%), with the others representing overlapping transcripts from exons or introns in both sense and anti-sense directions.

### *Altered mRNA expression profiles*

Up to 18,789 coding transcripts could be detected from twelve samples using 30,215 coding transcript probes. Compared with normal adrenal tissues, 881 mRNAs were up-regulated in APA, while 1103 mRNAs were down-

## lncRNA plays a role in APA through PCP4



**Figure 1.** The CNC network of mRNA PCP4. Blue nodes represent coding RNA (mRNA); pink nodes represent non-coding RNA (lncRNA). Red solid lines indicate positive correlations; blue dashed lines indicate negative correlations.

**Table 2.** lncRNAs both related to mRNA PCP4 and differentially expressed in microarray

mRNA	lncRNA	PCC	Correlation
PCP4	NR_003023	0.897755	+
PCP4	ENST00000417715	0.866329	+
PCP4	uc003zpr.2	0.852045	+
PCP4	ENST00000417262	0.862751	+
PCP4	ENST00000503380	0.871301	+
PCP4	ENST00000512207	-0.87945	-

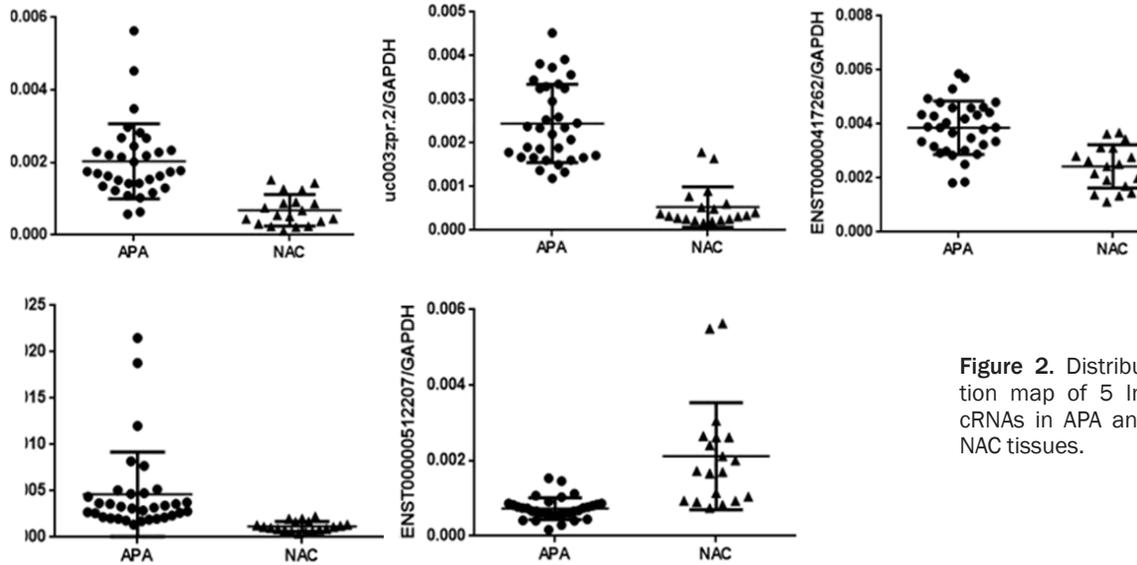
regulated. KCNJ5 was up-regulated in APA by 2.44-fold, while ATP1A1, ATP2B3, and CACNAID were not differentially expressed. GO and pathway analysis showed that the differentially

expressed mRNAs might involve the p53 signaling pathway, the MAPK signaling pathway and some other oxidation- and peroxidation-associated signaling pathways. They might also involve the metabolism of some important substances, for instance, steroid biosynthesis, bile secretion and so on. These results could provide us with greater insight into the pathogenesis and important functions of APA.

### Construction of the coding-non-coding gene co-expression network

Increasing evidence has indicated that lncRNAs play important roles in gene expression. Thus, we explored the correlations between the

## lncRNA plays a role in APA through PCP4



**Figure 2.** Distribution map of 5 lncRNAs in APA and NAC tissues.

expression of lncRNAs and protein coding genes. We hoped to find some lncRNAs able to explain the mechanism of APA. We chose mRNA PCP4, which was differentially expressed between the APA and NAC tissue by 5.11-fold. After the CNC network analysis, we obtained 83 lncRNAs related to PCP4 mRNA (Figure 1). These related lncRNAs were screened in “differentially expressed lncRNAs” in the microarray, which we had already previously obtained. Finally, we found 6 lncRNAs that were both related to PCP4 mRNA and differentially expressed in the microarray. They were NR\_003023, ENST00000417715, uc003zpr.2, ENST00000417262, ENST00000503380 and ENST00000512207.

### Real-time PCR verification

Real-time PCR was conducted on these 6 target lncRNAs in expanded samples (APA tissues: 33; NAC tissues: 19) with double tubes to verify whether there were still significant differences between the experiment group and the control group, and the data from two experiments were not statistically significant. Finally, 5 of the 6 target lncRNAs showed that the differences between the APA and NAC tissues were significant and consistent with the results of CNC network analysis and microarray analysis ( $P < 0.05$ ). They were NR\_003023, uc003zpr.2, ENST00000417262, ENST00000503380 and ENST00000512207. The real-time PCR results of lncRNA ENST00000512207 were consistent with the results of CNC network analysis and

microarray analysis but without a significant difference (Table 2). lncRNA uc003zpr.2 was the most specific lncRNA when our study graphed a distribution map with APA and NAC tissues (Figure 2). In contrast, the significance of mRNA between APA and NAC was also verified using real-time PCR in the aforementioned tissues with double tubes. The results of real-time PCR showed that PCP4 mRNA was significantly differentially expressed between APA and NAC ( $P = 0.01$ ).

### Discussion

PA is the most common form of endocrine hypertension, and APA comprises a frequent subtype of this disease. The removal of adenomas from patients with APA normalizes the excessive aldosterone secretion that is characteristic of this condition and as a consequence, cures or markedly improves the hypertensive status. It would be very helpful if we could diagnose APA rapidly and accurately. Thus, it is very instructive to gain deep insight into the pathogenic mechanisms of APA.

Increasing evidence has confirmed lncRNAs to be among the most important factors controlling gene expression [15]. Our study detected 5 lncRNAs (NR\_003023, uc003zpr.2, ENST00000417262, ENST00000503380 and ENST00000512207) related to PCP4 using microarray, CNC network and real-time PCR, and they might well involve the regulation of aldosterone synthesis and secretion. Further-

more, these 5 lncRNAs have not been reported before. We hope that these lncRNAs provide novel paths for better understanding of the molecular basis of APA.

PCP4 is an acronym of Purkinje cell protein 4, which is a 7.6 kDa protein with an IQ motif that binds to calmodulin [17]. PCP4 expression was recently reported to be up-regulated in APA, either compared with APA-adjacent adrenal glands or compared with NAC tissues [18]. Basset et al discovered that PCP4 was regulated by Ang-II as part of the Ca<sup>2+</sup>/CaM pathway and was involved in the regulation of CYP11B2 and of aldosterone production in human adrenocortical cells. A significantly positive correlation was detected in APA between PCP4 and CYP11B2 [19, 20]. Transient transfection with a PCP4 DNA vector produced a significant increase in CYP11B2 expression in PCP4-transfected H295R when Ang-II was added to the cell media [21-23]. In contrast, when PCP4 was knocked down by siRNA, the CYP11B2 mRNA demonstrated less expression following incubation with Ang-II [21]. Therefore, we hypothesized that the four lncRNAs might be involved in the pathogenesis of APA through regulation of the expression of PCP4. However, the precise function of these lncRNAs requires more direct experimental approaches.

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### Disclosure of conflict of interest

None.

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## lncRNA plays a role in APA through PCP4

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