Original Article

Gene Expression Profile of Persistent Postoperative Hypertension Patients with Aldosterone-producing Adenomas

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Abstract

Background: Hypertension often persists after adrenalectomy for primary aldosteronism (PA). Many studies have analyzed the outcomes of adrenalectomy for aldosterone-producing adenomas (APA) to identify predictive factors for persistent hypertension. However, differentially expressed genes in persistent postoperative hypertension remain unknown. Our aim was to describe gene expression profile of persistent postoperative hypertension patients with APA.

Methods: In this study, we described and compared gene expression profiles in persistent postoperative hypertension and postoperative normotension in Chinese patients with APA using microarray analysis. Confirmation was performed with quantitative real time-polymerase chain reaction analysis. Bioinformatic analysis (gene ontology analysis, pathway analysis and network analysis) was used for further research.

Results: Microarray analysis identified a total of 99 differentially expressed genes, including 18 up-regulated and 81 down-regulated genes. Among the dysregulated genes were fat atypical cadherin 1 as well as fatty acid binding protein 4 and other genes that have not been previously studied in persistent postoperative hypertension with APA. Bioinformatics analysis indicated that differentially expressed genes were associated with lipid metabolic process, metal ion binding, and cell differentiation. Pathway analysis determined that five pathways corresponded to the dysregulated transcripts. The mRNAs-ncRNAs co-expression network was composed of 49 network nodes and 72 connections between 18 coding genes and 31 noncoding genes.

Conclusions: This study revealed differentially expressed genes in persistent postoperative hypertension with APA and provided a resource of candidate genes for exploration of possible drug targets and prognostic markers.

Key words: Aldosterone-producing Adenomas; Hypertension; Microarray; Pathway

INTRODUCTION

Primary aldosteronism (PA), the most common form of secondary hypertension, is defined by autonomous aldosterone over-production with an estimated prevalence of 6%–12% in hypertensive patients and up to 20% in patients with resistant hypertension.[1] Abnormally high production of aldosterone can lead to suppression of plasma renin, sodium retention, hypertension, cardiovascular damage, and potassium excretion.[2] PA is mainly caused by idiopathic hyperaldosteronism (IHA) and aldosterone-producing adenomas (APA), both of which account for 95% of all reported cases.[3,4] Traditionally, APA is treated by surgery and IHA is treated with mineralocorticoid-antagonists.[5] Nonetheless, several patients with APA experience persistent hypertension after adrenalectomy.[6] Previous studies show

that the average long-term recovery rates of hypertension after unilateral adrenalectomy for APA range from 30% to 72%.[7,8] Some researchers have analyzed the outcomes of adrenalectomy for APA to identify predictive factors for hypertension resolution. The most important factors are the age at the time of operation, preoperative use of ≤2 antihypertensive agents, and the duration of the hypertension.[9-11] Previous studies have shown that chromosomal or genetic alterations may be associated with persistent hypertension in APA. Lenzini used whole transcriptome analysis to show that patients with low expression of aldosterone synthase (CYP11B2) in APA were less likely to be cured than those with a high expression.[12] Our previous research showed that DNA polymorphisms at the CYP11B2 locus may result in susceptibility of patients with APA to postoperative persistent hypertension.[13,14] However, few studies have been specifically designed to identify gene expression profiles that can predict the

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management of hypertension after adrenalectomy for APA to date.

In the present paper, we demonstrate that some genes are significantly differentially expressed in postoperative hypertensives than postoperative normotensives with APA. This finding suggests that the altered expression levels of genes may contribute to the occurrence of postoperative persistent hypertension with APA and may provides a resource of candidate genes for exploration of possible drug targets and prognostic markers.

METHODS

Patient profiles

We retrospectively selected clinical data from 94 patients with APA who had undergone complete unilateral laparoscopic adrenalectomy at the General Hospital of Chinese People's Liberation Army from June 2009 to July 2014. All medications were withdrawn 2 weeks (for spironolactone, at least 6 weeks) before evaluation. Potassium chloride was given to patients to maintain serum potassium levels of >3.0 mmol/L. Diagnosis of PA was carried out as previously described.[1] For PA screening, a cut-off upright ratio of plasma aldosterone concentration to plasma renin activity (aldosterone-to-renin ratio [ARR]) of over 30 in the presence of >15 ng/dl aldosterone and suppressed PRA were used. When ARR was >30, patients were subjected to saline infusion, captopril test, furosemide stimulation test, and/or orthostasis test for confirmation. In these patients, computed tomography scanning, magnetic resonance imaging of the adrenal glands, or an adrenal venous sampling was performed to differentiate between APA and IHA. All patients were subjected to histological examination to confirm adrenocortical adenoma.

The blood pressure (BP) levels of 94 patients were measured over a mean follow-up period at 2 years (range: 0.17–5) to determine whether or not values returned to normal (measured at least three times by an electronic sphygmomanometer at different times throughout the day). Postoperative BP <140/90 mmHg without any antihypertensive agent was considered as postoperative resolution of hypertension (noted as "normotension"); opposite findings were considered "hypertension." Patient demographics and clinical characteristics are summarized in Table 1.

RNA extraction and quality control

Tissue specimens were obtained during surgery under sterile conditions, immediately frozen in liquid nitrogen, and then stored at –80°C until extraction. Twenty-one tissue samples (9 hypertensive and 12 normotensive) were used for microarray. To isolate total-RNA, frozen tissues were minced with a homogenizer (IKA, Germany) and resuspended in Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA obtained by Trizol extraction was purified by processing with mirVana™ RNA isolation kit according to the manufacturer's instructions. Quantification and quality check were performed using Nanodrop and Agilent 2100 Bioanalyzer (Agilent

Table 1: Demographics and clinical characteristics of APA patients before adrenalectomy

Characteristics	Hypertension (n = 43)	Normotension (n = 51)	P
Age, year	48.19 ± 8.90	45.2 ± 12.10	0.186
Female/male, n	21/22	27/24	0.69
BMI, kg/m²†	25.83 ± 3.11	23.89 ± 2.84	0.002
Duration of hypertension, months*	127.88 ± 90.53	93.33 ± 75.14	0.048
Family history of hypertension, %	65.2	34.8	0.864
Treatment with ≥2 antihypertensive agent, %†	79.1	41.2	0.000
Systolic BP, mmHg	179.5	170	0.131
Diastolic BP, mmHg	100	100	0.377
Plasma aldosterone, ng/dl	731.84 ± 200.97	779.87 ± 272.17	0.369
PRA, ng·ml⁻¹·h⁻¹	0.3	0.20	0.142
Aldosterone/PRA ratio	75.2	142.2	0.316
Urinary potassium excretion, mmol/24 h	20.79	25.54	0.622
Urinary aldosterone excretion, mmol/24 h	23.80	30.50	0.378
Triglycerides, mmol/L	1.31	1.115	0.050
Total cholesterol, mmol/L	4.46 ± 0.98	4.23 ± 0.89	0.246
LDL cholesterol, mmol/L*	2.82 ± 0.81	2.48 ± 0.73	0.038

Data are shown as mean ± SD, percentages, or medians, and compared by *t*-test or by Mann–Whitney *U*-test. *$P < 0.05$, †$P < 0.01$. BP: Blood pressure; SD: Standard deviation; BMI: Body mass index; LDL: Low-density lipoprotein.

Technologies) instruments, respectively. RNA quantity was measured by a NanoDrop ND-2000.

Microarray analysis

Microarray analysis was performed by a commercial company (Oebiotech, PRC), using GeneChip® Human Transcriptome Array 2.0 (Agilent Technologies) designed to contain approximately 245349 array protein coding transcripts and 40914 array nonprotein coding transcripts derived from authoritative databases, including RefSeq, Ensemble, UCSC, Vertebrate Genome Annotation (Vega) database, www.noncode.org, lncRNA db, Mammalian Gene Collection (MGC) (version 10), and the Broad Institute.

Sample labeling, microarray hybridization, and washing were performed according to the manufacturer's standard protocol. Briefly, samples were used to synthesize cDNA. Labeled cRNA was then synthesized and hybridized to the microarray. After washing and staining, arrays were scanned by a Affymetrix Scanner 3000 (Affymetrix). Affymetrix GeneChip Command Console (version 4.0, Affymetrix) software was used to extract raw data. Expression Console (version 1.3.1, Affymetrix) software offered RMA normalization for gene analysis.

Microarray results analysis and construction of the co-expression network

After quantile normalization, raw signals from the microarray were log2 transformed. Differential expression

of genes was defined by the absolute value of fold change (FC) >1.5 and $P < 0.05$ (Student's t-test). Differentially expressed mRNAs were submitted to the DAVID database (*http://david.abcc.ncifcrf.gov*) for classification into different Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes annotation groups. Then, we constructed a coding-noncoding gene co-expression network that included the differentially expressed mRNAs and ncRNAs. The noncoding and coding genes that had Pearson correlation coefficients equal to or greater than 0.80 were chosen to draw the network using Cytoscape.

Gene ontology and pathway analysis

Gene ontology and pathway analyses were used to determine the roles of the closest coding genes in biological GO terms or pathways. GO analysis was performed to determine gene and gene product enrichment in biological processes, cellular components, and molecular functions. Fisher's exact test was used to determine whether or not the overlap between the differentially expressed gene list and the GO annotation list was greater than that expected by chance ($P < 0.05$ was used).

Real-time quantitative polymerase chain reaction validation

Total RNA was extracted from APA tissue of 40 consecutive patients, 21 of which were used in microarray. Integrity was evaluated using agarose gel electrophoresis stained with ethidium bromide.

Quantification was performed with a two-step reaction process including reverse transcription (RT) and polymerase chain reaction (PCR). Each RT reaction consisted of 0.5 μg RNA, 2 μl of Primer Script Buffer, 0.5 μl of oligo dT, 0.5 μl of random 6 mers, and 0.5 μl of Primer Script RT Enzyme Mix I (TaKaRa, Japan) in a total volume of 10 μl. Reactions were performed in a GeneAmp® PCR System 9700 (Applied Biosystems, USA) for 15 min at 37°C, followed by heat inactivation of RT for 5 s at 85°C. The 10 μl RT reaction mix was then diluted 10-fold in nuclease-free water and held at –20°C.

Real-time PCR was performed using a LightCycler® 480 II real-time PCR instrument (Roche, Switzerland) with 10 μl of the PCR reaction mixture including 1 μl of cDNA, 5 μl of 2 × LightCycler® 480 SYBR Green I Master (Roche), 0.2 μl of forward primer, 0.2 μl of reverse primer, and 3.6 μl of nuclease-free water. Reactions were incubated in a 384-well optical plate (Roche) at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Each sample was run in triplicate for analysis. At the end of the PCR cycles, melting curve analysis was performed to validate the specific generation of the expected PCR products. Primer sequences were designed in the laboratory and synthesized by Generay Biotech (PRC) based on the mRNA sequences obtained from the National Center for Biotechnology Information (NCBI) database and noncoding RNA sequences obtained from the NCBI database, as follows: 5'-AGTCTCACAGTGTCTTTAACTC-3' for ANGPTL1; 5'-TTAAATCATGGCCCACCTTAC-3' for matrix Gla

Protein (MGP); 5'-CTCTGCCGGACATACAAG-3' for FAT1; 5'-GTCAAGAGCACCATAACCT-3' for FABP4; 5'-CACATGGACTGAATCACATAGA-3' for COLEC12; 5'-TATAACAAGGCCACTGAAGATG-3' for CST2; 5'-TGTCTGCTGTGTGTTTGT-3' for OGN; 5'-AGCGGTAGTAACTTTGTGT-3' for n335562; 5'-TAGTGACCGTAATGCACCTT-3' for n337481. The expression levels of mRNA and ncRNA were normalized as required against meiotic nuclear division 5 homolog B (RMND5B) and calculated using the $2^{-\Delta\Delta Ct}$ method.[15]

Statistical analysis

Data are expressed as mean ± standard deviation, percentages, or medians. Univariate comparisons between groups were performed using t-test for variables with normal distribution or Mann–Whitney U-test when necessary. $P < 0.05$ was considered statistically significant. Statistical tests were performed using SPSS 17.0 (SPSS Inc., USA).

Results

Demographic characteristics

All patients were biochemically cured by the time of follow-up. Serum potassium normalized and plasma aldosterone concentrations were all in the normal range. A total of 94 follow-up patients with APA were grouped based on postoperative resolution of hypertension (normotension and hypertension). The clinical profiles of follow-up patients before adrenalectomy are summarized in Table 1. Compared with the normotension group, the hypertension group had higher preoperative BMIs ($P = 0.002$), higher low-density lipoprotein (LDL) cholesterol levels ($P = 0.038$), more antihypertensive medications for preoperative BP management ($P = 0.000$), and longer durations of hypertension ($P = 0.048$). According to multivariable Logistic regression analysis, preoperative use of two or more antihypertensive agents (odds ratio, 2.75; 95% confidence interval [CI], 1.25–6.09; $P = 0.012$) and high LDL level (odds ratio, 2.21; 95% CI, 1.01–4.83; $P = 0.048$) were independently associated with the persistence of hypertension.

General gene expression profiles

In the mRNA expression profiling data, we found 48 differentially expressed mRNA transcripts between hypertensive and normotensive samples after unilateral adrenalectomy for APA; 12 of these transcripts were up-regulated whereas 36 were down-regulated. Among the dysregulated mRNA transcripts, uc004edk. 1 (probe TC0X001181.hg.1) was the most up-regulated with an FC of 3.209, whereas fat atypical cadherin (FAT1) (TC04001824.hg.1) was the most down-regulated with an FC of 2.321.

Using the same criteria for mRNA, we found dysregulated 41 lncRNA transcripts; 4 of these transcripts were up-regulated whereas 37 were down-regulated. The most up-regulated and down-regulated lncRNA transcripts were n335785 (TC11002587.hg.1) and n341200 (TC07002818.hg.1) with FCs of 1.87 and 2.67, respectively.

We found 10 differentially expressed small noncoding RNAs transcripts; here, 2 transcripts were up-regulated whereas 8 were down-regulated. The most up-regulated transcript was RN5S348 (Probe TC11000967.hg.1), an rRNA with an FC of 1.76. The most down-regulated transcript was snRNA ENST00000459279 (TC03001561.hg.1) with an FC of 2.23. Tables 2 and 3 show the top 10 up-regulated and down-regulated mRNAs and ncRNAs in hypertensives compared with normotensives, respectively.

Differentially expressed mRNAs and ncRNAs were used to generate a heat map during unsupervised hierarchical clustering analysis and the RNAs clearly self-segregated into hypertension and normotension clusters, as shown in Figure 1. Subsequently, a co-expression network of differentially expressed ncRNAs-mRNAs genes was constructed. We used Pearson's correlation coefficients, equal to or greater than 0.80, to identify the ncRNAs and mRNAs and drew the network using Cytoscape. Our data showed that the co-expression network was composed of 49 network nodes and 72 connections between 18 coding genes and 31 noncoding genes [Figure 2].

Real-time quantitative polymerase chain reaction confirmation

We selected nine differentially expressed genes for qRT-PCR to identify expression levels in 40 APAs who had undergone adrenalectomy. These genes can be divided into two groups. The first group included genes that were randomly chosen, namely COLEC12, fatty acid binding protein 4 (FABP4), ANGPTL1, n337481, and OGN. In the second group, n335562 and MGP were chosen because they may be a paired ncRNA-mRNA [Discussion, Figure 2]. FAT1 was chosen because it was the most down-regulated mRNA [Table 2].

Results demonstrated that FABP4, CST2, and COLEC12 were over-regulated and that FAT1 and MGP were under-regulated in hypertensive compared with normotensive by qRT-PCR. Notable differences in expression were also observed ($P < 0.05$). Whereas, at variance, n335562, OGN, ANGPTL1 were under-regulated consistent with microarray but showed no differences. Unexpectedly, n337481 was over-expressed by qRT-PCR. Real time RT-PCR is apparently a more sensitive measure of actual differences between samples than microarray (Student's t-test, $P < 0.05$), as seen in Figure 3.

Table 2: Top 10 down-regulated and up-regulated mRNAs in persistent postoperative hypertensives for APA

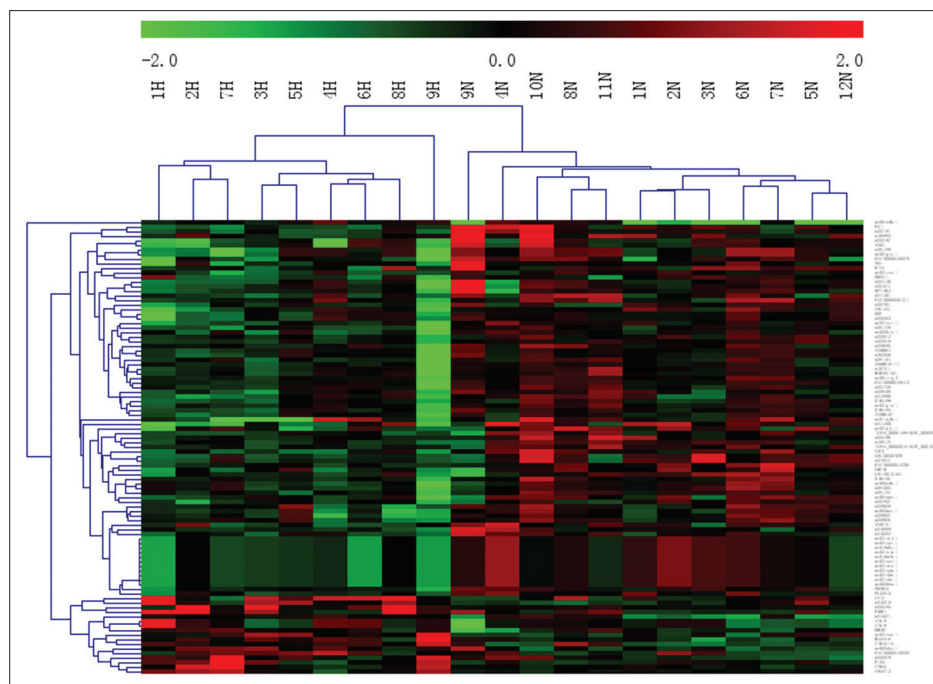| Down-regulated mRNA	FC	Up-regulated mRNA	FC
FAT1	2.321189	uc004edk. 1	3.209024
uc001pye. 1	2.277474	CST2	2.115898
uc011ajh. 1	2.093342	FABP4	1.78502
ANK2	2.006042	F13A1	1.703536
OGN	1.956325	STK19	1.649805
NAV3	1.939531	uc021sme. 1	1.608703
uc021sfj. 1	1.888356	uc003ekz. 1	1.547078
uc021sgr. 1	1.888356	ENST000004 40528	1.527321
uc010ubo. 1	1.888356	CYB5A	1.514503
uc021siu. 1	1.888356	DHCR7	1.50766

FC (abs): Absolute fold change; APA: Aldosterone-producing adenomas; FAT1: Fat atypical cadherin 1; FABP: Fatty acid binding protein 4.

Figure 1: Heat map of differentially expressed genes from hypertensive compared with normotensive. Each row represents one gene, and each column represents one tissue sample. The relative gene expression is depicted according to the color scale. Red indicates up-regulation, green indicates down-regulation. 2.0, 0, and − 2.0 reflect fold changes in the corresponding spectrum. 1–9 H represent hypertensive tissues 1–9, whereas 1–12 N represents normotensive tissues 1–12.

Gene ontology and pathway analysis

Using GO analysis, we found that the highest enriched GOs targeted by dysregulated transcripts were cell differentiation (ontology: Biological process), extracellular region (ontology: Cellular component), and metal ion binding (ontology: Molecular function), as shown in Figure 4. Pathway analysis determined that five pathways corresponded to the dysregulated transcripts [Figure 5] and that the most enriched network was the glycerophospholipid metabolism network, which is composed of two targeted genes.

The present study focused on APA. All of the patients in the study were diagnosed by a strict process, as described previously. All selected patients were hypertensive before adrenalectomy.

In this study, the rate of hypertension resolution after adrenalectomy was 54.5%, similar to previous studies where hypertension resolution was defined as a BP lower than 140/90 mm Hg with no use of antihypertensive agents at follow-up.[9] Univariate analysis indicated that
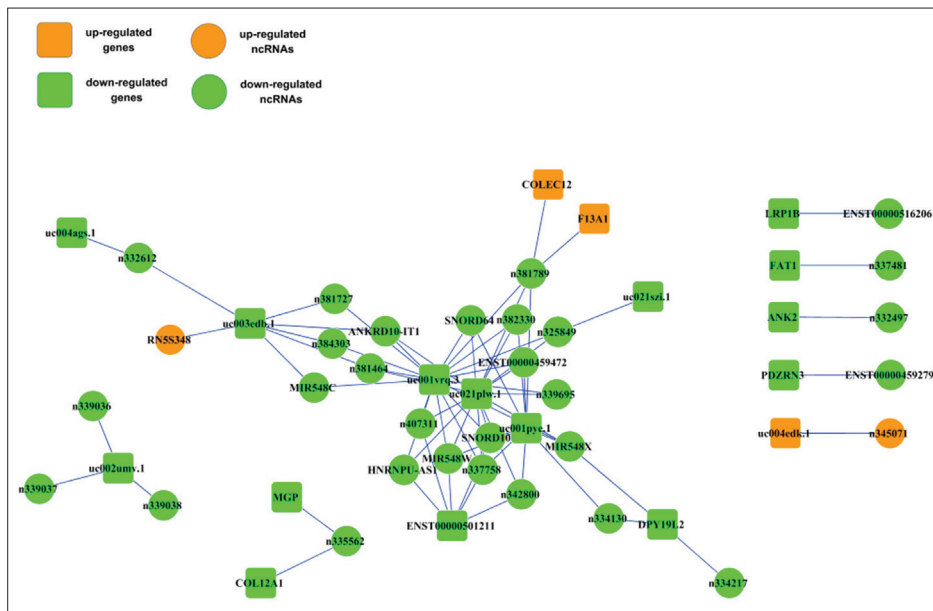

Figure 2: Differently expressed mRNA-ncRNA network consisting of 72 pairs of mRNA and ncRNA.
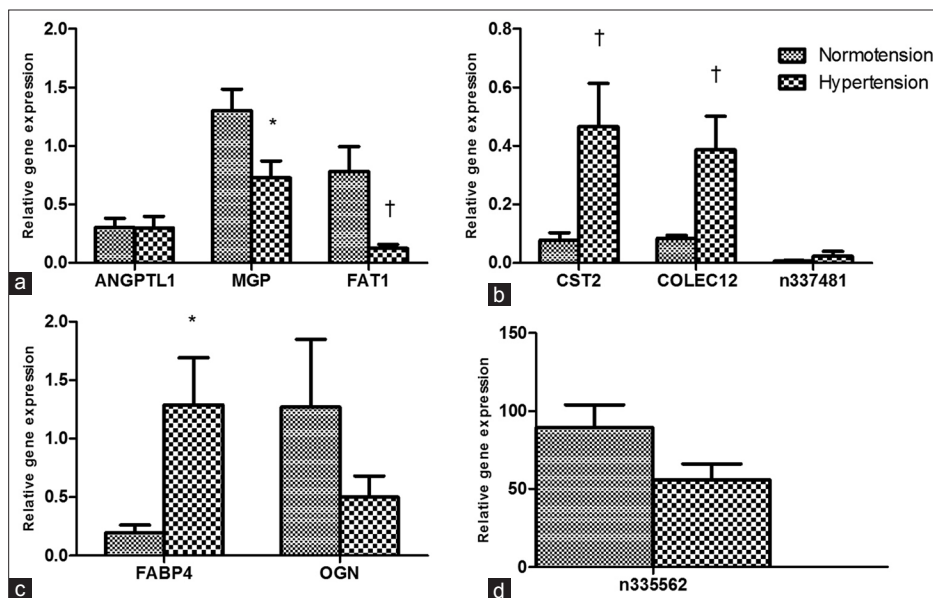

Figure 3: QRT-polymerase chain reaction validation of differentially expressed genes from hypertensive compared with normotensive. (a) ANGPTL1, matrix Gla Protein, and fat atypical cadherin 1 expression levels (b) CST2, COLEC12, and n337481 expression levels (c) fatty acid binding protein 4 and OGN expression levels (d) n335562 expression level. Values are normalized to RMND5B mRNA. Bars represent means ± standard error of mean and asterisks denote *$P < 0.05$, †$P < 0.01$, (Student's t-test).
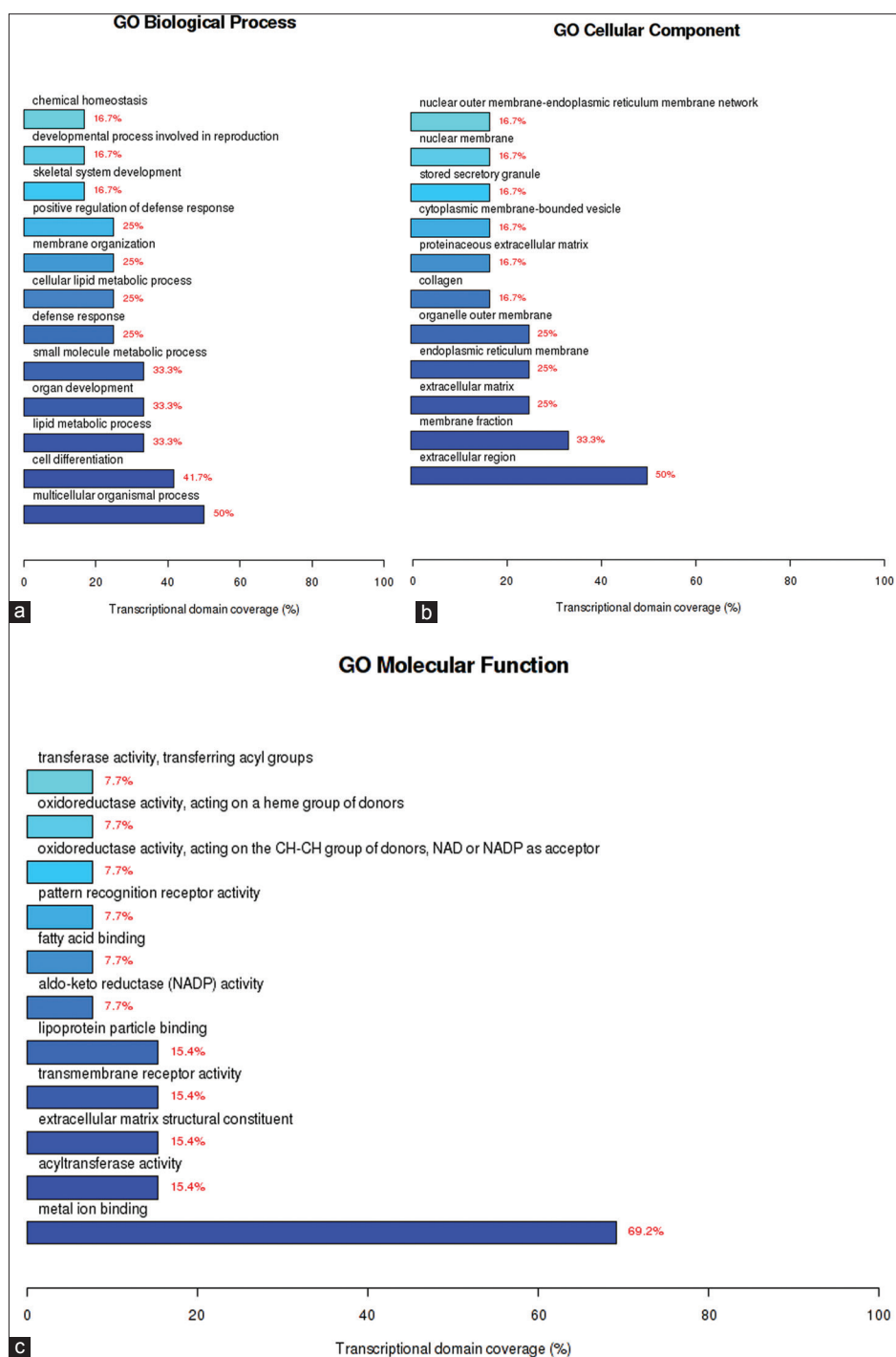
Figure 4: Gene ontology (GO) enrichment analysis of differently expressed mRNAs. (a) GO analysis of mRNAs according to biological process. (b) GO analysis of mRNAs according to cell component. (c) GO analysis of mRNAs according to molecular function.

BMI, durations of hypertension, LDL cholesterol level, and ≤2 antihypertensive agents were adequate predictors of surgical curability of APA. This result is consistent with a previous study.[16] Multivariate Logistic regression analysis demonstrated that the persistence of hypertension is related to preoperative use of two or more antihypertensive agents, as previously demonstrated in retrospective studies.[17,18] Moreover, we found that high LDL level is a highly important risk factor in postoperative persistence hypertension.

Genome-wide mRNA and ncRNA expression patterns in postoperative persistent hypertension patients with APA were assessed by microarray for the first time. Approximately 99 genes are differentially expressed in hypertensive tissues compared with normotensive ones. Nine of the differentially expressed genes were validated by qRT-PCR. Bioinformatics analysis indicated that some genes may be prognostic markers which involved in the pathogenesis of persistent postoperative hypertension of APA.

Table 3: Top 10 down-regulated and up-regulated ncRNAs in persistent postoperative hypertensives for APA

Down-regulated ncRNA	FC	Up-regulated ncRNA	FC
n341200	2.670407	n335785	1.868246
n381789	2.368042	n340210	1.848122
ENST00000459 279	2.23182	RN5S348	1.762958
n332497	2.151604	n336879	1.694456
n337481	1.946281	VTRNA1-3	1.608513
n408882	1.885581	n345071	1.511055
LOC100507039	1.819869		
n334130	1.803181		
n378347	1.773656		
n339037	1.755273		

FC (abs): Absolute fold change; PRA: Plasma renin activity; APA: Aldosterone-producing adenomas; FC: Fold change.
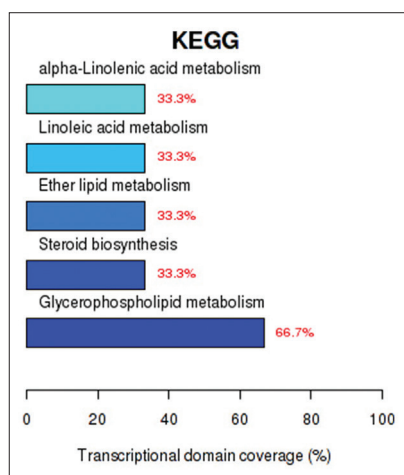
Figure 5: Pathway enrichment analysis.

The FAT1 gene is an intrinsic membrane protein classified as a member of the cadherin superfamily. Vascular smooth muscle cell (VSMC) proliferation has been suggested to be critical in the pathogenesis of atherosclerosis, hypertension, and restenosis. Hou demonstrated that FAT1 may control VSMC functions that are central to vascular remodeling by facilitating migration and limiting proliferation.[19] In the present microarray analysis, FAT1 was the most down-regulated mRNA. Decreases in FAT1 expression may mediate cell proliferation and vascular remodeling, which may be associated with postoperative persistent hypertension. Interestingly, FABP4 was identified as another potential predictor; this gene is mainly expressed in adipocytes and regenerated endothelial cells in arteries and an important component of insulin sensitivity regulation and atherosclerosis development.[20,21] A previous study demonstrated that FABP4 contributes to BP elevation and that FABP4 elevation is predisposed by a family history of hypertension;[22] our results are in accordance with this previous study. Circulating FABP4 may modulate endothelial functions and affect VSMC contraction, thereby modulating BP. The last gene that may be associated with persistent hypertension is MGP, which is an inhibitor of mineralization that has been implicated in vessel and cartilage calcification.[23] Recent studies show that MGP performs an inhibitory function in vascular calcification and may be a biomarker for cardiovascular calcification.[24,25] During a microarray analysis, MGP was linked to atherosclerosis and vascular calcification in senescent VSMC.[26] Our present study agrees with the previous study. Under-expression of MGP may be associated with vessel mineralization, which could be involved in the occurrence of postoperative persistent hypertension. Microarray analysis further revealed down-regulated lncRNA-n335562 located near MGP. lncRNAs are known to exert their functions through interactions with coding transcripts and proteins. LncRNAs may be critical in cardiovascular disease and can predict clinical outcomes of primary tumors.[27,28] Although n335562 was not significantly differentially expressed based on real-time PCR, the trend observed was in accordance with microarray results. Hence, our findings are preliminary and further work is necessary to investigate the relationship between n335562 and MGP. Our results must also be validated using a larger cohort group of samples.

To understand the functions of differentially expressed genes further, we applied GO and pathway analysis to study differentially expressed mRNAs and found that five pathways correspond to dysregulated transcripts. One of these pathways, the steroid biosynthesis signal pathway [Figure 6] was previously reported to be involved in the occurrence of hypertension in postoperative APA.[12] As for the four other pathways corresponding to differentially expressed transcripts, some or all may be involved in the persistence of hypertension after adrenalectomy for APA. Further research on this topic is recommended.
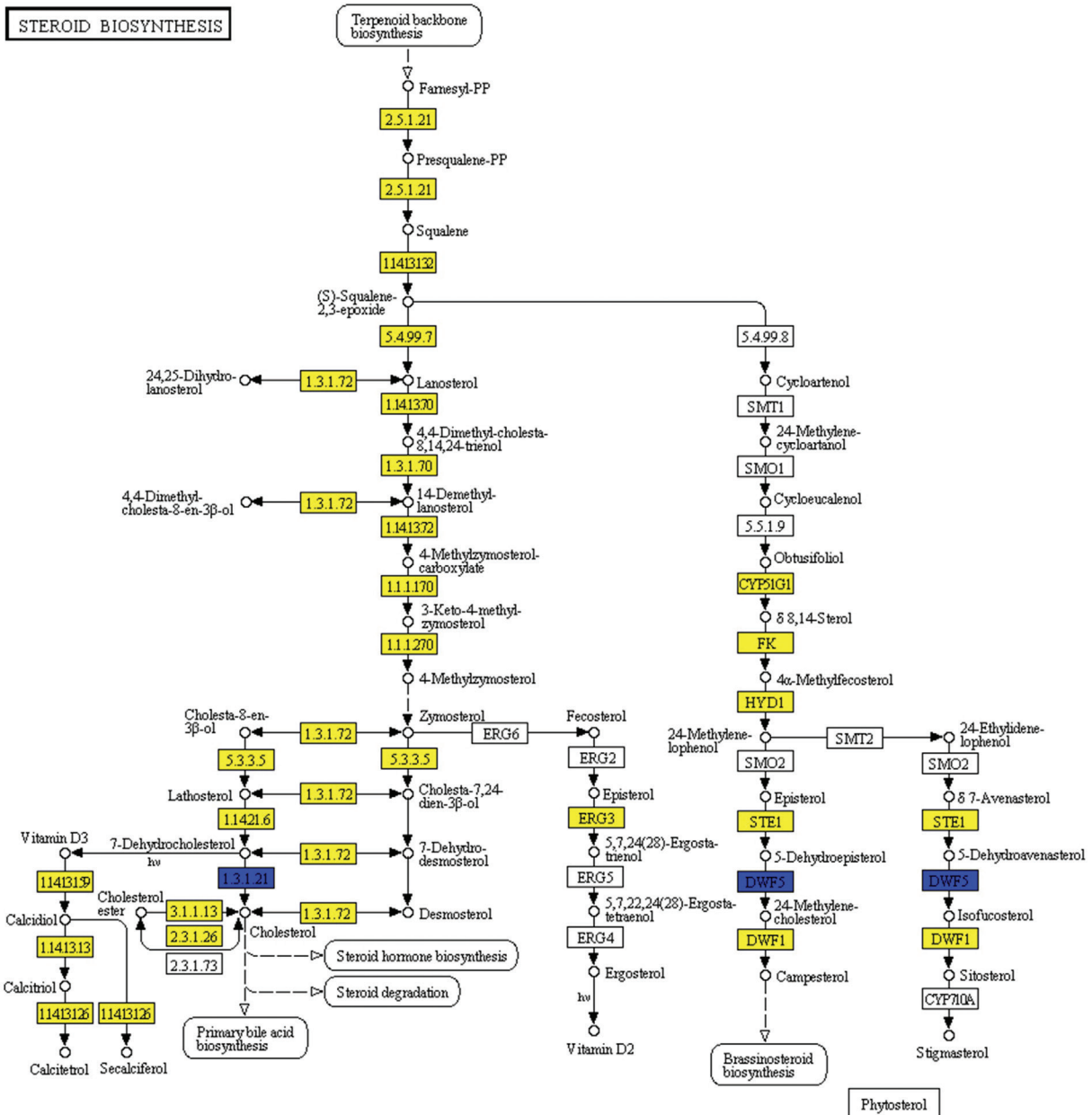
The main limitations of our study include the limited sample size and duration of follow-up during retrospective analysis; these limitations may present recall and referral bias. Thus, related studies with larger sample sizes are recommended.

In conclusion, differentially expressed genes between APA patients with postoperative persistent hypertension and normotension were reported for the first time. Our study revealed that nearly 99 genes are differentially expressed between hypertensive and controls. Some genes may be associated with biological pathways related to hypertension. Our study lays the foundation for future functional studies of genes as reliable prognostic biomarkers and provides a guide for new therapeutic strategies.

Figure 6: The "steroid biosynthesis" signal pathway shows is associated with an outcome of postoperative aldosterone-producing adenomas. Pathway analysis is a functional analysis that maps genes to KEGG pathways.

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