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Organ-differential responses to ethanol and kynurenic acid, a component of alcoholic beverages in gene transcription

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Abstract

Excessive alcohol (ethanol) use has long been known to affect human health negatively. However, the underlying molecular basis is incompletely understood. Moreover, consumption of alcohol is often mixed with kynurenic acid (KYNA), an abundant tryptophan metabolite produced during fermentation. The combined effect of ethanol and KYNA on host gene expression has not been investigated. The current study used mice as the model to interrogate the impact of ethanol and/or KYNA on global gene transcription. Adult male mice were administered with 2g/kg ethanol and/or 0.1mg/kg KYNA by gavage once a day for a week. Three organs: brain, kidney, and liver were collected and their total RNAs extracted for transcriptome sequencing and quantitative real-time PCR. Gene ontology, Kyoto encyclopedia of genes, and genomes pathway analyses revealed that alcohol affects the three organs differentially. Furthermore, the gene expression profile from alcohol and KYNA co-administration was significantly different from that of alcohol or KYNA administration alone. Strikingly, Indolamine 2,3-dioxygenase 1, a rate-limiting enzyme in tryptophan metabolism, was significantly increased in the brain after a combined exposure of alcohol and KYNA, suggesting that Trp metabolism was skewed towards the kynurenine pathway in the brain. Our systemic analysis provides new insights into the mechanism whereby alcohol and KYNA affects organ functions.

Keywords: alcohol; mouse; transcriptome analysis; tryptophan; kynurenine; kynurenic acid

1. Introduction

Alcohol has long been used and abused in human history. It is intensively investigated that excessive alcohol intake impairs the abuser's immune system and central nervous system (CNS) (Dolganiuc et al., 2003; Montesinos et al., 2016). Both acute and chronic consumptions of alcohol damage complement and coagulation activation, and antigen processing and presentation, which undermine the host defense capacity (Molina et al., 2010). Thus, alcohol abuse leads to immunodeficiency and autoimmunity, which are associated with an increased likelihood of infectious diseases (Cook, 1998; Romeo et al., 2007). Chronic alcohol ingestion has been shown to suppress the host immune reaction during sepsis (Margoles et al., 2016). In addition to the impact on host immunity, excessive alcohol use affects the nervous system (Sullivan and Pfefferbaum, 2014), often leading to aggressive and assaultive behaviors. Alcohol exposure during brain development dysregulates the redox status in adult brains (Brocardo et al., 2016).

Kynurenic acid (KYNA) is a metabolite of tryptophan formed enzymatically along kynurenine pathway in bacteria, fungi, plants and animals. During alcohol fermentation, abundant KYNA is produced (Yılmaz and Gökmen, 2018), which is naturally mixed in all alcoholic beverages. Moreover, KYNA has also been detected in tea, coffee and refreshments (Turska et al., 2018; Yılmaz and Gökmen, 2018; Turska et al., 2019). Therefore, co-exposure of alcohol and KYNA is unavoidable. Tryptophan (Trp) and its metabolites are known to play crucial roles in the immune system and CNS (Munn et al., 1999; Vécsei et al., 2013). Importantly, levels of Trp and its metabolites are heavily influenced by both acute and chronic alcohol intakes

(Neupane et al., 2015). Previous human studies have demonstrated that plasma Trp availability for uptake into the brain is decreased after acute exposure to ethanol and whisky (Badawy et al., 1995; Markus et al., 2004). As a result, serotonin synthesis and turnover reduces in the brain, whereas the concentration of plasma kynurenine is dramatically elevated. (Badawy et al., 2009). Not only are the concentrations of Trp and its metabolites are altered by acute alcohol exposure, the enzymes of the kynurenine pathway are affected as well. Indolamine 2,3-dioxygenase 1 (IDO1) and Trp 2, 3-dioxygenase (TDO) are rate-limiting enzymes, and the initial catalysts in the kynurenine pathway. IDO and TDO catalyze the conversion of Trp to N-formylkynurenine (Rafice et al., 2009). It was found that acute alcohol exposure activates TDO, but chronic alcohol exposure inhibits TDO activity (Badawy, 2002). Trp intake decreases alcohol consumption by animals, and it has always been speculated that serotonin formed from Trp mediates the reduction (Sellers et al., 1992). There are two metabolic pathways of kynurenine: one is that kynurenine, 3-hydroxykynurenine (3-HK) and 3-hydroxyanthranilic (3-HAA) are catalyzed by kynurenine 3-monooxygenase, kynureninase and 3-hydroxyanthranilate 3,4-dioxygenase to form 3-HK, 3-HAA and quinolinic acid, respectively; the other is that kynurenine is catalyzed by kynurenine aminotransferases to form KYNA (Han et al., 2010). Two pathways have very different physiological roles. For example, quinolinic acid is an agonist of N-methyl-D-aspartate receptor and free radical (Vécsei et al., 2013), while KYNA is an antagonist. (Lee et al., 2016).

Even though alcohol influence of global gene expression is widely investigated,

the combined effect of alcohol and KYNA has not been studied. In the current study, we have profiled gene transcriptomes of mouse brain, liver and kidney which had been exposed to alcohol and/or KYNA. Independent and synergistic effects of the two substances on these organs are compared. Several pathways, especially tryptophan metabolism, were differentially altered. The data provide new insight into alcohol toxicity at the transcriptional level.

2. Materials and methods

2.1. Animals, and ethanol and/or KYNA administration

The care and use of mice in this study were approved by the Hainan University Institutional Animal Care and Use Committee. Adult male mice of Kunming (KM) strain (Tianqin Biological Technology Co. Ltd, China), weighing between 25 and 27g were acclimatized in our animal facility for one week before experiments (Hogan et al., 2010). A total of 49 mice were randomly divided into four groups: 1) alcohol alone, 2) KYNA alone, 3) alcohol and KYNA in combination, and 4) non-treatment control. Detailed grouping and treatments were shown in table 1. Based on the highest concentration of KYNA found in alcohol beverages (38 μg/100mL in Mead) (Turska et al., 2019), we decided to use 100 μg/kg KYNA . We also selected the alcohol dose to be 2 g/kg. Alcohol and/or KYNA were orally administered to mice daily for a week, and the non-treatment controls were given vehicle of the same amount of sterile deionized water. For alcohol solution, absolute ethanol (99.7%, reagent AR, Macklin Company) was diluted to 20%v/v solution using sterile deionized water before use.

KYNA (Selleck Company) was in sterile deionized water and DMSO (the final concentration <5%, Biofroxx).

2.2. Tissue collection and RNA extraction

Mice were quickly euthanized by cervical dislocation. Their liver, kidney and brain were immediately collected, and one-gram tissue from each organ was used for RNA extraction with Trizol reagent (Sangon biotech, Total RNA Extractor). One portion of the extracted RNAs from each organ were pooled within the same group for RNAseq analysis. The remaining RNA was kept individually for quantitative real-time PCR analysis. The purity and integrity of the total RNA (A_{260}/A_{280} ratio >1.8) was verified by using a NanoDrop spectrophotometer (Thermo, NanoDrop2000), and examined by electrophoresis on a 1.0% agarose gel.

2.3. cDNA library preparation and sequencing

mRNA was enriched by using poly-dT oligo attached to magnetic beads from total RNA samples (3 μg) and then fragmented at an elevated temperature. The target RNA was converted into double-strand cDNA (dscDNA) by using N6 random primer. The dscDNAs were repaired with phosphate at 5' end and formed stickiness 'A' at 3' end; then they were ligated by an adaptor with stickiness 'T' at 3' end. The ligation products were PCR amplified for library products used for sequencing by Illumina BGISEQ-500.

2.4. Data filtering and reads mapping

The sequence of adaptor, high content of unknown bases and low-quality reads were removed before downstream analysis, and the remaining reads were considered

clean reads. Bowtie2 (Langmead et al., 2009) and HISAT (hierarchical indexing for spliced alignment of transcripts) (Kim et al., 2015) were used to map clean reads to a reference gene and genome, respectively. In general, the higher the ratio of alignment indicates the closer the genetic relationship between sample and reference species.

2.5. Gene quantification and screening differentially expressed genes (DEGs)

Transcript quantification was analyzed through RSEM (RNA-Seq by Expectation Maximization)(Badawy et al., 2011), in which the RNA-Seq expression level read counts are normalized using Fragments Per Kilobase of transcript per Million mapped reads (FPKM). DEGs were identified by a strict algorithm between two samples at the absolute value of Log2 Ratio (control/exposure) ≥ 1 with a false discovery rate (FDR) $\leq 0.1\%$ (Benjamini and Hochberg, 1995). Genes with similar expression patterns usually mean functional correlation. We performed clustering analysis of DEGs with cluster (Eisen et al., 1998) and Java Treeview (Saldanha, 2004) softwares.

2.6. GO and KEGG analyses

We used GO enrichment analysis, a classifiable system that can comprehensively describe the properties of genes and their products. GO terms, which take the corrected p-value ≤ 0.05 as a threshold, are significantly enriched in DEGs. Genes usually interact with each other to play roles in certain biological functions. To further understand biological functions of genes, KEGG, the major public pathway-related database, is used to identify significantly enriched metabolic or signal transduction pathways in DEGs (Kanehisa et al., 2008). KEGG pathways with a Q-value ≤ 0.05 are significantly enriched in DEGs.

2.7. Quantitative real-time PCR (q-RT-PCR)

Each RNA sample was treated with PrimeScript RT reagent Kit with gDNA Eraser (Takara, Otsu, Japan) following the manufacturer's protocol, and subjected to reverse transcriptase reactions using oligo-dT primer. The q-RT-PCR reactions were performed in triplicate with ROCHE LightCycler96 using FastStart Essential DNA Green Master (Roche). The amplification conditions were 95℃ for 120 s, followed by 35 cycles of 95℃ for 10 s and 72℃ for 30 s. Melting curve analysis was performed to confirm specific amplification. $2^{-\triangle\triangle Ct}$ method was used to calculate the relative expression quantity of each target gene (Livak and Schmittgen, 2001). Detailed information on primers is listed in table S1 in the supplementary data.

3. Results

3.1 Reads mapping and sequencing saturation analysis

RNA-seq libraries of the pooled samples were generated and sequenced. On average, 22,965,743 raw reads per library were generated, and 22,926,703 clean reads retained after filtering adaptors, poly-N stretches and low quality reads (Table S2 in the supplementary data.). An average of 81.76% and 94.23% clean reads were mapped with reference to gene and genome, respectively. The specific results of each sample were shown in Table S3 and Table S4 in the supplementary data.

3.2. Gene quantification

Based on the quantitative RNA-seq data, we calculated the gene expression distribution of each sample, as shown in Fig. S1 (a-f). Comparison of gene expression profiles of the same organ among the treatment and control gave rise to significant

differences. To examine sample correlation, we calculated the correlation coefficient between each two samples and drew the correlation heatmap shown in Fig. S2. The highest coefficient of correlation occurred in brain, followed by kidney and liver. This indicated that liver is the most affected organs among the three. Meanwhile, the cluster tree was built by Euclidean distance algorithm to present the distance and difference between samples more visually (Fig. S3).

3.3. Screening DEGs and clustering analysis

DEGs between samples were analyzed using Poisson distribution (Audic and Claverie, 1997). The absolute value of Log2 Ratio (control/treatment) ≥ 1 and FDR ≤ 0.1% were regarded as the criterion to judge the significance of the DEGs. The scatter diagram was drawn to present the distribution of DEGs in Fig. S4 (Silva-Adaya et al.). In addition, the statistics of significant DEGs was shown as Fig. S5. DEGs are highest in the kidney, followed by the liver and then the brain. A heatmap indicating genes that showed significantly different expression was drawn to display changes in gene expression more clearly (Fig. 1).

3.4. GO analysis and KEGG pathways

To determine the function of DEGs after alcohol treatment in different organs, all of the genes which are expressed differently with statistical significance have been mapped to terms in the GO database and KEGG. These genes were assigned to three main categories to help understand the distribution of gene functions from the macro level, covering biological processes, cellular components and molecular functions (Fig. S6 - Fig. S8 in the supplementary data). There was a similar trend of enriched DEGs in

the three organs. The terms “single-organism process”, “cellular process” and “metabolic process” were the main categories of biological processes. As for cellular functions, “cell”, “cell part” and “organelle” were significantly enriched. Molecular functions were highly enriched for binding function and catalytic activity.

KEGG analysis was used to discover changes of biological pathways when the mice were exposed to acute alcohol. The top 20 pathways, mainly significantly enriched KEGG pathways ($p < 0.05$), were picked (Fig. S9 - Fig. S11 in the supplementary data) and expressed by the ratio of DEG numbers annotated in this pathway term to all annotated gene numbers. Some of metabolic pathways in all the three organs were effected by acute alcohol exposure, and liver had the most number of genes were affected at the transcriptional level.

3.5. Alcohol and KYNA synergistically affect tryptophan metabolism

We selected a total of 16 genes that were prominently elevated or reduced in comparisons between alcohol-treated and control mice to confirm differences in mRNA levels of the selected genes by q-RT-PCR (Fig. 2). The first 11 genes were involved in the kynurenine pathway. In the brain, *IDO1* and the last five genes are the DEGs in RNA-Seq. The significant differential expressions of these genes were also shown by q-RT-PCR. Three genes, *TDO2*, *kynureninase* and amino carboxymuconate semialdehyde decarboxylase *(ACMSD)* were comparable in expression by q-RT-PCR, and the p-values of the remaining genes were greater than 0.05. The results regarding gene expression changes of all 16 genes using q-RT-PCR and RNA-seq were roughly

the same in the kidney and liver. And the q-RT-PCR results of genes in tryptophan pathway of the mice after exposed to alcohol and/or KYNA were shown in Fig. 3, Fig. 4, Fig. 5 and Fig. 6.

4. Discussion

It is well established that alcohol has profound impact on gene expression at the system level (Corrao et al., 2004; Arbaizar et al., 2010; Ronksley et al., 2011; Bagnardi et al., 2015). However, the combined effect of alcohol and KYNA on gene expression has not been reported. The rationale for the current study is that KYNA is found in almost all alcoholic beverages on the market. (Yılmaz and Gökmen, 2018; Turska et al., 2019). Thus, alcohol partaking is unavoidably blended with KYNA. Our data clearly demonstrated that alcohol and KYNA exposure led to a distinct transcriptome that is different from that of the alcohol exposure alone or the KYNA-exposure alone. More importantly, we found that combination of the two substances severely altered tryptophan metabolism.

4.1 Responses in Trp metabolism of mouse exposed to alcohol

The effect of alcohol on Trp metabolism through kynurenine pathway and serotonin synthesis has been studied previously, suggesting that acute alcohol treatment could induce less serotonin availability to the brain, which can trigger more aggressive and assaultive trends than normal (LeMarquand et al., 1994; Crabbe et al., 1996; Ramboz et al., 1996). Through KEGG pathway analysis, we found two genes attached to “serotonergic synapse” (ko04726) in the brain were significantly down-regulated, including Trp 5-monooxygenase (TPH2), the first enzyme in the transformation of Trp

into serotonin and serotonin neurotransmitter transporter, which transports serotonin into the glial cells and recycles serotonin after nerve conduction (Ziegler et al., 1993; Lesch et al., 1994). Our findings may explain the reason for the decrease of serotonin in the brain after acute alcohol administration.

Since the expression of TPH2 was decreased in brain, there were more Trp through kynurenine metabolism. IDO and TDO play critical roles in regulating the physiological flux of Trp into subsequent metabolic pathways. Many studies have shown that acute, chronic ethanol administration to animals and humans could change TDO activity and therefore affect serotonin metabolism (Curzon and Knott, 1974; Badawy, 2002). The serotonin decrease could be induced by activation of the hepatic enzyme TDO that uses heme as a cofactor (Badawy, 2013). 5-aminolevulinate synthase (ALAs) is the first and a rate-limiting enzyme in the heme biosynthesis, which is known to be feedback repressed by heme (Hamilton et al., 1991). After a week of acute alcohol treatment, we found the mRNA expression of ALAs was up-regulated in the liver, but ALAs was reduced in the brain. Doss et al. have evaluated that alcohol can augment the inducibility of ALAs (Doss et al., 2000). Subsequently, Ren and Correia suggested that heme deficiency can impair TDO synthesis at the transcriptional level (Ren and Correia, 2000).

Contrarily, the concentration of cortisol was down-regulated in the liver because of the decreased expression of 3beta-hydroxy-Delta5-steroid dehydrogenase and steroid Delta-isomerase, which are involved in cortisol biosynthesis. However, the expression of these two enzymes were both increased in the kidney. The early studies

have demonstrated that acute alcohol treatment markedly increases plasma glucocorticoid levels, and the activity of TDO enhances with the increase of glucocorticoid since dexamethasone exposure can increase the promoter activity of TDO2 gene (TABAKOFF et al., 1978; Rose et al., 2010; Soichot et al., 2013). Consequently, the activity of TDO was reduced in the liver and brain and enhanced in the kidney because of the cortisol or glucocorticoid level changes, which the expression of TDO was decreased in brain and liver ($P<0.05$), and was not changed significantly in kidney (Fig. 3c).

kynurenine 3-monooxygenase is an effective drug target for several neurodegenerative and/or neuroinflammatory diseases, especially Huntington's, Alzheimer's, and Parkinson's diseases (Miranda et al., 1997; Campesan et al., 2011; Silva-Adaya et al., 2011; Zwilling et al., 2011). kynurenine 3-monooxygenase is a class A FAD monooxygenase, which catalysis is dependent upon FAD reduction by NADH or NADPH (Van Berkel et al., 2006). We have found the activity of kynurenine 3-monooxygenase in liver was enhanced obviously after acute alcohol administration (data not shown). In this experiments, there was no significant change in expression level, but NADH dehydrogenase (E7.1.1.2; E1.6.99.3) and NADH dehydrogenase (ubiquinone) Fe-S protein 5 (NDUFS5) were down-regulated. In nicotinate and nicotinamide metabolism, purine-nucleoside phosphorylase (PNP) and 5'-nucleotidase (EC: 3.1.3.5) were up-regulated, while NAD+ glycohydrolase (NGA) was reversed. Changes in the expression of the above genes lead to increased level of NADH and

NADPH, which may explain the raised activity of kynurenine 3-monooxygenase in liver.

4.2 Responses in Trp metabolism of mouse exposed to KYNA

Previous studies have implicated people intake KYNA on a daily basis when they drink something that requires fermentation. KYNA can modify neuronal function through antagonizing the glycine site of the N-methyl-D-aspartate receptors and/or the neuronal cholinergic α7 nicotine receptors, which nM concentrations of KYNA may reduce the activity of brain excitatory transmission (Németh et al., 2004; Cristiane et al., 2007; Kessler et al., 2010; Moroni et al., 2010; Russi et al., 2010). It has been confirmed that 3-HK, 3-HAA and KYNA can induce aversion to alcohol by suppressing the liver mitochondrial low Km aldehyde dehydrogenase (ALDH) activity *in vitro* and *in vivo* (1mg/kg body wt.) (Badawy and Morgan, 2007; Badawy et al., 2011), whereas we found that the expression of ALDH was increased in kidney, but decreased in liver when the mice were exposed to KYNA (Fig. 6b), which may demonstrate that the inhibited expression of liver ALDH may induce the increased expression of ALDH in kidney. Additionally, THP2 was up-regulated in the brain of the mice exposed to KYNA and alcohol and KYNA, in which the prior is increased more than 10 times. However, the three experimental groups were all down-regulated in kidney. KYNA has the most significant effect on regulating the expression of TPH2 (Fig. 6a).

4.3 Responses in Trp metabolism of mouse exposed to alcohol and KYNA

When the mice were exposed to alcohol and KYNA, TDO was dramatically up-regulated in kidney of the mice exposed KYNA, but the expression was decreased in

kidney of the mice exposed alcohol and KYNA ($P<0.05$).The decrease of peripheral TDO activity induce kynurenine increase in serum that contributed to the increase in brain KYNA (Kanai et al., 2009; Sekine and Fukuwatari, 2019). What's more, we found that the expression of IDO1 was increased in brain of the mice exposed to alcohol and alcohol and KYNA (Fig.3a). Therefore, Trp metabolism moved toward kynurenine pathway in brain after acute alcohol and alcohol and KYNA administration.

The expression of kynureninase was lowered significantly, while kynureninase was up-regulated in kidney of the mice only administrated alcohol (Fig. 4b). 3-HK, the substrate of kynureninase, is one of the metabolisms that can cross the blood-brain barrier in KP. The concentration of 3-HK may be lifted in brain of alcoholism, when they drink alcohol beverage containing KYNA habitually, which will rise the concentration of quinolinate leading to increased excitotoxicity. What's more, the expression of DRD1 in the mice administrated KYNA and alcohol and KYNA were significantly up-regulated. Interestingly, that of DRD1 in the mice exposed alcohol and KYNA was higher much than others (Fig. 6c). There probably is a synergistic effect between alcohol and KYNA in regulation the expression of DRD1. Therefore, the alcohol beverage containing KYNA may make the drinker more exhilarated.

Our data also shows that there are some large differences among the organs in response to alcohol and/or KYNA. After alcohol exposure, we found that the number of genes whose expression was down-regulated in liver was most, but the up-regulated genes were maximum in the kidney, the DEGs in both the liver and kidney were shown in Fig. 7. We speculate that organs have different sensitivity to acute alcohol. Liver

seems to have more vulnerability than kidney and brain, acute alcohol exposure may restrain liver function. TDO is strongly expressed constitutively in the liver (Michael et al., 2014), while the expression of TDO decreased in the liver and had a sharp increase in the kidney after KYNA administration. The expression of ALDH had the same change. The reasons why KYNA can cause the significant differences in different organs remain to be assessed in future studies.

5. Conclusions

In summary, there were variable expression levels between different organs after acute alcohol exposure, whereas more expression differences in tryptophan pathway were found after KYNA exposure. When the mice exposed alcohol and KYNA simultaneously, Trp metabolism shifted toward kynurenine pathway in the brain, which may cause mice excitation and excitotoxicity because of the increased expression of DRD1 and the reduced expression of kynureninase.

Conflicts of interest

There are no conflicts of interest to declare.

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Appendix

Supplementary data

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Figure legends

Fig. 1 Union heatmap of DEGs for each cluster

Each column represents a group and each row represents a gene. The differences of expression were shown in different colors. The red crolor stands for increase and the blue for decrease. Gradient color barcode at the right top indicates log2(FC) value (FC, Foldchange of expression in treatment case to expression in control case).

Fig. 2 Comparison of the results of RNA-seq

Gene expression changes revealed by q-RT-PCR are represented by blue bars and the resuls obtained through RNA-seq were represented by redbars. Y-axis indicates log2(ratio) value (ratio, the ratio of experiment group to control group). The data for the brain (a), kidney (c) and liver (b) were shown in different panels.

Fig. 3 The relative quantitative results of genes for rate-limiting enzyme in tryptophan pathway

Different colors represent different treated groups, IDO1 (a), IDO2 (b), TDO (c). Y-axis indicates the ratio of the gene expression of treated group to that of control group.

Fig. 4 The relative quantitative expression results of genes in kynurenine pathway

Different colors represent different treated groups, kynurenine 3-monooxygenase (a), kynureninase (b), 3-hydroxyanthranilate 3,4-dioxygenase (c). Y-axis indicates the

ratio of the gene expression of treated group to that of control group.

Fig. 5 The relative quantitative expression results of genes of kynurenine aminotransferases (KATs)

Different colors represent different treated groups, KAT1 (a), KAT2 (b), KAT3 (c), KAT4(d). Y-axis indicates the ratio of the gene expression of treated group to that of control group.

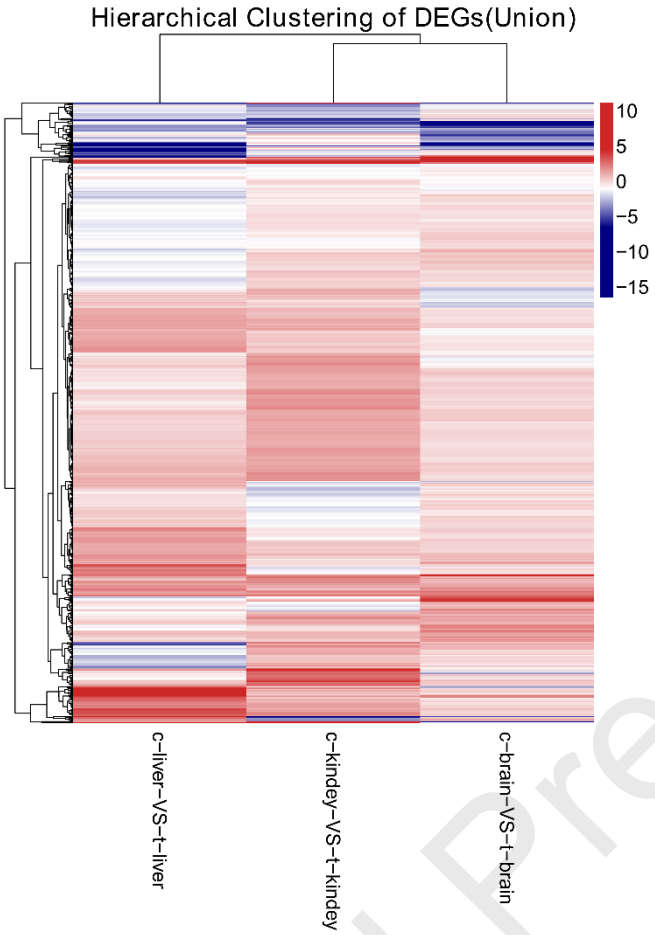
Fig. 6 The relative quantitative expression results of genes

Different colors represent different treated groups, TPH2(a), ALDH (b), DRD1 (c) in the brain. Y-axis indicates the ratio of the gene expression of treated group to that of control group.

Fig. 7 Heatmap of the DEGs in both liver and kidney

Each column represents a group and each row represents a gene. Expression differences were shown in different colors. Gradient color barcode at the right top indicates log2(FC) value(FC, Foldchange of expression in treatment case to expression in control case). The red crolor stands for increase and the blue for decrease. The tree on the left shows the results of cluster analysis of different genes.

On the right of heatmap is an abbreviation of each gene name.



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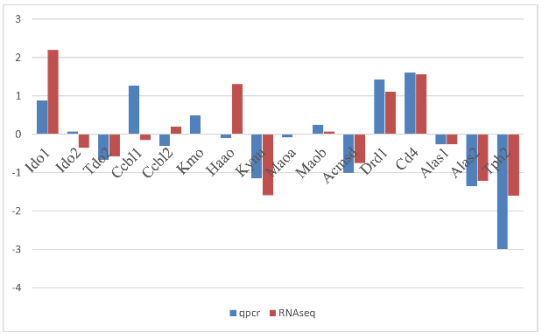

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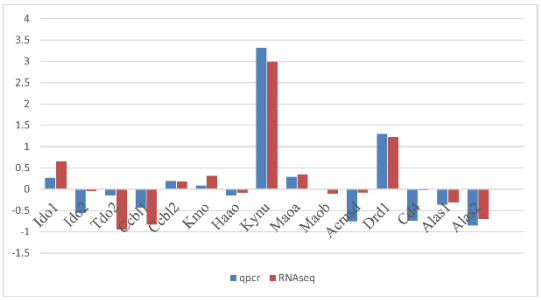

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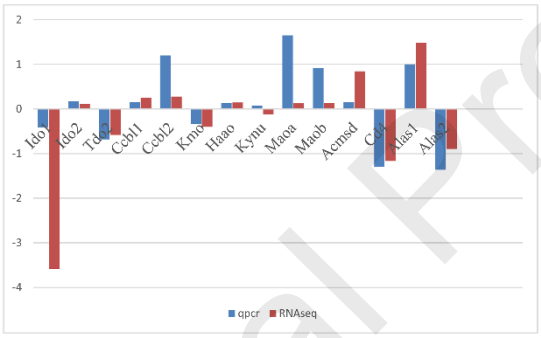

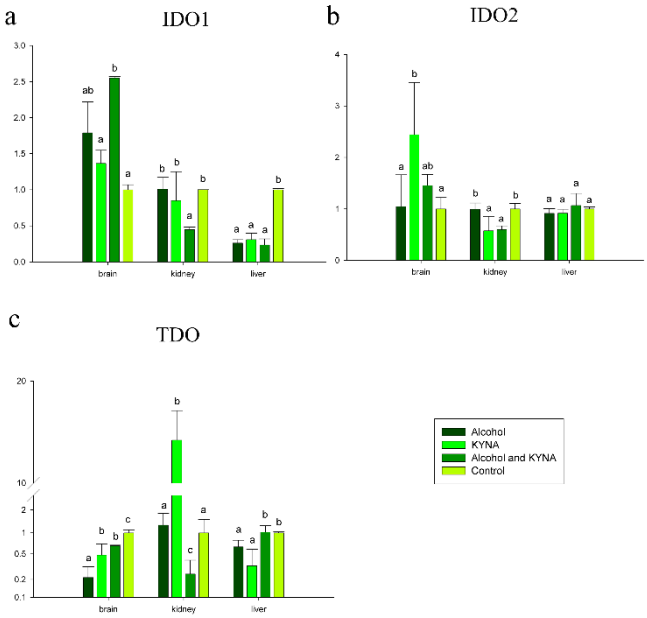

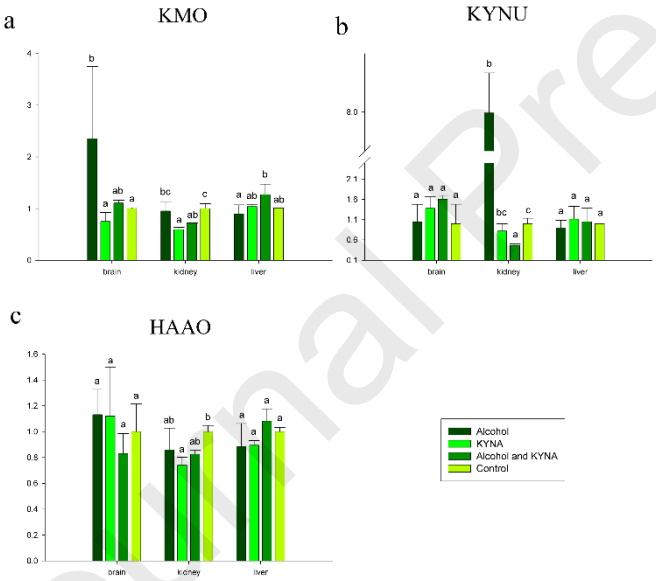

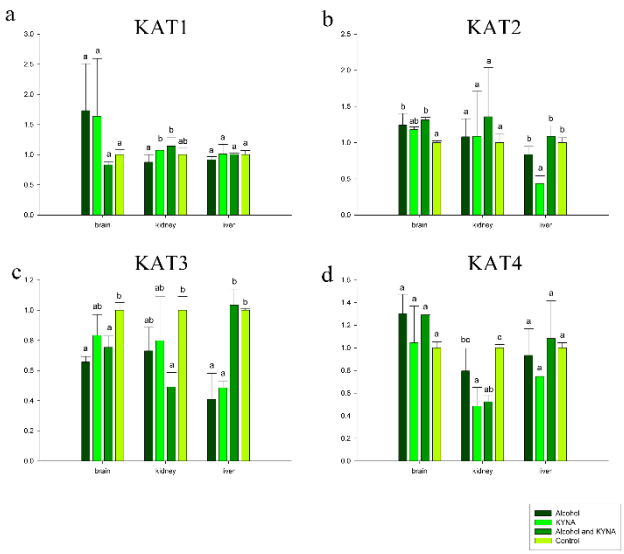

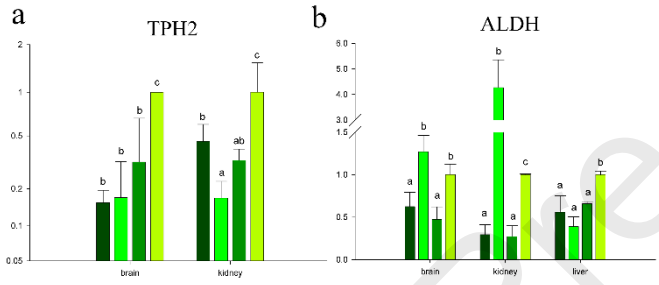

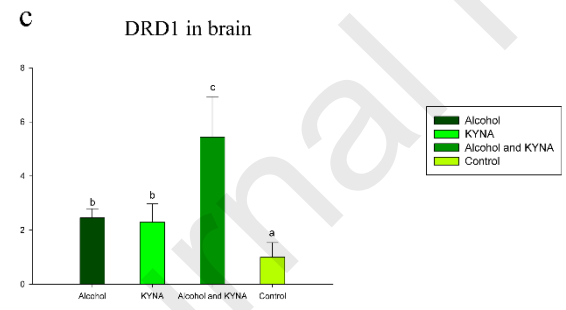

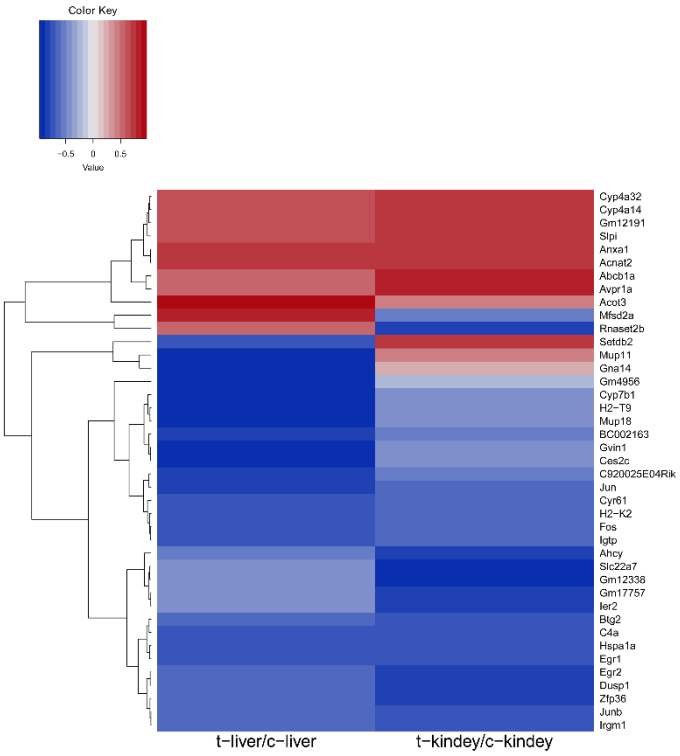

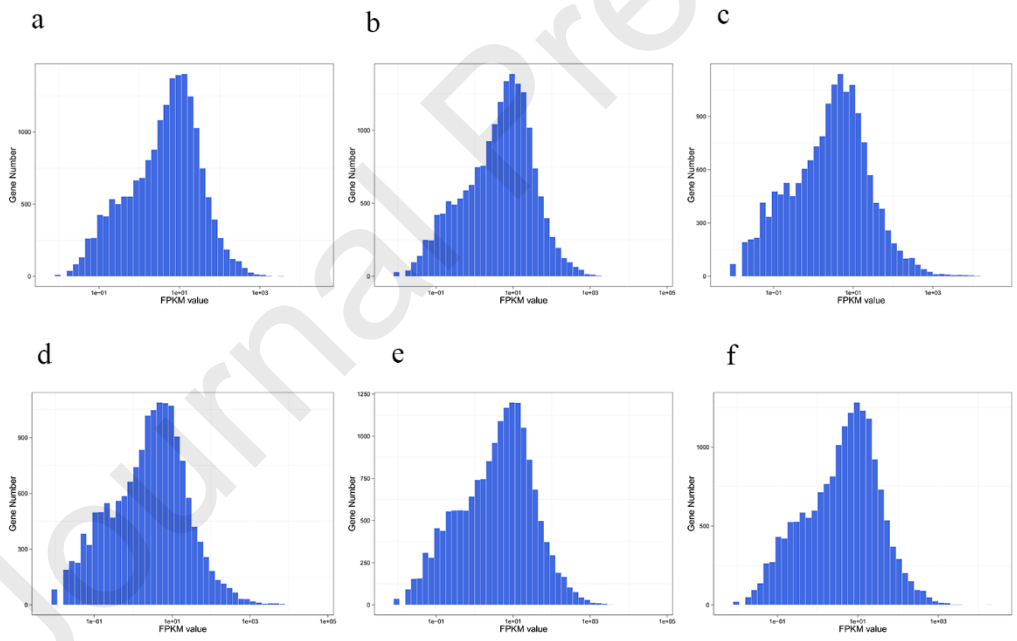

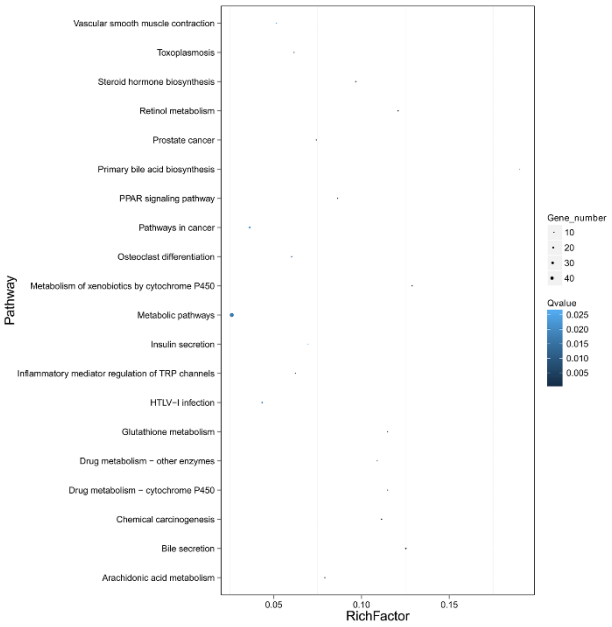

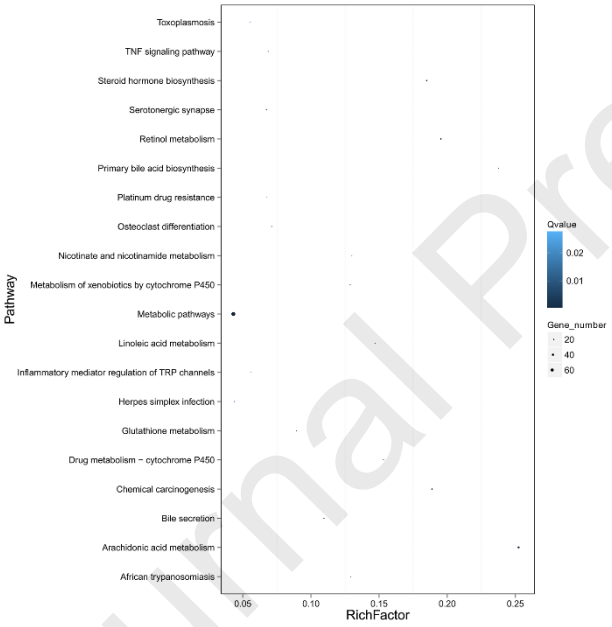

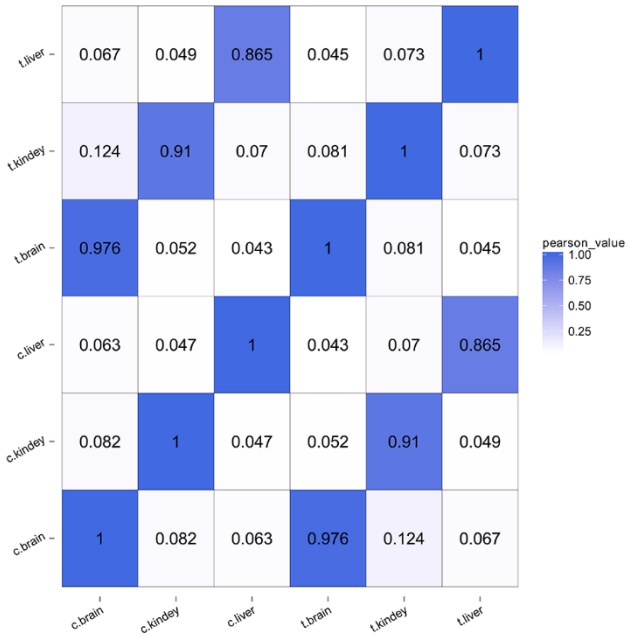

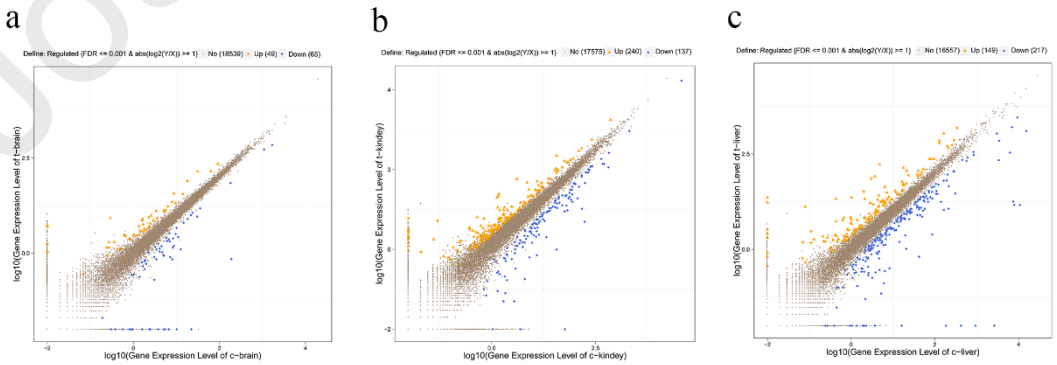

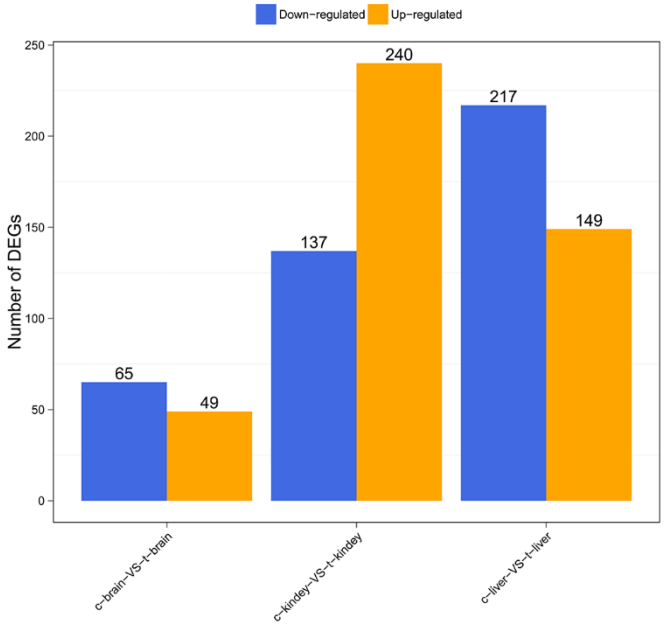

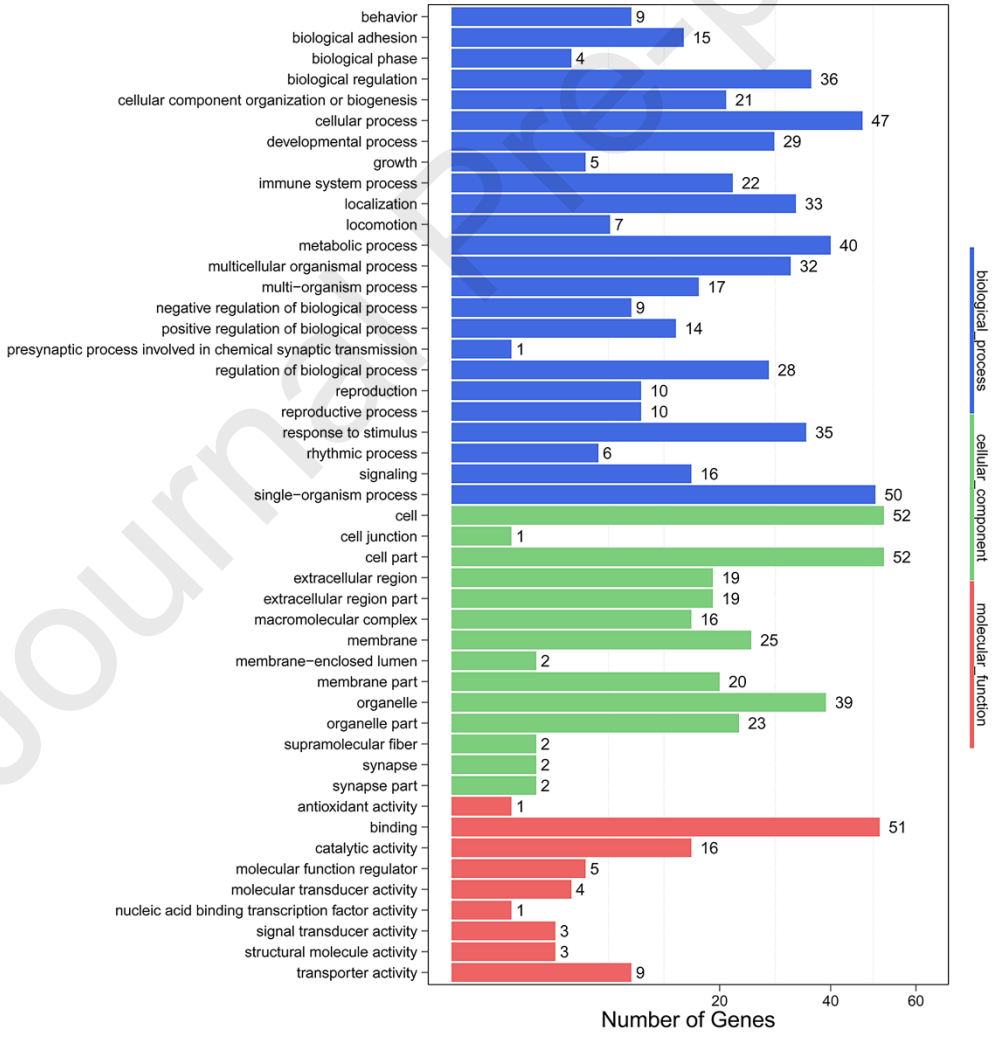

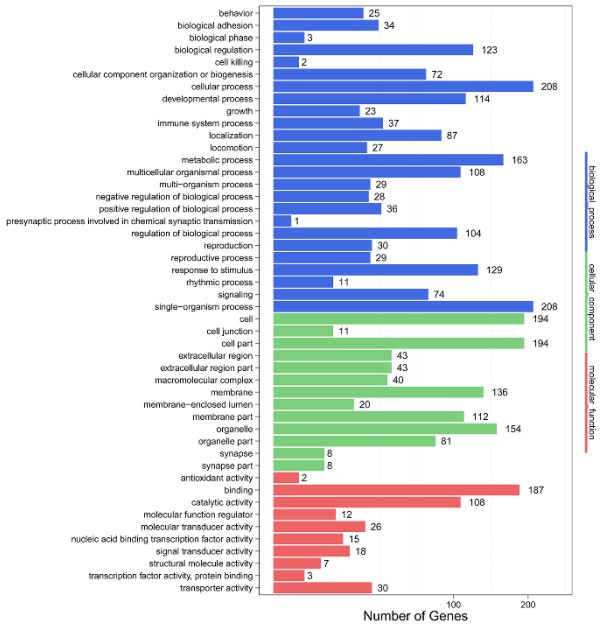

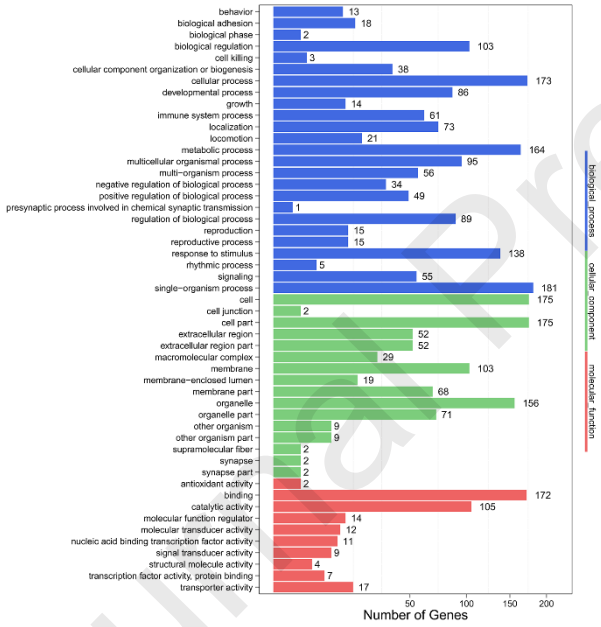

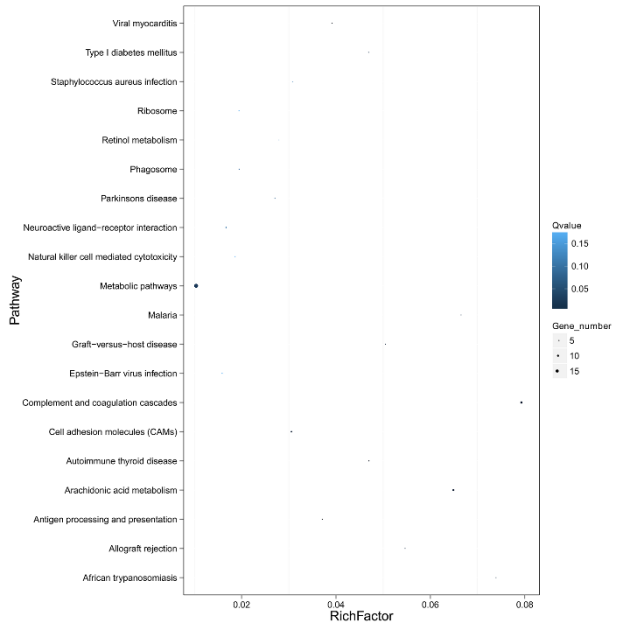

Author Contributions

Conceived and designed the experiments: QH, GW. Performed the experiments: CY. Analysed the data: CY, YZ, CL, HZ, XD. Assisted in some experiments: YZ, CL. Wrote the paper: CY, QH, GW.

Highlights

• RNAseq transcriptomes analyses revealed that alcohol affects the three organs differentially.

• The gene profiles from alcohol and KYNA co-administration were significantly different from those of alcohol or KYNA alone.

• Trp metabolism was skewed towards the kynurenine pathway in the brain, which is predicted to enhance alcohol excitotoxicity.

Abbreviations

Kynurenic acid	KYNA
Central nervous system	CNS
Tryptophan	Trp

Kynurenine pathway	KP
Indolamine 2,3-dioxygenase 1	IDO1
Trp 2, 3-dioxygenase	TDO
3-hydroxykynurenine	3-HK
3-hydroxyanthranilic	3-HAA
Trp 5-monooxygenase	TPH2
Aldehyde dehydrogenase	ALDH

Supplementary Information

Organ-differential responses to ethanol and kynurenic acid, a component of alcoholic beverages in gene transcription

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Transcriptional changes other than tryptophan metabolism genes

1. Nervous responses of mouse exposed to alcohol

The comparison of transcriptome data between different tissues can also offer some information related to the nervous system. We obtained 36 DEGs, associated with the nervous system, across 10 different neural signaling pathways based on GO and KEGG analyses. Both serotonergic synapse (ko04726) and dopaminergic synapse (ko04728) were affected in the brains, livers and kidneys of mice. 6 genes related to "dopaminergic synapse" (ko04728), such as dopamine receptor D1 (*DRD1*) were significantly up-regulated. Previous evidence has suggested that systemic administration of alcohol to rodents increased the dopamine levels in tissue, which can induce the personality dimension of impulsiveness [1]. The increase of *DRD1* expression is closely related to addictive behaviors, including alcoholism (ko05034), cocaine addiction (ko05030) and morphine addiction (ko05032) [2, 3], which explains why alcoholics are unable to wean themselves off alcohol.

In addition, the other significantly affected pathways related to the nervous system such as glutamatergic synapse (ko04724) and retrograde endocannabinoid signaling (ko04723) were affected by alcohol treatment. Glutamate and γ-amino butyrate (GABA) are symbols of excitatory and inhibitory amino acids, respectively [4-6]. In this study, we found that the expression of glutamate transporter (EAAT1) was up-regulated, and the expression of GABA receptor was down-regulated, which may therefore affect the balance of excitatory and inhibitory neurotransmission and explain the hyper excitability observed during ethanol withdrawal.

2. Immune responses of mouse exposed to alcohol

The damages to immune system via drinking alcohol, including acute and chronic consumption, were reported previously [7]. The consequences of the immunologic injures may increase sensibility to pneumonia, hepatitis, and other infectious diseases. Moreover, circulating auto-antibodies are often present in chronic alcoholic individuals, and the liver is the major organ having alcohol-induced injury [8, 9]. Dissections of alcohol's effects in many aspects of the immune system provide insight into the factors that lead to a greater risk of infection in the alcohol abusing population [10]. In this study, genes in four common immune-related pathways: complement and coagulation cascades (ko04610), allograft rejection (ko05330), antigen processing and presentation (ko04612) and graft-versus-host disease (ko05332) all had significant differential expressions in the brains, livers and kidneys of the mice taking alcohol. Twenty one genes classified as "Complement cascades" had significant differential expressions. Complement component 4 (C4), which is a fibrin synthesis initiator, was significantly down-regulated. C4 could be cleaved with C2 to form C4bC2a, which serves as C3 convertase to cleave C3. When the degree of C3 cleavage is reduced, the ability of the complement system to clear apoptotic cells and lyse bacteria diminishes [11, 12]. Down-regulation of some inhibitors such as plasminogen activator inhibitor 1 (PAI1) and tissue factor pathway inhibitor (TFPI) could lead to increased degradation of fibrin. To elicit a response from the cell mediated arm of the adaptive immunity, antigens need to be presented to the CD4+ and CD8+ T cells. Class I major histocompatibility complex (MHC) is an important component of the antigen processing and presentation pathway. MHC expression reduction may lead to certain types of viruses' infection [13, 14]. In our

present study, we found that MHC and T-cell surface glycoprotein (CD4) were significantly down-regulated in the mouse liver. Therefore, the above genes expression changes in four common immune-related pathways could help us understand the mechanism by which alcoholic liver disease causes inflammation [15].

Supplementary Tables

Table S1. Animal grouping, treatments and assays

	Group	No. of mice	Repeat	Treatment	Assay
Experiment 1: Alcohol globle effects	Alcohol alone	8	1	Intragastric administration of 20%v/v solution of ethanol in a 2 g/kg dose daily for a week	RNA-seq, q-RT-PCR validation (three replications)
	The control	8	1	Intragastric administration of 13ml/kg of sterile deionized water daily for a week	RNA-seq, q-RT-PCR validation (three replications)
Experiment 2: Effects of Alcohol and KYNA co-administration on tryptophan metabolism	Alcohol alone	3 x 3	3	Intragastric administration of 20%v/v solution of ethanol in a 2 g/kg dose daily for a week	q-RT-PCR
	KYNA alone	3 x 3	3	Intragastric administration of 8 μg/mL solution of KYNA (sterile deionized water) in a 100 μg/kg dose daily for a week	q-RT-PCR
	alcohol and KYNA	3 x 3	3	Intragastric administration of 8 μg/mL solution of KYNA (20%v/v solution of ethanol) in a 100 μg/kg dose daily for a week	q-RT-PCR
	The control	3 x 2	3	Intragastric administration of 13ml/kg of sterile deionized water daily for a week	q-RT-PCR

Table S2. Primer sequences of genes used in the q-RT-PCR analysis

gene ID	Gene name	Primers
15930	*Indolamine 2,3-dioxygenase 1 (IDO1)*	F:CCCAGTCCGTGAGTTTGTCA R:CTCTTCCGACTTGTCGCCAT
209176	*Indolamine 2,3-dioxygenase 2 (IDO2)*	F:CGGGGCTTCATCCTAGTGAC R:TAGCAGGGTGTCCTGACTGT
56720	*Trp 2, 3-dioxygenase (TDO)*	F:GGGGGATCCTCAGGCTATCA R:TGTCACTGTACTCGGCTGTG
70266	*kynurenine aminotransferase 1 (KAT1)*	F:GGATCGATCACAACCCCTGG R:ACGCTGAGGTGTACTGGTTG
229905	*kynurenine aminotransferase 3 (KAT3)*	F:AGAGAGATCGGATGGTCCGT R:TCCGAGAGGTCAGCACCTAA
23923	*kynurenine aminotransferase 2 (KAT2)*	F: CCAAGATGGTCTCTGTAAGG R: CAGCCTAGTGGTTTCATAGC
270627	*kynurenine aminotransferase 4 (KAT4)*	F:CACCGTGAGCTTATACAGGAAG R:TGGAGGAGGAGCATAGTCATAG
11670	*aldehyde dehydrogenase (ALDH)*	F: CACTTCCAGCGGGTCATAAA R: TAGGATGGTGGGAGCTATGT
98256	*kynurenine 3-monooxygenase (KMO)*	F:GACTGCCGTGGAGTCCTATG R:GGAACCTTGTCAGGTCCGAG
70789	*Kynureninase (KYNU)*	F: ATTCCCAAAATGCGGGACCT R: CCCTACAATCCAAGGGCGTT
17161	*monoamine oxidase A (Maoa)*	F:TTCTGGAAGCCCGGGATAGA R:TGGGTTGGTCCCACATAAGC
109731	*monoamine oxidase B (Maob)*	F: CTCTGCAGCCCGTCCATTAT R:GGCTGACGTAGAACCCTTCC
107766	*3-hydroxyanthranilate 3,4-dioxygenase (HAAO)*	F:ACTGTAAGGACCTTGGCACG R:GACCGTGTATTCAGCGGGAA
266645	*Amino carboxymuconate semialdehyde decarboxylase (ACMSD)*	F: ACAATGACCTAGCTGCCACC R:CTAGCGCCTTAACACAACGC
13488	*dopamine receptor D1 (Drd1)*	F:GGTTGAGCAGGACATACG R:TTGCTTCTGGGCAATCC
12504	*CD4 antigen (Cd4)*	F:TGAAGGAAACGCTCCCACTC R:TGCAGTGTCCCTTTGTCCAG
11655	*aminolevulinic acid synthase 1 (Alas1)*	F:GGAGAGCACAAATCTTCCC R:GTCATTACTGCACCAGACC
11656	*aminolevulinic acid synthase 2 (Alas2)*	F:AGCAAGATTGTGCAGAGG R:CTTCGGGTGGTTGAATCC
216343	*Trp 5-monooxygenase (Tph2)*	F:CGACCATCCAGGATTTAAGG R:TGGGCTGACCATATTTATAGC

Table S3. Summary of sequencing data for each sample

Sample	Sequencing Strategy	Raw Data Size (bp)	Raw Reads Number	Clean Data Size (bp)	Clean Reads Number	Clean Data Rate (%)
c-brain	SE50	1165142650	23302853	1163184850	23263697	99.83
c-kidney	SE50	1176583350	23531667	1174840600	23496812	99.85
c-liver	SE50	1171959950	23439199	1170397000	23407940	99.86
t-brain	SE50	1162811400	23256228	1161533100	23230662	99.89
t-kidney	SE50	1039397800	20787956	1036621950	20732439	99.73
t-liver	SE50	1173827950	23476559	1171433500	23428670	99.79

Table S4. Alignment statistics of reads align to reference gene

Sample	Total Reads	Total Mapped Reads (%)	Unique Match (%)	Multi-position Match (%)	Total Unmapped Reads (%)
c-brain	23263697	80.99	77.27	3.73	19.01
c-kidney	23496812	77.76	73.06	4.7	22.24
c-liver	23407940	86.57	71.34	15.23	13.43
t-brain	23230662	81.65	77.81	3.84	18.35
t-kidney	20732439	76.68	72.21	4.48	23.32
t-liver	23428670	86.89	77.53	9.36	13.11

Table S5. Alignment statistics of reads align to reference genome

Sample	Total Reads	Total Mapped Reads (%)	Unique Match (%)	Multi-position Match (%)	Total Unmapped Reads (%)
c-brain	23263697	94.64	74.3	20.34	5.36
c-kidney	23496812	95.68	71.35	24.33	4.31
c-liver	23407940	90.71	66.8	23.91	9.29
t-brain	23230662	94.71	73.79	20.92	5.3
t-kidney	20732439	95.18	70.53	24.65	4.82
t-liver	23428670	94.47	72.89	21.58	5.53

Supplementary Figures

Fig. S1. The gene expression distributions

X-axis and Y-axis represent FPKM values and number of genes, respectively. (a), distributions in the brain from the mice without giving alcohol; (b), in the brain with alcohol treamtment; (c), in the liver without alcohol treamtment; (d), in the liver with alcohol treamtment; (e), in the kidney without alcohol treamtment; (f), in the kidney with alcohol treamtment.

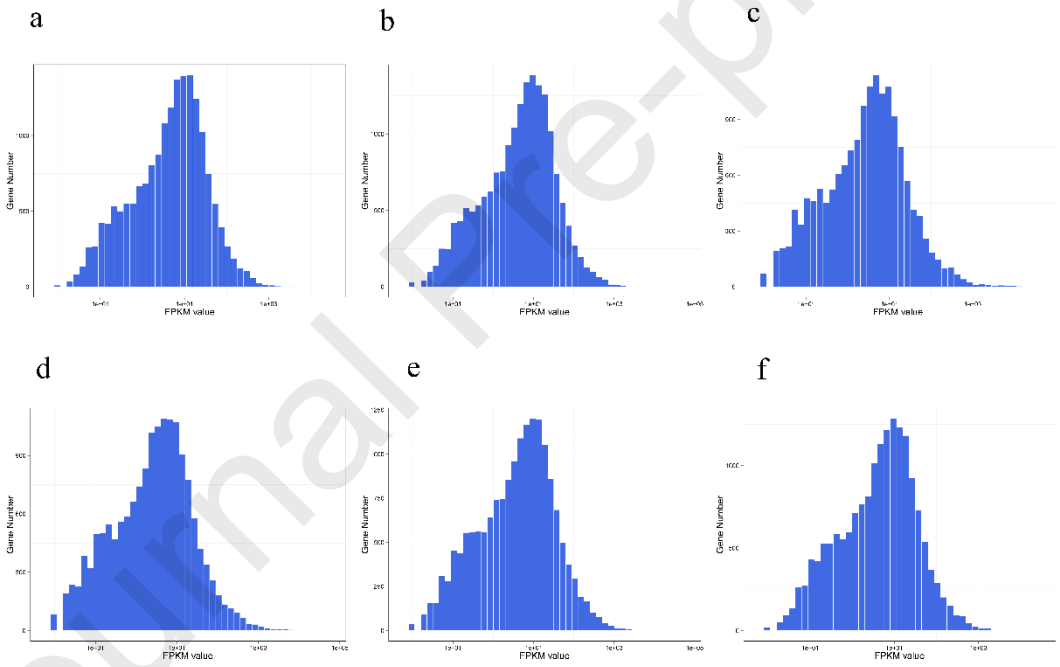


Fig. S2. Heatmap of correlation coefficient values crossing samples

Gradient color barcode at the right top indicates the minimum value in white and the maximum in blue. If one sample is highly similar with another one, the correlation value between them is very close to 1. The correlation values were labeled in each comparison box.

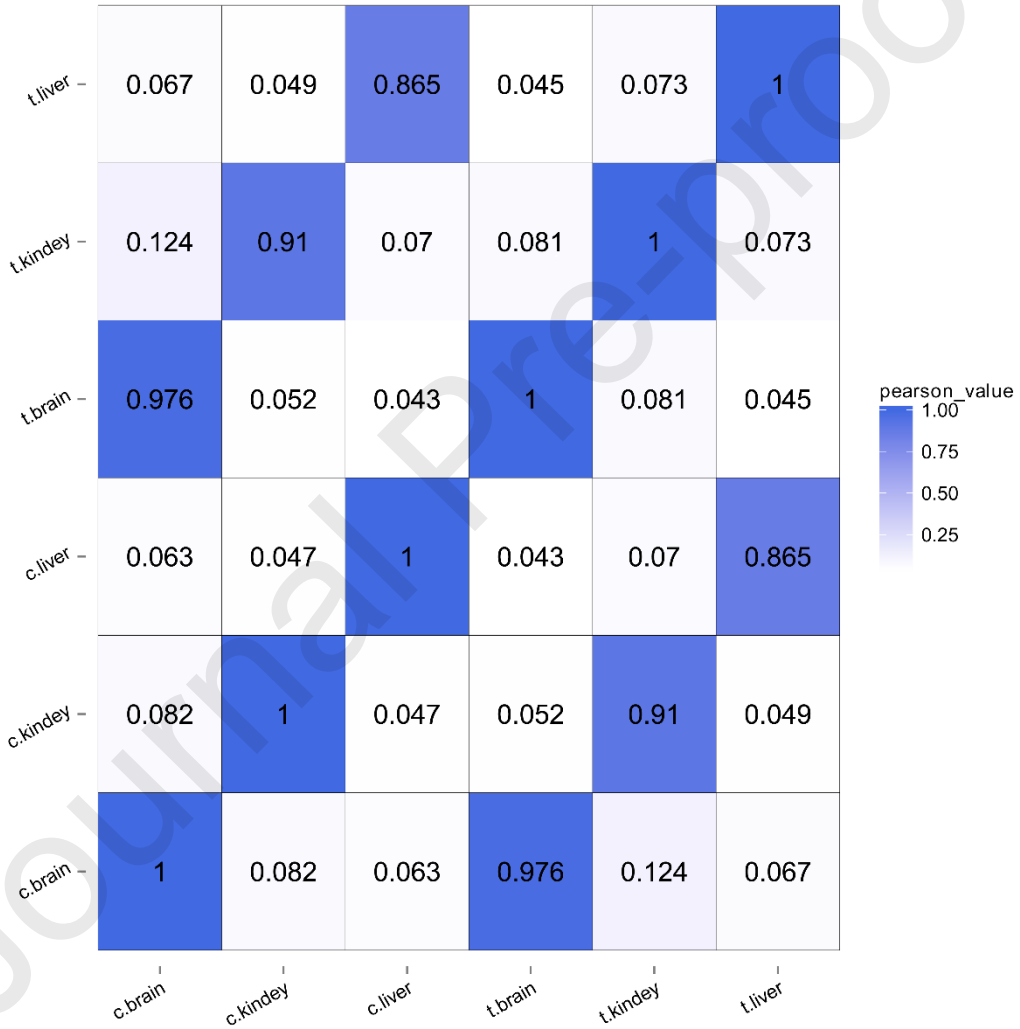


Fig. S3. Cluster tree of all samples.

The distances of expressed genes are calculated by Euclidean method. The vertical axis represents the height of the cluster tree. If samples have similar height values, they are easily to be gathered.

Fig. S4. Scatter plots of all expressed genes in each pairwise

X-axis and Y-axis represent log2 values of gene expression of control and treatment groups, respectively for the brain (a), liver (b) and kidney (c). The blue color marked are down-regulated genes, the orange color marked are up-regulated genes and brown color marked are non-significantly differently expressed genes.

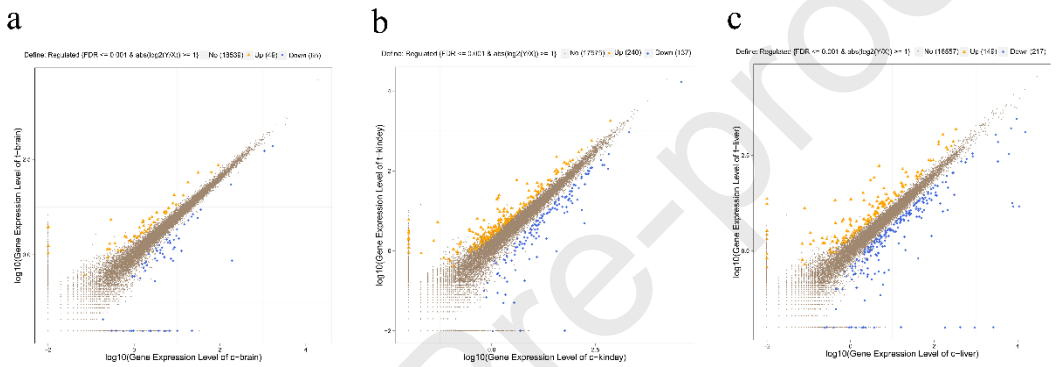


Fig. S5. Statistics of significant DEGs

X axis represents pairwise and Y axis represents number of screened DEGs. Blue bar

denotes down-regulated genes and orange bar for the up-regulated.

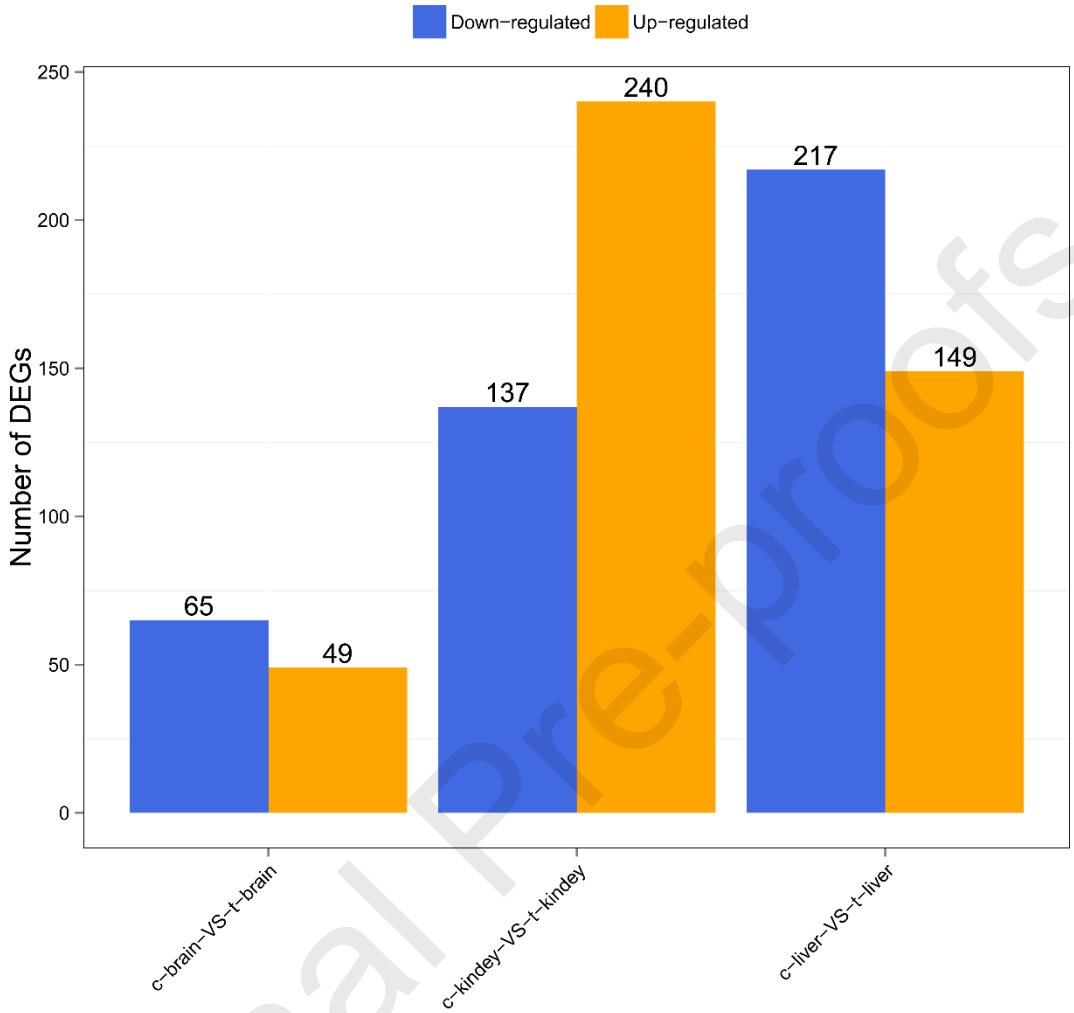


Fig. S6. Gene ontology classification of DEGs in brain

X axis is for the number of DEGs represented by its square root value, and Y axis

represents GO terms. All GO terms were grouped into three ontologies: The blue colored is for biological process, the brown colored is for cellular component and the orange colored is for molecular function.

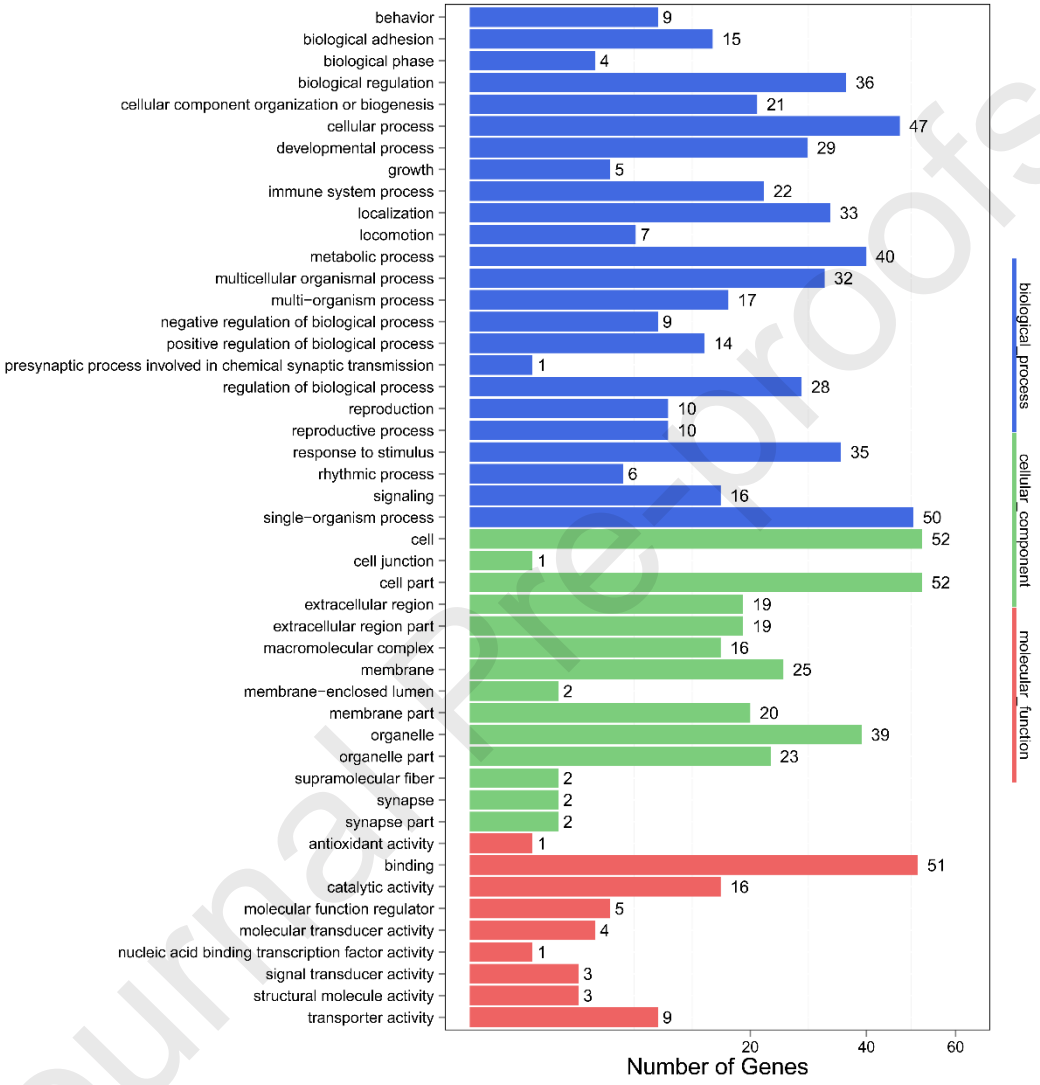


Fig. S7. Gene ontology classification of DEGs in kidney

X axis is for the number of DEGs represented by its square root value, and Y axis represents GO terms. All GO terms were grouped into three ontologies: The blue colored is for biological process, the brown colored is for cellular component and the orange colored is for molecular function.

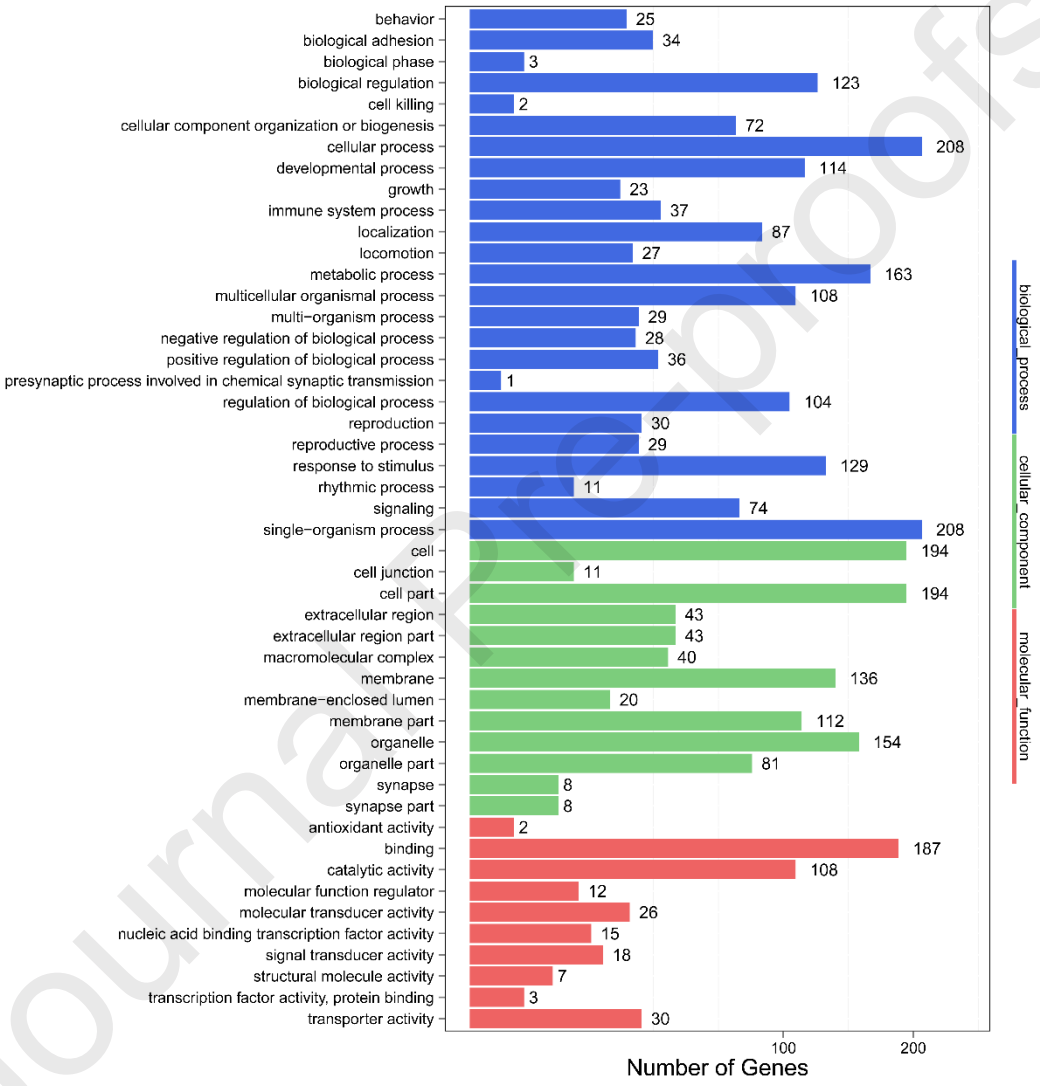


Fig. S8. Gene ontology classification of DEGs in liver

X axis is for the number of DEGs represented by its square root value, and Y axis represents GO terms. All GO terms were grouped into three ontologies: The blue colored is for biological process, the brown colored is for cellular component and the orange colored is for molecular function.

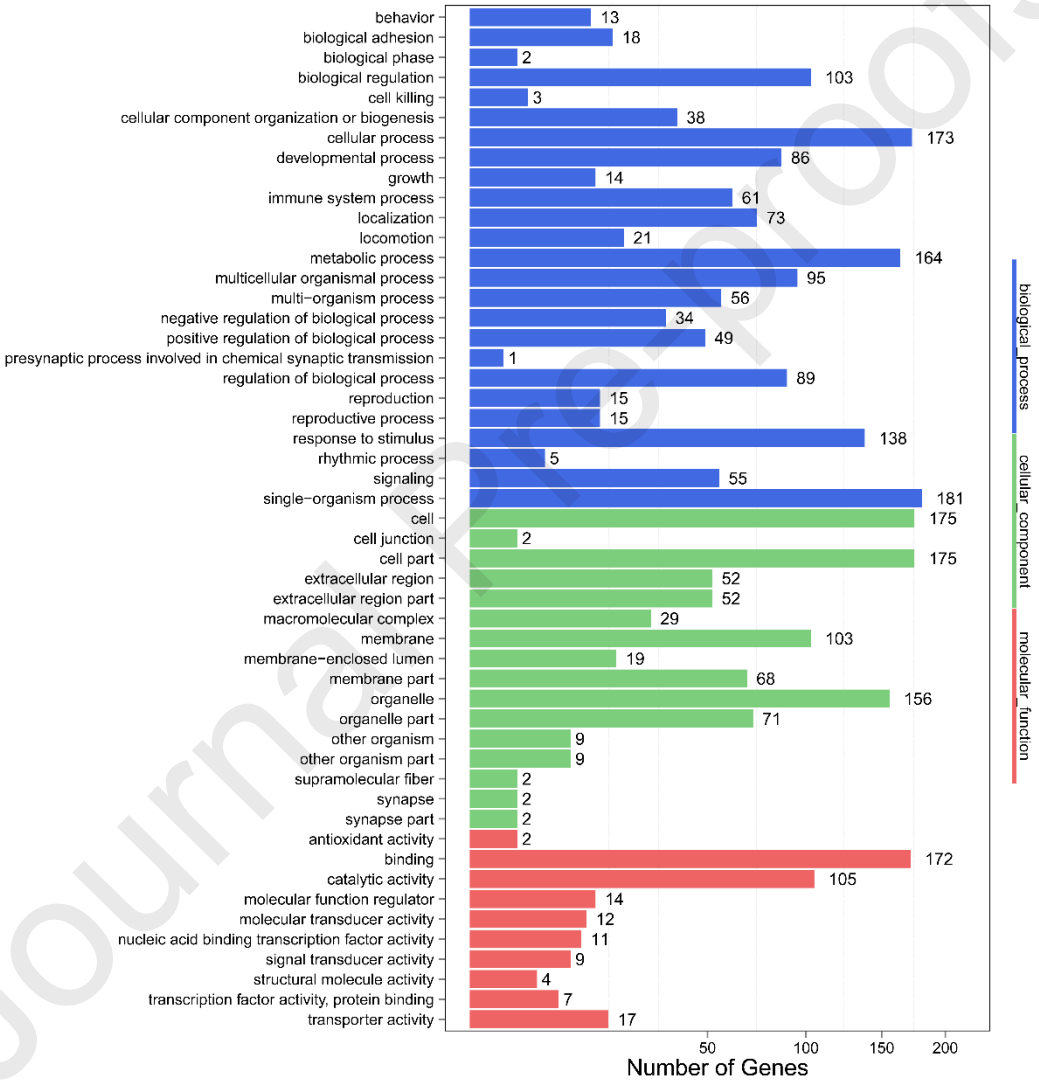


Fig.S9. Statistics of pathway enrichment of DEGs in brain

Q-value is corrected p value ranging from 0~1, and less Q-value means greater intensiveness. Y axis is for the name of pathway and X axis represents the rich factor (the degree of KEGG pathway enrichment). The statistics for the brain (a), kidney (b) and liver (c) data were shown in different panels.

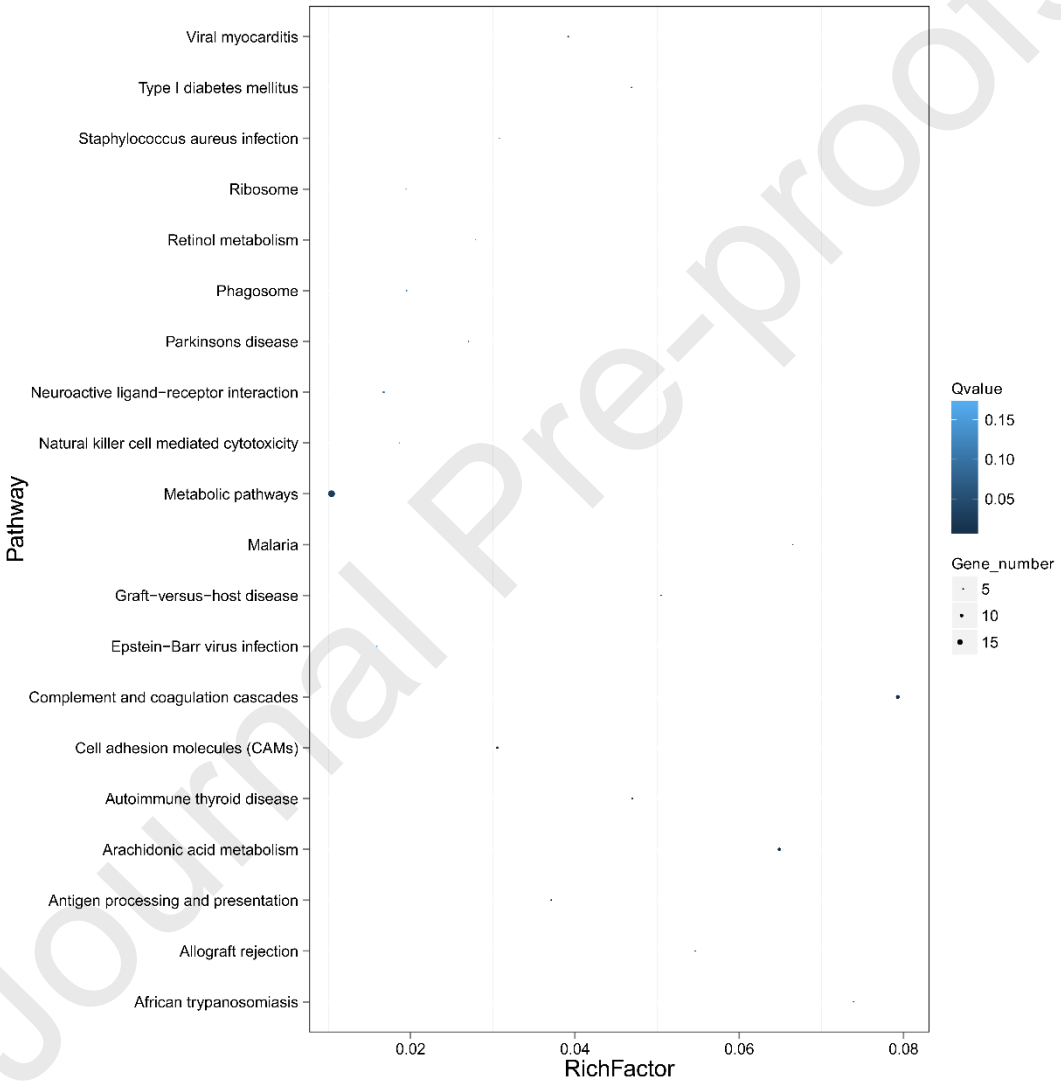


Fig.S10. Statistics of pathway enrichment of DEGs in kidney

Q-value is corrected p value ranging from 0~1, and less Q-value means greater intensiveness. Y axis is for the name of pathway and X axis represents the rich factor (the degree of KEGG pathway enrichment).

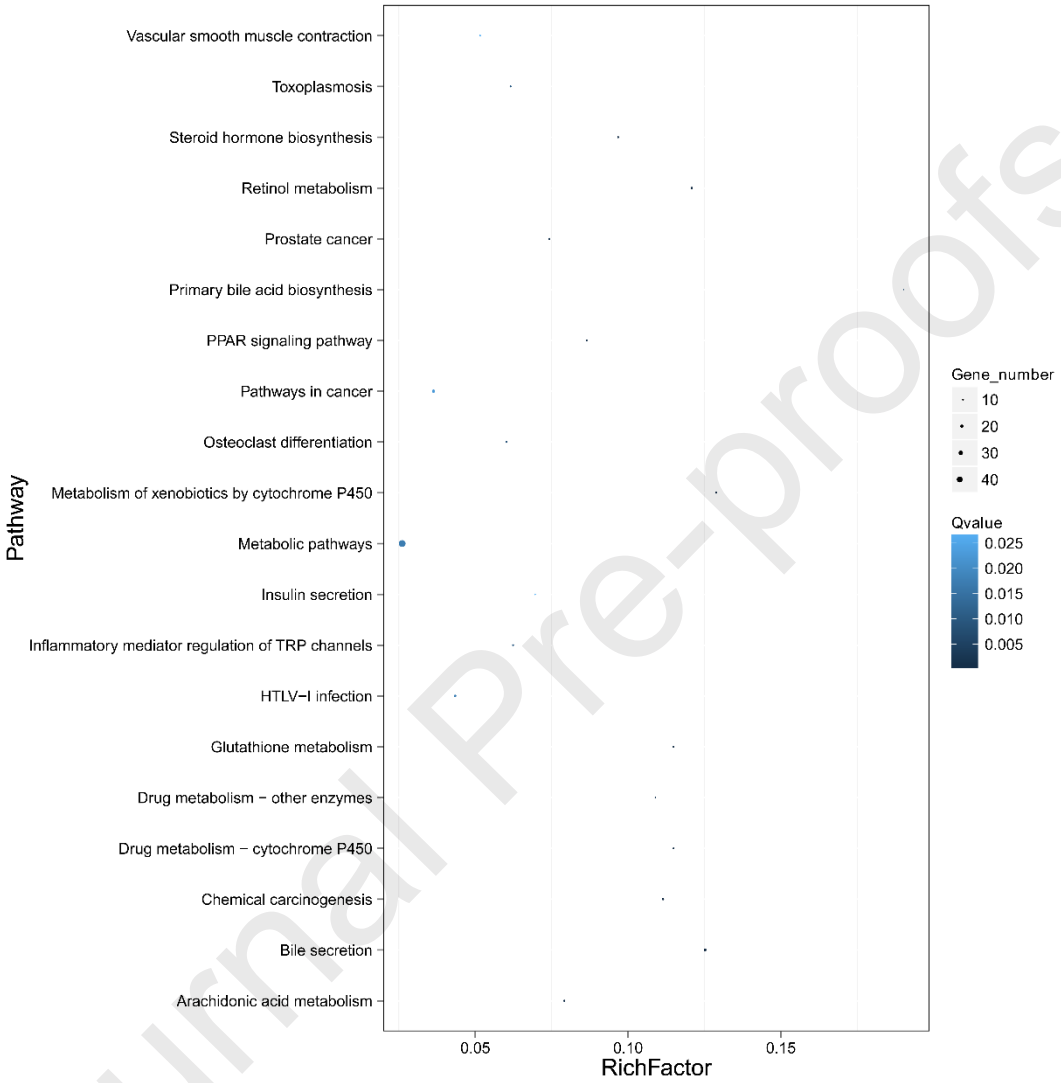
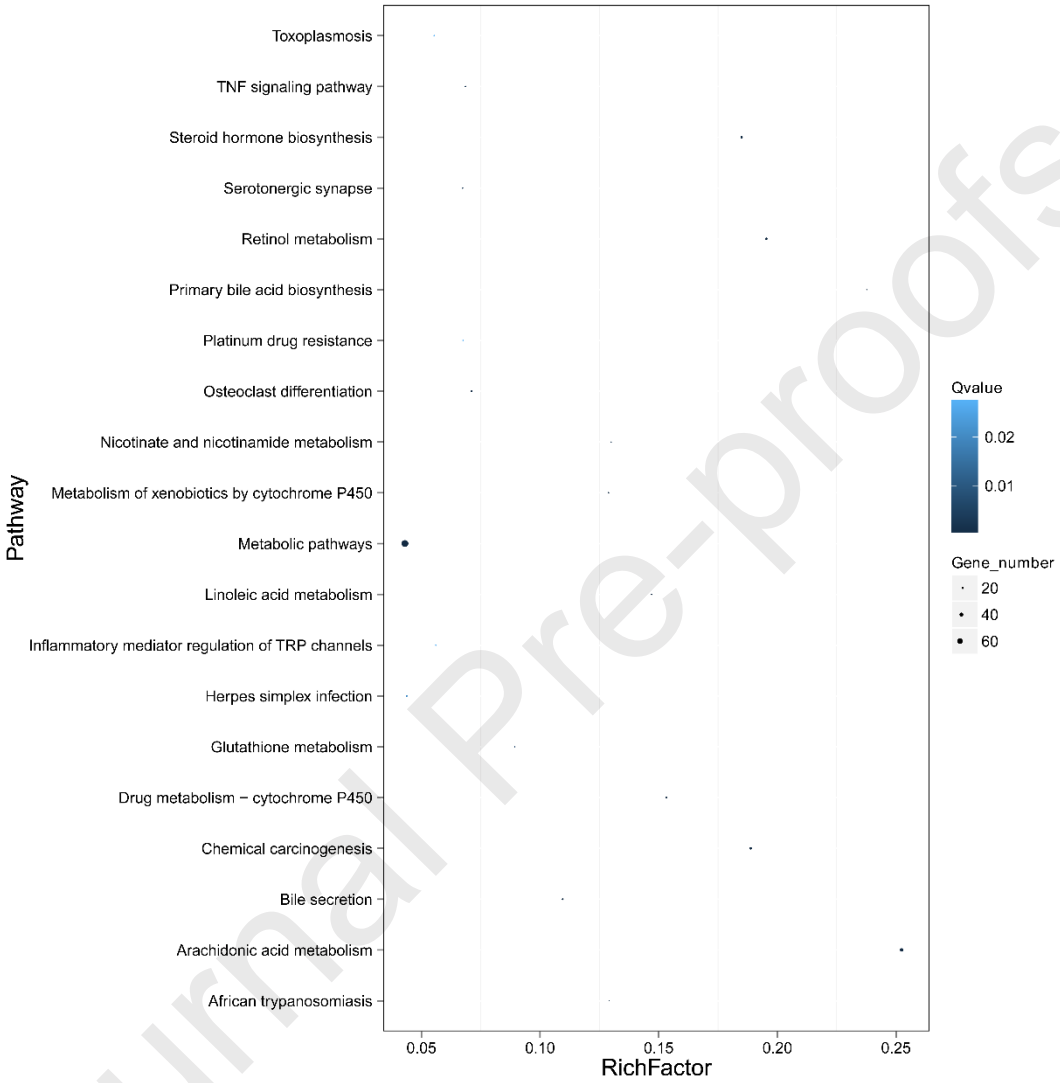


Fig.S11. Statistics of pathway enrichment of DEGs in liver

Q-value is corrected p value ranging from 0~1, and less Q-value means greater intensiveness. Y axis is for the name of pathway and X axis represents the rich factor (the degree of KEGG pathway enrichment).



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Organ-differential responses to ethanol and kynurenic acid, a component of alcoholic beverages in gene transcription

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Conflicts of interest

There are no conflicts of interest to declare.