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 (IncRNA) 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 IncRNA in aldosterone synthesis. We analyzed the mRNA and IncRNA 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 IncRNAs, we performed Coding-non-coding Gene Co-expression (CNC) network analysis and real-time PCR of expanded samples (APA: 33; NAC: 19). Altered IncRNA 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) ≥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+) 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...
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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.

IncRNAs 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 IncRNAs exert significant roles in both normal development and diseases [14, 15]. In particular, the regulatory roles of IncRNAs in the expression, activity and localization of protein-coding genes have attracted greater attention. It has been increasingly recognized that IncRNAs 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 endocrinial 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 IncRNA coding gens, screening by PCC and drawing the CNC network using
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Table 1. List of primers

<table>
<thead>
<tr>
<th>LncRNA primer</th>
<th>Sequence</th>
<th>Product length (bp)</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH total F</td>
<td>GGGAAACTGTGGCGGTAT</td>
<td>299</td>
<td>qPCR</td>
</tr>
<tr>
<td>GAPDH total R</td>
<td>GAGTGGGTGTCCCTGTTGAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR_003023 total F</td>
<td>CCGTGGCTGTGGGGTTGTTG</td>
<td>215</td>
<td>qPCR</td>
</tr>
<tr>
<td>NR_003023 total R</td>
<td>CTTTCTTCCGGGTGGTCGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENST00000417715 total F</td>
<td>GCTGAAACACTGATGTTTGG</td>
<td>188</td>
<td>qPCR</td>
</tr>
<tr>
<td>ENST00000417715 total R</td>
<td>GCTTATGGGATTTTGGGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>uc003zpr.2 total F</td>
<td>AAGTGGGTGACACACTGTCCC</td>
<td>165</td>
<td>qPCR</td>
</tr>
<tr>
<td>uc003zpr.2 total R</td>
<td>TCTGCTCTATGCTTCATCCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENST00000417262 total F</td>
<td>TCTCTGCTTGGTCGTGCC</td>
<td>217</td>
<td>qPCR</td>
</tr>
<tr>
<td>ENST00000417262 total R</td>
<td>CAAGCTTTGTTGAGTTTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR605514 total F</td>
<td>AGGTGAAGCATCAACATGGAAC</td>
<td>115</td>
<td>qPCR</td>
</tr>
<tr>
<td>CR605514 total R</td>
<td>GACAATGAAAGACGAGGACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENST00000503380 total F</td>
<td>CCCAGCAGTCATCCACATAA</td>
<td>162</td>
<td>qPCR</td>
</tr>
<tr>
<td>ENST00000503380 total R</td>
<td>GCGAACAGGAAAGGAAACCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENST00000512207 total F</td>
<td>TGTGGGGCCAGAGGTTTGGT</td>
<td>104</td>
<td>qPCR</td>
</tr>
<tr>
<td>ENST00000512207 total R</td>
<td>TGGTGTTGGAGATTGGTGCTGGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cytoscape. Thus, the CNC network showed the IncRNAs 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 SuperScriptTM III Reverse Transcriptase Kit (Invitrogen), according to the manufacturer's recommendations. Target IncRNAs were measured by real-time PCR using an Applied Biosystems ViiA 7 Real-time PCR System. The expression values of individual IncRNAs 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 IncRNA 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 IncRNA and mRNA profiles. After normalization, the distributions of log2-ratios among the tested samples were nearly the same. The scatter plot was used for assessing the IncRNA and mRNA expression variations between the APA and NA arrays. Hierarchical clustering is one of the most widely used clustering methods for analyzing IncRNA 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 IncRNAs expression levels between the 6 APA tissues and NAC tissues. To identify differentially expressed IncRNAs 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 IncRNAs differentially expressed between the APA and normal control groups, among which 1181 IncRNAs were up-regulated, and 628 were down-regulated. The majority of differentially expressed IncRNAs in the APA group were from intergenic regions (~60 antisense to protein-coding loci ~20%), with the others representing overlapping transcripts from exons or introns in both sense and antisense 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, and 628 were down-regulated in APA, while 1103 mRNAs were down-
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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 peroxidization-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

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

<table>
<thead>
<tr>
<th>mRNA</th>
<th>IncRNA</th>
<th>PCC</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCP4</td>
<td>NR_003023</td>
<td>0.897755</td>
<td>+</td>
</tr>
<tr>
<td>PCP4</td>
<td>ENST00000417715</td>
<td>0.866329</td>
<td>+</td>
</tr>
<tr>
<td>PCP4</td>
<td>uc003zpr.2</td>
<td>0.852045</td>
<td>+</td>
</tr>
<tr>
<td>PCP4</td>
<td>ENST00000417262</td>
<td>0.862751</td>
<td>+</td>
</tr>
<tr>
<td>PCP4</td>
<td>ENST00000503380</td>
<td>0.871301</td>
<td>+</td>
</tr>
<tr>
<td>PCP4</td>
<td>ENST00000512207</td>
<td>-0.87945</td>
<td>-</td>
</tr>
</tbody>
</table>

Increasing evidence has indicated that lncRNAs play important roles in gene expression. Thus, we explored the correlations between the
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Expression of IncRNAs and protein coding genes. We hoped to find some IncRNAs 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 IncRNAs related to PCP4 mRNA (Figure 1). These related IncRNAs were screened in "differentially expressed IncRNAs" in the microarray, which we had already previously obtained. Finally, we found 6 IncRNAs 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 IncRNAs 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 IncRNAs showed that the differences between the APA and NAC tissues were significant and 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 IncRNA 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 adeno-mas 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 IncRNAs to be among the most important factors controlling gene expression [15]. Our study detected 5 IncRNAs (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.
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 Ca²⁺/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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