The Bioinformatics Analysis of Aldosterone-Producing Adenoma and Verification of Differentially Expressed Genes

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Abstract

Background

Previous studies have investigated the transcriptional modulations of aldosterone overproduction of aldosterone-producing adenomas (APAs), and several potential genes were found with high expressions.

Purpose

We aimed to systematically study the genes and pathways associated with molecular mechanism underlying APA by bioinformatics analysis and experimental validation for the expression profile.

Methods

This study was performed based on three gene expression profiles (GSE64957, GSE8514, and GSE60042). Differentially expressed gene (DEG) investigation, function and pathway enrichment, as well as protein-protein interaction (PPI) network, were performed by the bioinformatics analysis. For the validation with quantitative PCR, tissues from 11 patients with non-functioning adrenal adenoma (NFA) and 13 with APA were included in our cohort.

Results

In this study, the bioinformatics analysis was performed and 182 upregulated and 88 downregulated DEGs were identified. As expected, the upregulated DEGs were primarily involved in calcium ion homeostasis (GO: 0055074, n = 3, p = 2.00×10^{-4}). In the KEGG pathway analysis, calcium signaling pathway (hsa04020, n = 8, p= 4.38×10^{-6}) and the aldosterone synthesis and secretion (hsa04925, n = 6, p = 8.73×10^{-6}) were enriched. Moreover, quantitative PCR was performed to detect the expression of 7 upregulated genes (PCP4, ATP2A3, CYP11B2, CLCN5, HTR4, VDR and AQP2) among the intersection of DEGs. The mRNA levels of CYP11B2, HTR4 and AQP2 were significantly increased in APA samples compared to NFA (24.420 folds of NFA, p<0.001, 3.753 folds of NFA, p=0.002 and 11.487 folds of NFA, p=0.018).

Conclusion

In summary, the present study showed several candidate genes with high expression from bioinformatics analysis and our cohort. And the DEGs were enriched in aldosterone synthesis and secretion and calcium signaling pathway as expected.

Introduction

Primary aldosteronism (PA) is the most common form of endocrine hypertension with a prevalence of 5–20% in patients with hypertension and is characterized by the excessive production of aldosterone[1, 2]. PA

is mainly caused by either the aldosterone-producing adenoma (APA) or bilateral adrenal hyperplasia (BAH)[3].

Over the last decade, several studies investigated the gene expression profile of APAs compared to normal adrenals or adjacent adrenal cortexes with the aim of identifying transcriptional modulations of aldosterone overproduction[4, 5]. Genome-wide expression (microarray) and RNA-sequencing analysis (RNA-Seq) have become commonplace in the examination of gene expression of APA[6, 7]. Numerous genes, including the ones encoding steroidogenic enzymes like CYP11B2, CYP11B1, CYP21A1, CYP11A1, CYP17A and HSD3B2, few genes involved in calcium signaling or endoplasmic reticulum calcium storage like CALM2, CALR and CAMK-I, and several G-protein-coupled hormone receptors like receptors of GnRH, LH, vasopressin and serotonin, have been identified in previous studies as differentially expressed in APAs and the adrenal cortexes[5, 8].

In the present research, bioinformatics analysis and experimental validation for the expression profile of APAs compared with controls were studied (workflow diagram was in Fig. 1). First, the bioinformatics analysis was performed based on several gene expression profiles. Differentially expressed gene (DEG) investigation, function and pathway enrichment, as well as protein-protein interaction (PPI) network, were performed. We aimed to systematically investigate potential genes and pathways associated with the disease progression, which may aid in elucidating the molecular mechanism underlying APA. Additionally, several DEGs from databases were then verified in our cohort with 13 tissue samples from APA and 11 from non-functioning adrenal adenoma (NFA).

Materials And Methods

Data resource

Gene expression profile data (accession no. GSE64957, GSE8514, and GSE60042) were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). GSE64957 and GSE8514 dataset were produced on a GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array platform, and GSE60042 was produced on a GPL14550 Agilent-028004 SurePrint G3 Human GE 8x60K Microarray platform. A total of 47 tissue samples from APA patients and 39 normal tissue samples from their adjacent adrenal glands (AAG) were included in these datasets.

Data preprocessing and differential expression analysis

DEGs were obtained from GEO databases by a way of GEO2R analysis (http://www.ncbi.nlm.nih.gov/geo/geo2r/). The adj. P < 0.05 and |logFC| > 1.0 were set as DEGs cutoff criterion.

The intersection DEGs of these three datasets and any two of them were further considered more carefully, and all of the DEGs covered by these datasets were used for the enrichment analysis.

Gene ontology and pathway enrichment analysis of DEGs

The Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf,gov/) has facilitated the transition from data collection to biological analysis. The Geno Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were performed by KEGG Orthology Based Annotation System (KOBAS) online tool (http://kobas.cbi.pku.edu.cn/anno_iden.php). P < 0.01 was set as the cutoff criterion.

Integration of protein-protein interaction (PPI) network and modules selection

The Search Tool for the Retrieval of Interacting Genes (STRING, http://string.embl.de/) database was used to construct the PPI network for DEGs. The network view summarizes the network of predicted associations for a particular group of proteins. The network nodes are proteins, and edges represent the predicted functional associations. The cut off criterion of confidence score was set as > 0.7. Subsequently, the results were visualized using Cytoscape software. The sub-module of the PPI network was further identified using MCODE tool using the following parameters: degree cut off = 2, node score cut off = 0.2, k-core = 2, and max. depth = 100. The enrichment analysis of every module was further performed by KOBAS online.

Subjects and tissues in our cohort

Tissues from 11 patients with NFA and 13 with APA were recruited. The clinical and pathological diagnoses were made according to established criteria. Patients with NFA had normotension and no signs or symptoms of hormone excess, had normal serum potassium (K+) levels and displayed normal suppression of serum cortisol after low-dose dexamethasone treatment. We included only those patients where the discovery was incidental. All patients with APA had hypertension and hypokelamia, were diagnosed on the basis of an elevated plasma aldosterone concentration, suppressed plasma renin activity, and computerized axial tomography. All of the APA patients were detected with somatic KCNJ5 mutations by sequencing, and positive staining of CYP11B2 by immunohistochemistry (IHC). The tumor tissue samples were obtained from unilateral adrenalectomy, and snap-frozen using liquid nitrogen and stored at -80°C until use. The study received ethical approval from the ethics committee of Peking Union Medical College Hospital. Written informed consent was obtained from all the patients.

RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using the Qiagen RNeasy Mini Kit (74104, Qiagen, Hilden, Germany) according to the manufacturer's protocol. The quality and quantity of total RNA were determined using an ND-2000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). First-strand cDNA was synthesized from 1 ug of total RNA using PrimeScript RT (PK0446, TaKaRa, Kusatsu, Japan) and oligo (dT) primers. For the target genes including PCP4, ATP2A3, CYP11B2, CLCN5, HTR4, VDR and AQP2 identified by bioinformatics analysis above, a double-stranded DNA dye, SYBR-Green, was used with 10 ul of SYBR-Green PCR master mix (PK0445, TaKaRa, Kusatsu, Japan) and 100 nmol of each primer. PCR was

performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) with a total volume of 20 ul/reaction following the reaction parameters recommended by the manufacturer. All reactions were performed in triplicate. The primers used were in Supplement 1. RT- qPCR was conducted to analyze the mRNA expression of different genes in the tissues from different groups. Gene expression was analyzed by relative quantitation with the $2^{-\Delta\Delta Ct}$ method using GAPDH as an internal control. The results are expressed as the target/internal standard concentration ratio of each sample.

Statistical analysis

Normally and non-normally distributed continuous variables were presented as Mean ± SD and median (interquartile range), respectively. Independent t tests and non-parametric tests (the Mann-Whitney test) with IBM SPSS Statistics 22.0 software were used to assess differences between APA and NFA patients for the clinical characteristics. The fold change of qPCR data between APA and NFA samples were converted to logarithms, and then using the non-parametric tests. P value < 0.05 was considered to be significant.

Results

DEGs in APA samples compared with control AAG samples

As large amounts of data were included in the gene expression profiles, the original data were analyzed and filtered. A total of 182 upregulated and 88 downregulated DEGs were identified by GEO2R analysis. The intersection DEGs of the three datasets consisted of 6 genes, PCP4, ATP2A3, PPP4R4, CTNND2, CYP11B2 and CLRN1. More genes including IL17D, EDA2R, RAB3C, SCRN1, CLCN5, MTMR4, ABCB4, HTR4, QPCT, GBP2, NETO2, VDR, CBR1, ADAM23, FAM19A4 and AQP2 were covered in any two of these datasets. The Venn graph showed the intersection DEGs was in Fig. 2.

Functional enrichment analysis for DEGs

To further elucidate the roles of DEGs, GO functional and KEGG pathway enrichment analyses were performed. As expected, the upregulated DEGs were primarily involved in calcium ion homeostasis (GO: 0055074, n = 3, p = 2.00X10-4). And they also enriched in regulation of cardiac conduction (GO: 1903779), dendrite (GO: 0030425), synapse (GO: 0045202) and oligosaccharide metabolic process (GO: 0009311). In the KEGG pathway analysis, calcium signaling pathway (hsa04020, n = 8, p = 4.38X10-6, Supplement 2 (Ⓐ)) came first, and the aldosterone synthesis and secretion (hsa04925, n = 6, p = 8.73X10-6, Supplement 2 (Ⓑ)) was the second one. The detail information of upregulated DEGs enrichment was showed in Table 1. As for downregulated DEGs, the most common enrichments were protein binding (GO: 0005515), extracellular space (GO: 0005615), collagen-containing extracellular matrix (GO: 0062023), extracellular region (GO: 0005576) and extracellular exosome (GO: 0070062). And the downregulated DEGs were enriched in the KEGG pathways of cancer (hsa05200) and proteoglycans in cancer (hsa05205). The detail information of downregulated DEGs enrichment was showed in Table 2.

PPI network construction from DEGs

The DEGs were further analyzed using the STRING database to construct the PPI network, and the general PPI network with all of the 270 DEGs were showed in Fig. 3. Furthermore, the constructed PPI network was exported into Cytoscape software, and subjected to the sub-module PPI network construction using the MCODE tool. As shown in Supplement 3 ((□a) for Module 1, (□b) for Module 2 and (□c) for Module 3), three sub-modules with MCODE score greater than 4.0 were identified from the constructed PPI network. In the Module 3, the DEGs were primarily involved in positive regulation of cytosolic calcium ion concentration (GO: 0007204, n = 3, p = 1.45×10^{-6}), including PTGFR, CCKBR and TACR1 genes. In the KEGG pathway analysis of Module 3, calcium signaling pathway (hsa04020, n = 4, p = 7.60×10^{-8}) with PTGFR, CCKBR, HTR2B and TACR1 genes was enriched firstly. The detail information of Module 3 enrichment analysis was showed in Supplement 3 (□). Other modules (Module 1 and 2) were not enriched successfully due to the few nodes.

Clinical manifestations of study participants

Thirteen patients with diagnosed APA (ages: 27–69 years) and 11 patients with NFA (as control group) were recruited in this study, which used to confirm the mRNA expression of several DEGs by RT-qPCR. Clinical characteristics of these patients were summarized in Supplement 4. The systolic blood pressure (SBP), diastolic blood pressure (DBP), and aldosterone of APA group were all higher, and the plasma K+, PRA and tumor size were lower than that in NFA group. The results above showed that the clustering on clinical manifestations of these two groups was obvious, which suitable for the subsequent RT-qPCR analysis.

The mRNA expression of several DEGs by RT-qPCR

To confirm the results of bioinformatics analysis, RT-qPCR was performed to detect the mRNA expression of 7 upregulated genes (PCP4, ATP2A3, CYP11B2, CLCN5, HTR4, VDR and AQP2) among the intersection of DEGs, which related to aldosterone synthesis and secretion and calcium signaling regulation (the proteins encoded by the above seven genes and their biological functions were showed in Supplement 5). The mRNA levels of CYP11B2, a well-known upregulated gene, was also tested positively in our cohort (24.420 folds of NFA, p < 0.001). And also, the HTR4 and AQP2 were significantly increased in APA samples compared to NFA (3.753 folds of NFA, p = 0.002 and 11.487 folds of NFA, p = 0.018). The fold changes, p values of all the 7 genes and the box plots of 3 upregulated genes by RT-qPCR were showed in Table 3 and Fig. 4.

Discussion

In this study, the bioinformatics analysis was performed and 182 upregulated and 88 downregulated DEGs were identified. As expected, the upregulated DEGs were enriched in calcium signaling pathway and the aldosterone synthesis and secretion. The intersection DEGs of different GEO databases, which also related to the pathways above, included PCP4, ATP2A3, CYP11B2, CLCN5, HTR4, VDR and AQP2.

Furthermore, the mRNA levels of CYP11B2, HTR4 and AQP2 were significantly increased in 13 APA samples compared to 11 NFA samples from our cohort, which confirmed the high expression of these genes and the important role in the occurrence and development in APA.

It was the first study to explore the potential mechanism of APA through the method of bioinformatics analysis. With the comprehensive consideration of many researches about expression profiles in APA, the reliability of the final results was increased. By the functional enrichment analysis of DEGs, we further confirmed the relationship between APA and regulated pathways, including aldosterone synthesis and secretion, calcium signaling pathway, G protein-coupled receptor signaling pathway, cAMP signaling pathway, positive regulation of cytosolic calcium ion concentration, and regulation of cardiac conduction, which was consistent with the previous studies of APA transcriptome profiles[7, 9, 10]. Moreover, several upregulated pathways, like oligosaccharide metabolic process and response to muscle stretch from GO were enriched in APA samples, which were discovered for the first time.

There were 22 genes which highly expressed from at least two GEO databases about APA by our bioinformatics analysis. The most prominent of these, was CYP11B2 gene, which encodes aldosterone synthase and catalyzes the multi-step reaction of deoxycorticosterone to produce aldosterone. CYP11B2 is considered as a marker of aldosterone synthesis and widely believed with upregulated expression in APA. Besides, the other three DEGs, PCP4, HTR4 and VDR also have been reported to be involved in the occurrence and development of APA[11−13], and among them, the upregulated expression of HTR4 was verified by the qPCR in this study. HTR4 is a serotonin receptor subtype known to be expressed in adrenal gland, and serotonin acted on the receptor can increase aldosterone secretion in vivo and in vitro[14−16]. In addition, several other DEGs that might be related to calcium signaling pathway and aldosterone secretion, but so far, they have not been reported to participate in the progression of APAs, such as ATP2A3, CLCN5 and AQP2. And only the last one, AQP2, was verified in our cohort. The AQP2 is an ADH sensitive aquaporin. The recent studies said that, the binding of ADH and receptor can cause a transient increase of intracellular calcium[17, 18], which followed the increased aldosterone secretion. This phenomenon of upregulated expression of AQP2 in APAs was also found by Wang in 2011 without detail elucidation[8]. However, Niu et al found that, AQP2 was expressed in all the adrenal medullary tumors, but not in adrenal cortical tumors, which reflected the maintenance of water metabolism via AQP2 during tumorigenesis[19]. Therefore, whether the gene is related to the occurrence of APAs remains to be further studied.

Perhaps due to the limitation of samples and the specificity of selected tissues, the high expression of the other genes was not verified in our cohort and further researches are needed to confirm the gene expression from the related pathways. In this study, we made NFA tissues as the control group of APAs, which used more for discussing the secretion function and the related mRNA expression. Nevertheless, AAG tissues were used widely as the normal control in the previous studies and GEO datasets. Although reducing the difference of the intra group to a certain extent, it was not easy to obtain the AAG samples

from single zona glomerulosa of adrenal gland accurately. The cells in distinct zones might have different steroid hormone expression profiles.

Several limitations to our study should be acknowledged. First of all, the microarray data we included were generated from the NCBI website[15, 20, 21]. Besides the qPCR verification for mRNA expression of several significant genes, other experiments were needed to confirm their impacts on APA. Second, the three GEO databases all used AAG as control to explore the differences of transcription. In this study, we recruited the patients with NFA as control. It might be one of the reasons why the expression of some genes did not show upregulation as we found in the databases.

In summary, the present study showed candidate genes with high expression and part of them were verified in our cohort, which might predict the progress of APA. And all the DEGs were enriched in aldosterone synthesis and secretion and calcium signaling pathway. Nevertheless, future studies are warranted to determine the detailed molecular mechanisms underlying APA more than bioinformatics analysis.

Declarations

Funding

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of data and material

All the data and materials mentioned have been contained in the public database, and tables, figures and supplementary materials of this manuscript.

Code availability

Not applicable.

Authors' contributions

Conceived and designed the experiments: MN and AT; Collected clinical data: YZ, YC and AT; Performed the experiments: YG and YC; Analyzed Data: YG, XM and HW; Write the manuscript: YG and AT; Revise the manuscript: AT, MN and YZ. All authors have approved the final draft of the manuscript.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee (Ethics committee of Peking Union Medical College Hospital; S-K431) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication

All the authors and the patients included in this study have agreed the publication of this manuscript.

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Tables

Table 1. The detail information of upregulated DEGs enrichment in the functional enrichment analysis of APAs

ID	Analysis type	Process	Count	P-value	Genes
hsa04020	KEGG Pathway	Calcium signaling pathway	8	4.38×10^{-6}	CCKBR, PHKA1, HTR4, HTR2B, TACR1, ATP2A3, ATP2B2, ATP2B3
hsa04925	KEGG Pathway	Aldosterone synthesis and secretion	6	8.73×10^{-6}	MC2R, CYP11B2, ATP2B3, PDE2A, ATP2B2, ATP1B1
hsa04024	KEGG Pathway	cAMP signaling pathway	7	7.52×10^{-5}	CNGB3, MC2R, HTR4, ATP2B3, ATP2A3, ATP2B2, ATP1B1
GO: 1903779	Gene Ontology	regulation of cardiac conduction	4	1.37×10^{-4}	ATP1B1, ATP2A3, ATP2B3, ATP2B2
GO: 0030425	Gene Ontology	dendrite	9	1.71×10^{-4}	RELN, KCND3, HTR4, CYP46A1, SLC4A8, HTR2B, CTNND2
GO: 0045202	Gene Ontology	synapse	9	1.71×10^{-4}	GPC4, CHRNA5, CYFIP2, PMP22, HTR4, GJC1, HTR2B, CADM1, EEF1A2
GO: 0009311	Gene Ontology	oligosaccharide metabolic process	3	1.77×10^{-4}	ST3GAL6, NAGA, ST8SIA5
GO: 0055074	Gene Ontology	calcium ion homeostasis	3	2.00×10^{-4}	SGCD, WFS1, SNX10

DEG: differentially expressed gene; APA: aldosterone-producing adenoma

Table 2. The detail information of downregulated DEGs enrichment in the functional enrichment analysis of APAs

ID	Analysis type	Process	Count	P-value	Genes
hsa05200	KEGG Pathway	Pathways in cancer	9	3.49×10^{-6}	MMP2, PRKCA, GSTA2, EPAS1, PTGER3, FOS, JUN, GSTA5, HGF
hsa05205	KEGG Pathway	Proteoglycans in cancer	6	7.90×10^{-6}	LUM, MMP2, DCN, PRKCA, GPC3, HGF
hsa05166	KEGG Pathway	Human T-cell leukemia virus 1 infection	6	1.20×10^{-5}	HLA-DMA, ZFP36, FOS, JUN, ETS2, EGR1
GO: 0005515	Gene Ontology	protein binding	62	7.04×10^{-15}	ZNHIT2, USP2, NAALAD2…
GO: 0005615	Gene Ontology	extracellular space	23	5.70×10^{-13}	MMP2, SEMA3C, HBB…
GO: 0062023	Gene Ontology	collagen-containing extracellular matrix	13	3.59×10^{-12}	NID1, BGN, DCN…
GO: 0005576	Gene Ontology	extracellular region	21	6.49×10^{-10}	LUM, BGN, DCN…
GO: 0070062	Gene Ontology	extracellular exosome	21	5.66×10^{-9}	LUM, BGN, PRKCA…

DEG: differentially expressed gene; APA: aldosterone-producing adenoma

Table 3. The mRNA expression in samples of APA compared to ones of NFA

	gene name	fold change	P value
1	PCP4	1.593	0.331
2	ATP2A3	1.587	0.252
3	CYP11B2	24.420	<0.001
4	CLCN5	0.326	0.228
5	HTR4	3.753	0.002
6	VDR	1.754	0.424
7	AQP2	11.487	0.018

Figures
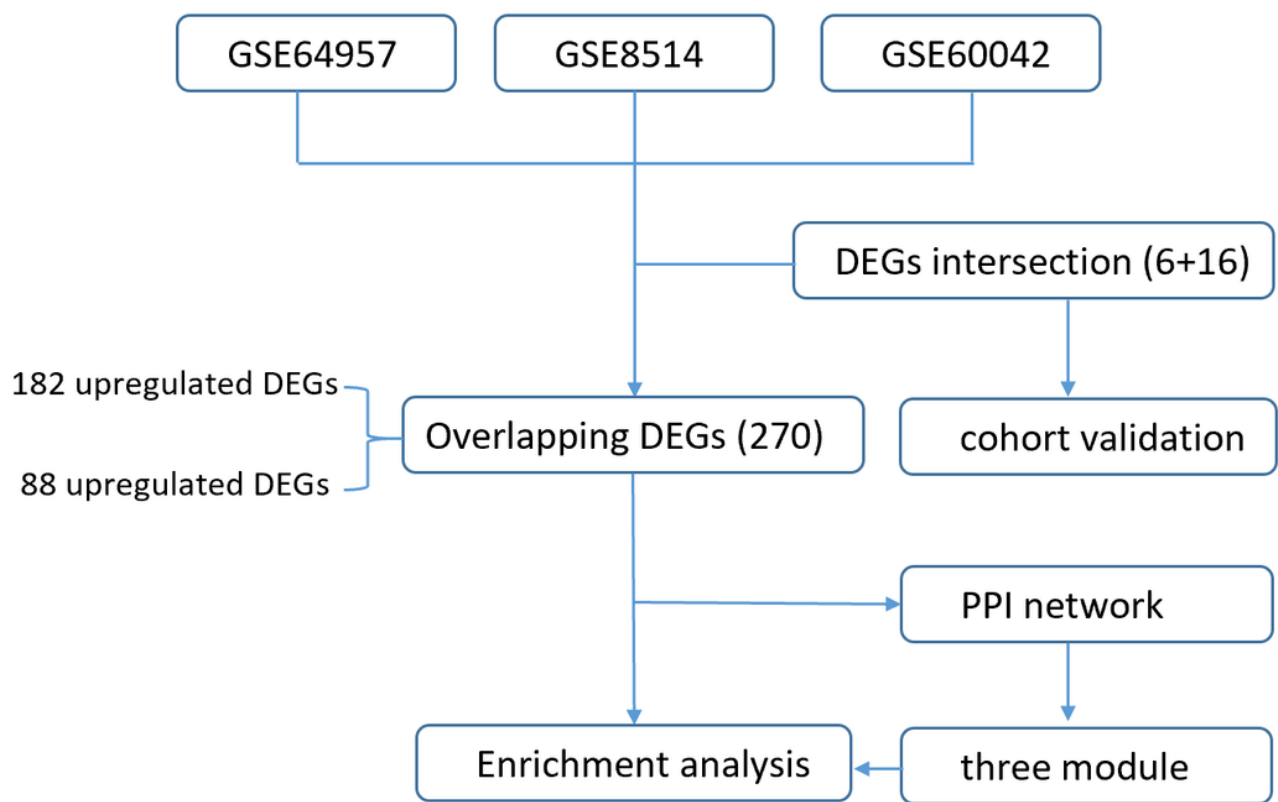
Figure 1

Workflow diagram
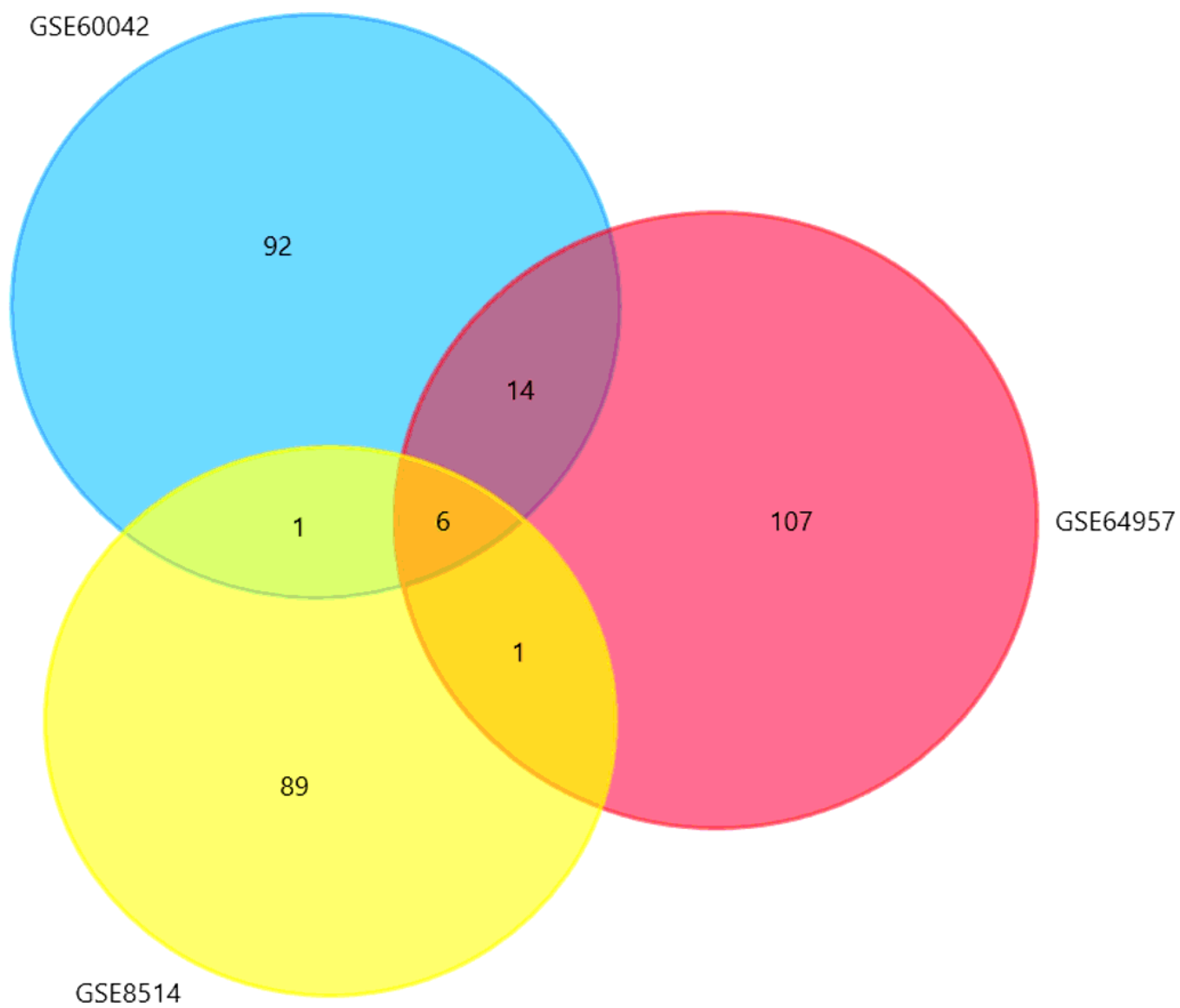
Figure 2

The Venn graph of three GEO databases

Figure 3

The protein-protein interaction network with all of the 270 differentially expressed genes The proteins were represented by the nodes, and the predicted functional associations were represented by the edges.
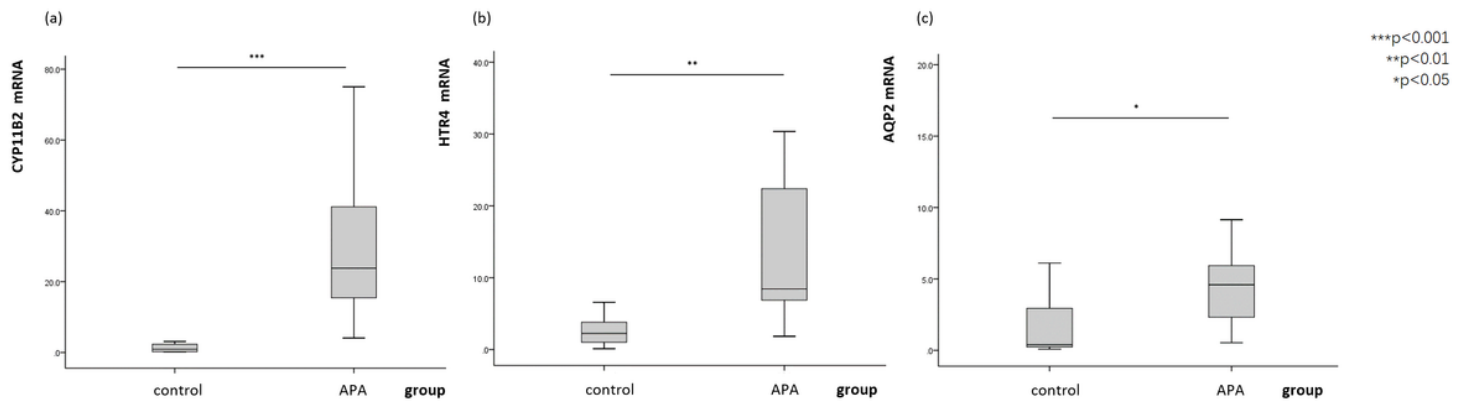
Figure 4

The mRNA expression levels of 3 upregulated genes CYP11B2, HTR4 and AQP2 between APAs and NFAs Y-axis represents test group (APA) and control group (NFA); X-axis represents the relative expression (fold change) of each mRNA. "***" means P<0.001, "**" means P<0.01, and "*" means P<0.05. The lines from top to bottom show the upper margin, the upper quartile Q3, the median, the lower quartile Q1, and the lower margin, separately. (A) The mRNA expression of CYP11B2 (B) The mRNA expression of HTR4 (C) The mRNA expression of AQP2

Supplementary Files

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