Analysis of differentially expressed genes and microRNAs in alcoholic liver disease

YING LIU[1*], SHAO-HUA CHEN[2*], XI JIN[2] and YOU-MING LI[2]

[1]Department of Gastroenterology, Heilongjiang Province Hospital, Harbin, Heilongjiang 150036;
[2]Department of Gastroenterology, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310003, P.R. China

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Abstract. The purpose of this study was to screen differentially expressed genes and microRNAs in order to find a new target for the accurate diagnosis and effective therapy of alcoholic liver disease (ALD) at the gene and microRNA levels. The total RNA of liver tissues was extracted from four groups of patients, ten subjects each. Microarrays were utilized to detect differentially expressed genes and microRNAs. According to gene values, significance levels and false discovery rate with a random variance model, gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, node genes and key microRNAs in networks were obtained and analyzed. A total of 878 differentially expressed genes and 26 microRNAs were found. In co-expression genetic networks, node genes modulating the network were Acyl-coenzyme A synthetase-3 (ACSF3), Frizzled-5 (FZD5), LOC727987 and C1orf222. In microRNA-gene networks, the key microRNAs were hsa-miR-570, hsa-miR-122, hsa-miR-34b, hsa-miR-29c, hsa-miR-922 and hsa-miR-185, which negatively regulated approximately 79 downstream target genes. In the course of ALD, we found 4 differentially expressed node genes and analyzed ACSF3 and FZD5. ACSF3 was significantly upregulated, and was involved in fatty acid and lipid metabolism and accelerated liver injury. These two genes were involved in fatty acids and lipid metabolism. FZD5 was downregulated and reduced the synthesis of membrane transport protein in the hepatic membrane and the membrane stability, and accelerated the liver cell apoptosis process. Six key microRNAs regulated numerous biological functions such as the immune response, the inflammatory response and glutathione metabolism. This finding provides valuable insight into the diagnosis and treatment of ALD.

Correspondence to: Dr You-Ming Li, Department of Gastroenterology, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310003, P.R. China
E-mail: hhd1118@163.com

*Contributed equally

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Introduction

Alcoholic liver disease (ALD) consists of a spectrum of diseases, including mild liver injury, alcoholic fatty liver, alcoholic hepatitis, and alcoholic cirrhosis that has the potential of progressing to hepatocellular carcinoma. China has a higher prevalence of viral hepatitis than western countries (1), although it has shown a declining trend year by year (2). Alcohol consumption has resulted in a gradual increase in morbidity and mortality of ALD (3). However, the epidemiology of ALD in China remains vague, with only few reports at the provincial level. Previous investigations showed the incidence of ALD was approximately 4-5% in Zhejiang, Xi'an, Guizhou and Taiwan (4). Due to numerous complications related to ALD and the heavy economic burden of its treatment, the pathogenesis of ALD has attracted the attention of much research. In several Chinese studies, rat models were used to investigate different genes, signaling pathways and different microRNAs in a certain stage of hepatic injury (5,6). Although marked progress has been made at the molecular level, the detailed differentially expressed pathogenic genes and microRNAs which downregulate these genes remain unknown.

High-throughput gene and microRNA microarray technology has been used to explore gene expression and regulation, transcription in various diseases (7). microRNAs often negatively regulate gene expression at the post-transcriptional level by incompletely binding to target sequences within the 3'-UTR, and generally do not affect the expression of mRNA (8). Therefore, we combined these two technologies to screen differentially expressed genes and microRNAs in ALD and analyzed their biological functions. To our knowledge, this is the first report on these differentially expressed genes and microRNAs in different stages during the course of ALD, using patient liver tissues. The results of our study may aid in exploring the pathological mechanisms of ALD, and may provide new targets for early diagnosis and gene therapy.

Materials and methods

Patients. Subjects from the Gastroenterology Department and the Outpatient Department of Heilongjiang Provincial Hospital (Harbin, China) were enrolled in the study from December 2010

Table I. Patient characteristics.

Specimen no.	Age	Gender	Drinking duration (year)	Daily alcohol consumption (g)
1. Hepatitis	35	M	10	150-200 liquor
2. Healthy	67	M	0	
3. Healthy	36	M	0	
4. Cirrhosis	68	M	40	250 liquor
5. Cirrhosis	55	M	25	150/2 bottles liquor/beer
6. Cirrhosis	53	M	30	200-300 liquor
7. Hepatitis	37	M	17	100 liquor
8. Hepatitis	40	M	15	4 bottles beer
9. Alcohol-free liver	45	M	13	3 bottles beer
10. Alcohol-free liver	56	M	27	80-100 liquor
11. Healthy	45	M	0	
12. Alcohol-free liver	49	M	11	120/2 bottles liquor/beer

Table II. Liver functions of ALD patients.

Specimen no.	AST (IU/l)	AST (IU/l)	GGT (IU/l)	ALP (IU/l)	TB (mmol/l)	Albumin (g/l)	PT (%)
1. Hepatitis	194	70	236	165	26.57	32	67
4. Cirrhosis	278.5	124.6	556.7	198.3	64.4	25	62
5. Cirrhosis	179.6	112	434	387.9	74.6	23	57
6. Cirrhosis	332.2	289.1	650.2	734.3	121.5	21	47
7. Hepatitis	208	123	765	531	57	38	71
8. Hepatitis	98.4	67.1	232.5	332.4	21.8	39	76

to January 2011. Total RNA of liver tissues was extracted from four groups of patients, with ten subjects in each group: healthy subjects, drinkers without liver disease, alcoholic hepatitis, and alcoholic cirrhosis (Tables I and II). The diagnostic criteria for ALD were based on the guideline for diagnosis and treatment established by the Division of Fatty Liver and Alcoholic Liver Disease, Chinese Society of Hepatology. The protocols of this study were approved by the institutional review board of the First Affiliated Hospital, Zhejiang University School of Medicine and Heilongjiang Province Hospital. Written informed consent was obtained from all participants.

Extraction and labeling of sample RNA. Total RNA of liver tissues was extracted from liver tissue using Invitrogen TRIzol, and then the RNeasy kit (Qiagen, Shanghai, China) was used for further purification of total RNA. Purified total RNA was then stored at -80˚C until use. Agar electrophoresis or the Agilent 2100 Bio Analyzer System (Agilent, Santa Clara, CA, USA) was used to analyze the integrality and quality of total RNA.

A total of 500 ng total RNA was used to synthesize double-stranded complementary DNA (cDNA) *in vitro* using T7-Oligo(dT) as a primer according to the manual of the Ambion Illumina TotalPrep RNA Amplification kit (Ambion, Austin, TX, USA). Double-stranded cDNA was transcribed and synthesized into complementary RNA (cRNA), which was further amplified and labeled with biotin. Labeled cRNA was purified and stored at -20˚C.

Quantitative measurement of the probe. The cRNA synthesized *in vitro* and labeled with biotin was quantitatively measured using a RiboGreen kit (Molecular Probes, Carlsbad, CA, USA).

Microarray hybridization and image scanning. A total of 1.5 μg purified and biotin-labeled cRNA was dissolved in 10 μl water and was then added into GEX-HYB buffer. The mixture was added to the microarray. Microarray hybridization was carried out at 58˚C in a hybridization oven. The microarray was incubated with EIBC solution and was washed at a high temperature; it was then rinsed with 100% ethanol, sealed with E1 buffer, stained with Cy3 dye, and dried. Microarray was scanned to extract the signals following hybridization using a high precision laser confocal scanner (Illumina scanner, 0.8 M).

Bioinformatic data processing. An Illumina error model was used for the analysis of differentially expressed genes. $P<0.05$ was considered to indicate statistically significant differences. The false discovery rate (FDR) was used to evaluate the significance of gene ontology (GO) and pathways. By combining the GO database with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, functional classifications and important pathways of related differentially expressed genes were analyzed. TargetScan and MicroCosm were used to analyze the differentially expressed microRNAs and their negatively regulated genes.

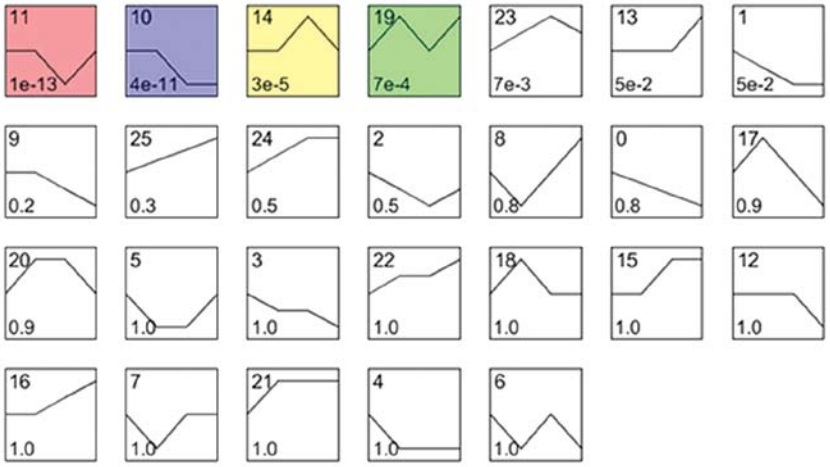

Figure 1. The four significant clustering trends of differentially expressed genes. Trend 11 of the gene expression was invariant at first, then downregulated, and it finally returned to normal level. The variation of trend 14 was contrary to that. Gene expression in trend 10 was invariant at first, then downregulated, then, to a certain extent, it remained unchanged; trend 19 was upregulated at first to the maximum, then downregulated, and when it was downregulated to the healthy level, it was upregulated again.

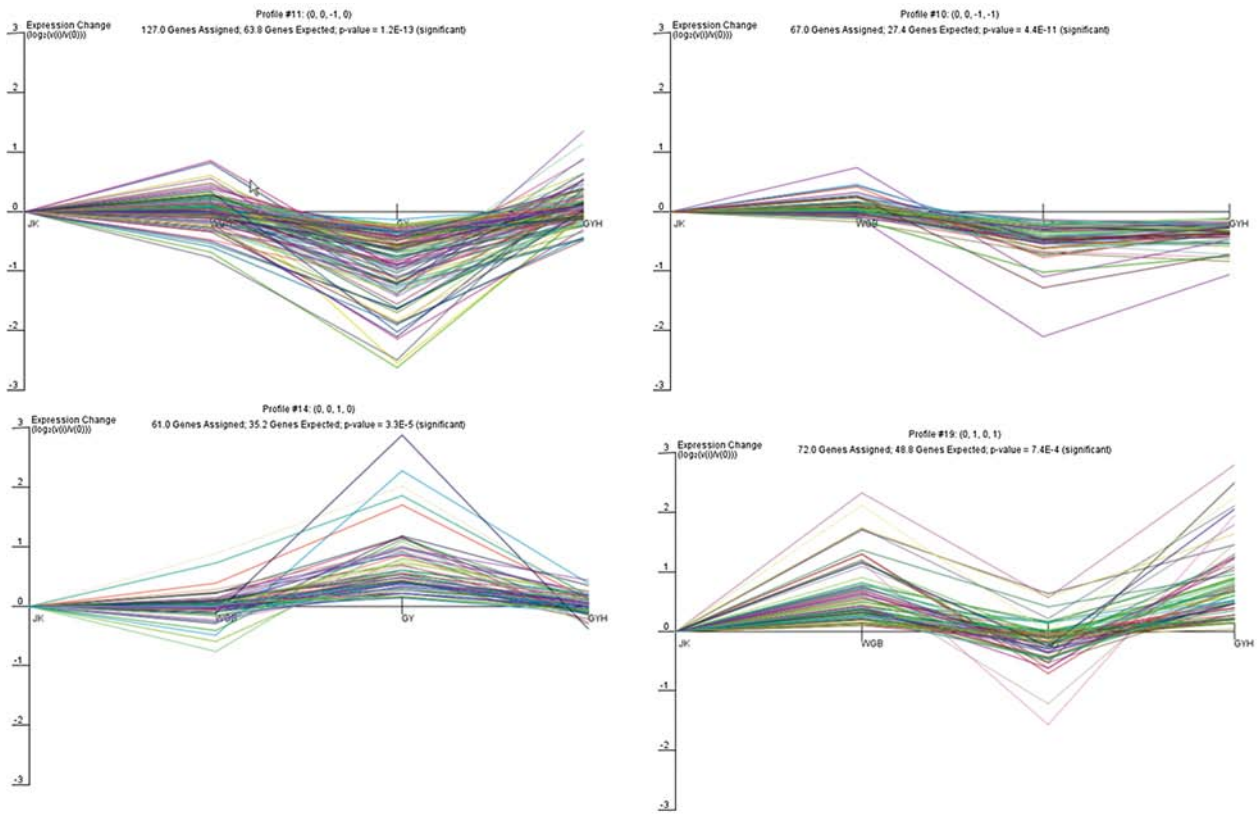

Figure 2. The curve of each significant clustering trend. The horizontal axis represents time (each stage of the disease), vertical axis represents gene expression, the healthy controls as the initial point, the logarithm of ratio of the latter and former as vertical coordinate. A line represents a gene.

Results

Identification of differentially expressed genes and microRNAs in four groups. In microarray scanning, there were 10 subjects in each group. We chose the random variance model to calculate P-value and FDR of microRNAs. P<0.05 was considered to indicate statistically significant differences. There were 878 differentially expressed genes and 26 microRNAs among the four groups during the stages of ALD.

Clustering trend of differentially expressed genes. Despite the large number of differentially expressed genes obtained by microarray scanning, not all have biological effects on the occurrence and development of ALD. Therefore, we analyzed the gene clustering trend based on the gene expression value. We found the genes with identical expression trends had similar functions or were involved in the same biological processes during ALD. Four significant trends of gene clustering were obtained (Fig. 1). We observed

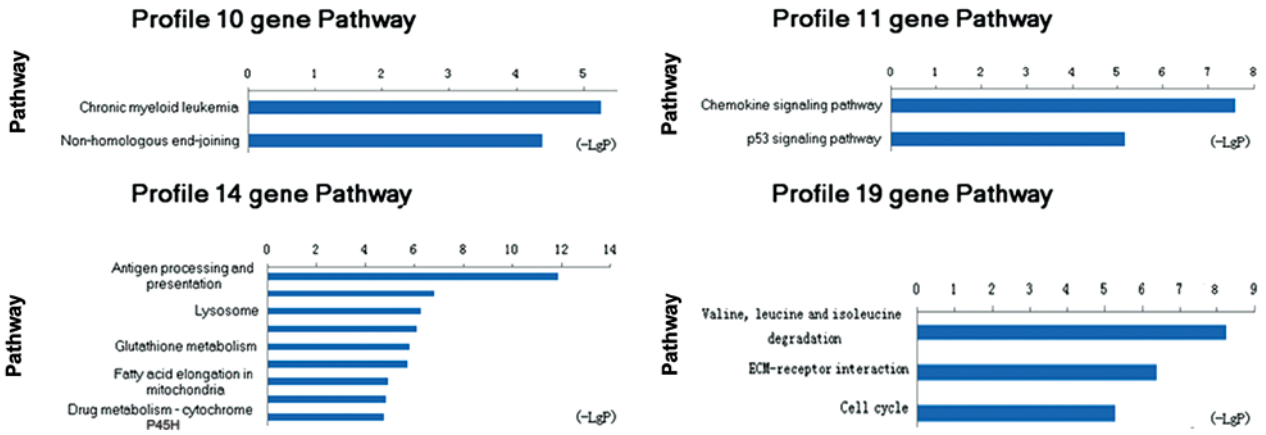

Figure 3. Significant levels of 16 pathways in four clustering trends. Logarithm of P-value of each pathway is the horizontal axis, each pathway name is the vertical axis..

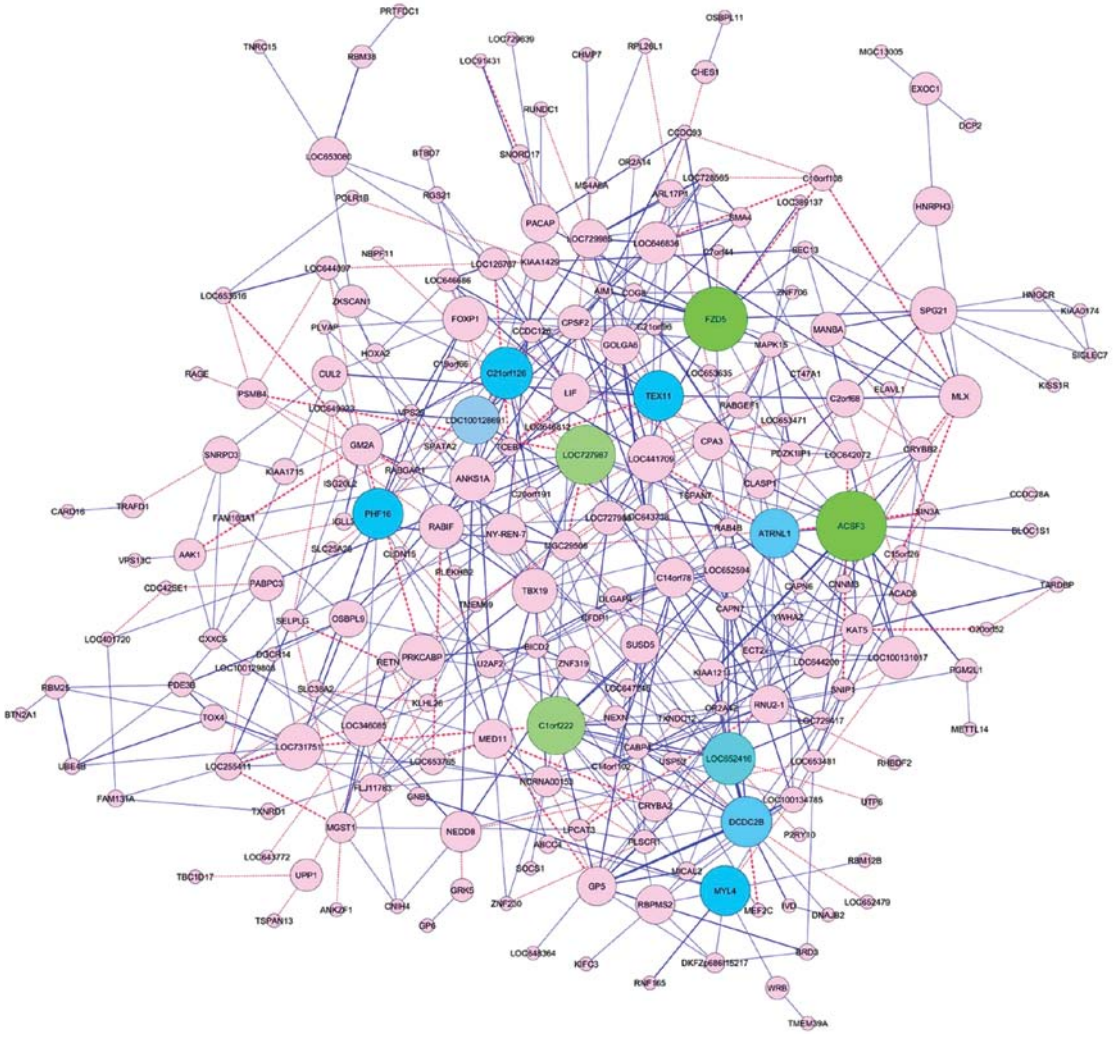

Figure 4. Regulation network of gene expression. The green points represent the node gene, the blue and the pink points represent the first and next stage genes which the node genes regulated, respectively. The area and depth of color of points represent the importance of genes in network. The line represents the interaction among genes, in which the solid line represents gene expressions which are positively correlated (activation), while dashed lines represent the negative correlation (inhibition).

the variation of four trends and found that trend 11 of the gene expression was invariant at first, then downregulated, and it finally returned to normal level, consisting of 127 significantly different genes. The trends 10, 14 and 19 had their corresponding variation of gene expression (Fig. 2), consisting of 67, 61, 72 significantly different genes, respec-

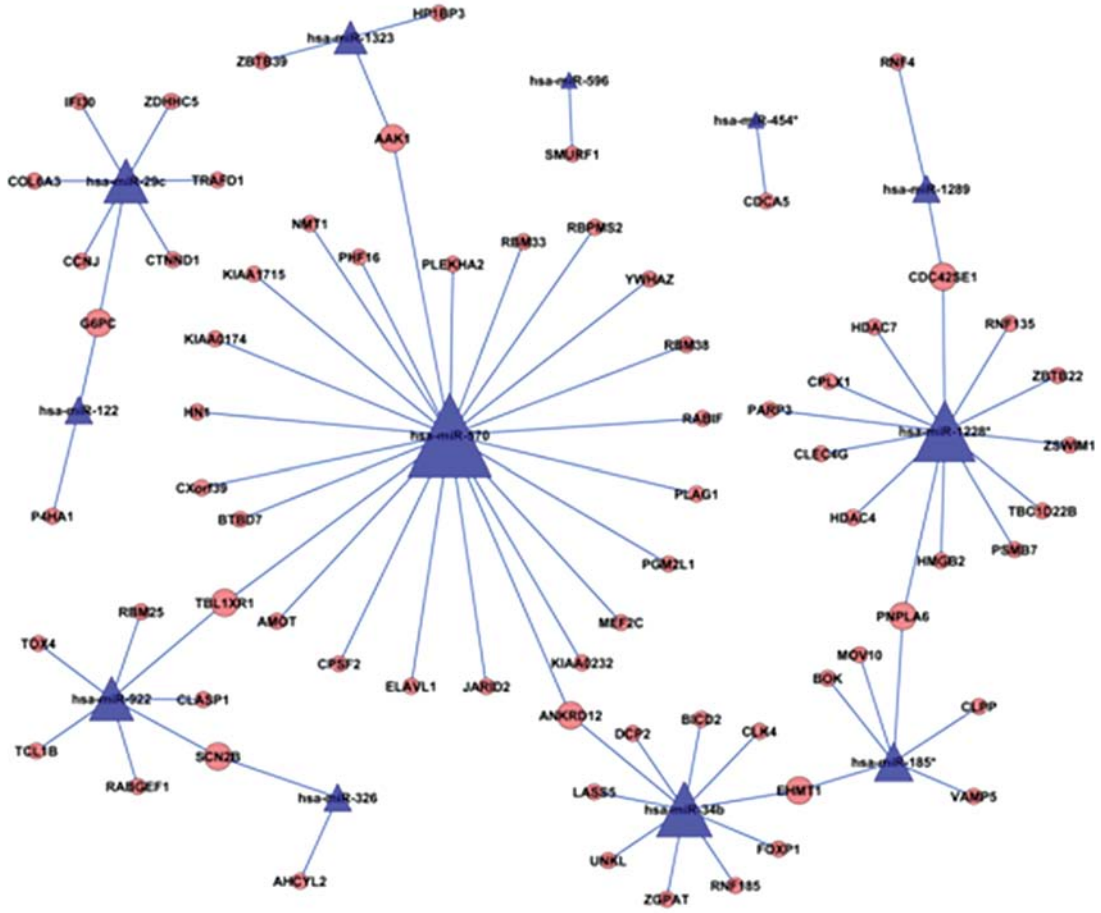

Figure 5. microRNA-gene regulation network. The blue triangles express different microRNAs, the pink circles express genes which were negatively regulated by microRNAs. The area of a triangle is used to determine the important position in the network.

tively. In total, 327 different genes were involved in the four signal clustering trends in ALD.

Significance analysis of gene function and pathway. The genes with identical expression trends had similar functions or participated in the same biological processes during ALD. Therefore, we analyzed functions and pathways of genes in profile clustering trends based on the GO and the KEGG databases. $P<0.05$ was considered to indicate statistically significant differences. The FDR was used to evaluate the significance of GO and pathway. We took the logarithm of P-value of each GO as the horizontal axis, representing significant level in the trend. The vertical axis represents each corresponding GO-term. In brief, 317 significantly different GOs and their significant level in four clustering trends were described. Sixteen significantly different pathways were found, where 31 corresponding genes participated in these 16 pathways (Table III). Logarithm of P-value of each pathway was the horizontal axis, each pathway name was the vertical axis; the significant level of 16 pathways in four trends is described (Fig. 3).

Construction of the co-expression network of genes. The expression values of genes showing significant trends were used to calculate the co-expression correlation coefficients, which, together with significance level, were used to construct the co-expression network (Table IV and Fig. 4). Betweenness centrality of each gene in the co-expression network was calculated, and describes the degree of importance of the nodes in the whole network. The bigger the betweenness centrality is, the more important the position in the network of genes. The node genes modulating the network were Acyl-coenzyme A synthetase-3 (ACSF3), Frizzled-5 (FZD5), LOC727987 and C1orf222. ACSF3 had the maximal betweenness centrality in the entire gene-modulating network, with a betweenness centrality of 0.102935, and was downregulated by 21 microRNAs. The second was FZD5, downregulated by 17 microRNAs, whose betweenness centrality was 0.087. The other two were 0.07683 and 0.073813, downregulated by 13 and 16 microRNAs, respectively.

Joint analysis of differentially expressed genes and microRNAs. Based on the TargetScan and MicroCosm database, the obtained genes, which had both target regulation and negative expression to microRNAs were used for the microRNA gene regulation network (Fig. 5). The key microRNAs in the gene co-expression network were hsa-miR-570, hsa-miR-122, hsa-miR-34b, hsa-miR-29c, hsa-miR-922, hsa-miR-185, the total number of genes that they negatively regulated was 79. Hsa-miR-570 was the most important, downregulating 24 genes such as AAK1, TBL1XR1 and ANKRD12.

Table III. Significant pathways and genes they involve.

Pathway ID	Pathway name	Differences in pathway	P-value	FDR	Contributing genes
path:hsa04062	Chemokine signaling pathway	4	0.0052204307278	0.16183335256	CXCL5, GNB5, GRK5, RASGRP2
path:hsa04115	p53 signaling pathway	2	0.028061295046	0.43495007322	MDM4, SIAH1
path:hsa00280	Degradation of valine, leucine and isoleucine	2	0.0032905901894	0.055940033219	ACAD8, IVD
path:hsa04512	ECM–receptor interaction	2	0.011866746986	0.10086734938	GP5, GP6
path:hsa04110	Cell cycle	2	0.025762276056	0.14598623098	CDC14B, YWHAZ
path:hsa05220	Chronic myeloid leukemia	2	0.026257111723	0.42340164739	CBL, TGFBR2
path:hsa03450	Non-homologous end-joining	1	0.047803391687	0.42340164739	LOC731751
path:hsa04612	Processing and presentation of antigen	4	0.00026849550669	0.013156279828	CTSB, IFI30, PSME1, TAP1
path:hsa05020	Prion diseases	2	0.0090623148839	0.15612302009	C1QC, EGR1
path:hsa04142	Lysosome	3	0.013201362888	0.15612302009	CTSB, GM2A, NPC2
path:hsa03050	Proteasome	2	0.014701427334	0.15612302009	PSMB4, PSME1
path:hsa00480	Glutathione metabolism	2	0.017962637503	0.15612302009	GSTK1, MGST1
path:hsa04120	Ubiquitin-mediated proteolysis	3	0.019117104501	0.15612302009	SOCS1, TCEB1, UBE2L6
path:hsa00062	Elongation of fatty acids in mitochondria	1	0.032767838442	0.19798547604	HADHA
path:hsa00980	Metabolism of xenobiotics by cytochrome P45H	2	0.034559330627	0.19798547604	GSTK1, MGST1
path:hsa00982	Drug metabolism-cytochrome P45H	2	0.036364679273	0.19798547604	GSTK1, MGST1

Pathway name represents the detailed pathway. P-value represents the significant level, and $P<0.05$ is considered to indicate statistically significant differences. When the false discovery rate is smaller, the error of judgment for each pathway is smaller. Differences in pathway refer to the number of contributing genes being different in each pathway.

Table IV. Node genes in network.

Gene	Definition	Betweenness	Degree
ACSF3	Homo sapiens acyl-CoA synthetase family member 3 (ACSF3), mRNA	0.102935	21
FZD5	Homo sapiens frizzled homolog 5 (Drosophila) (FZD5), mRNA	0.087438	17
LOC727987	Predicted: Homo sapiens similar to protein C21orf70 homolog (LOC727987), mRNA	0.07683	13
C1orf222	Homo sapiens chromosome 1 open reading frame 222 (C1orf222), mRNA	0.073813	16

Betweenness centrality of each gene in network describes the degree of importance of the nodes in the whole network. When the betweenness centrality is bigger, the position in the network of gene is more important. Degree stands for the number of microRNAs which negatively regulate the node genes.

Discussion

ALD consists of a series of liver diseases induced by long-term and excessive consumption of alcohol. ALD also belongs to the 10 most common causes of mortality in North America and Europe (9). The pathogenesis of ALD has yet to be fully elucidated, although three hypotheses have been postulated (10); the alcohol dehydrogenase (ADH) pathway, microsomal ethanol oxidase system (MEOS), and the catalase (CAT) pathway. Several studies have focused on activated hepatic stellate cell (11), gene polymorphism and mRNA expression in patients (12), lipid peroxidation (LOP) reaction (13), and apoptosis. However, they lacked the detailed mechanism at the genetic function level and microRNA regulation. In addition, most experiments utilized rat models. Therefore, the accuracy and the pathogenesis of ALD remain controversial.

In our study, we employed high work accuracy and efficiency microarray technology, we used hepatic tissue of patients diagnosed with ALD by clinical examination, and we identified 878 differentially expressed genes and 26 microRNAs. This comprised 317 significant GOs, 16 significant pathways and obtained the 5 node genes in the co-expression network and six key microRNAs in the microRNA-gene regulated network.

Acyl-coenzyme A synthetase-3 (ACSF3) was the first key node gene in the gene-modulating network, which was located at 16q24.3 (14). It included nine exons and two transcript variant districts, encoding 576 amino acids (15). ACSF3 binds to thioester and CoA to activate fatty acids for the formation of acyl-coenzyme A, which is involved in the metabolism of fatty acids and lipids as well as ATP binding (16). During the development of ALD, ACSF3 may promote lipid peroxidation, it may interfere with the metabolism of fatty acids (17) and it may aggravate hepatocyte injury and ATP consumption. These effects are unfavorable for hepatocyte repair (18). By calculating gene values, we found that, during dynamic development of ALD, clear upregulation of gene expression of ACSF3 was displayed. Furthermore, the maximal betweenness centrality in the entire gene-modulating network was 0.102935, indicating its controlling capability and importance. Continuous upregulation of ACSF3 maintains transcriptional activation and, subsequently, strengthens the binding of ACSF3 to thioester and CoA to activate fatty acids, which results in the irreversible advancement of ALD from hepatitis to cirrhosis.

Frizzled-5 (FZD5) was the second key node gene in the gene-modulating network. It is a multi-transmembrane glycoprotein composed of 585 amino acids. FZD is the receptor of the Wnt protein and has important effects on the Wnt signaling pathway (19,20). If the Wnt signaling pathway is activated, Wnt binds to the FZD receptor and inhibits the degeneration of β-catenin. The latter is kept at a stable level in the cytoplasm, is gradually transported to the nucleus after accumulation and is related to modulating apoptosis and development (21,22). The gene values of FZD5 in alcoholic hepatitis and cirrhosis were clearly downregulated. The second highest value of betweenness centrality in the entire gene-modulating network was 0.087. A possible mechanism may be that continuous downregulation of FZD5 during advancement of alcoholic hepatitis to cirrhosis decreased gene activities and reduced the syntheses of multi-transmembrane transport proteins. This would reduce the binding capability of FZD5 with the Wnt protein receptor

as well as the number and stability of β-catenin in the cytoplasm. The number of β-catenin molecules in hepatocyte nucleus and therefore promoted hepatocyte apoptosis (23).

The specific biological functions of the LOC727987 and C1orf222 genes remain unclear and warrant further exploration.

There were six key microRNAs that negatively regulated 79 genes as their downstream targets. Hsa-miR-570 is the most important in the network and controls 24 downstream genes, including Homo sapiens AP2 associated kinase 1 (AAK1). The mu2 subunit of the AP2 complex is known to be phosphorylated *in vitro* by a copurifying kinase, and it has previously been demonstrated that mu2 phosphorylation is required for transferrin endocytosis (24). Homo sapiens transducin (β)-like 1X-linked receptor 1 (TBL1XR1) is a multifunctional co-repressor of transcription. The structure of this family of molecules is highly conserved and closely related co-repressors have been found in all eukaryotic organisms. Regulation of co-repressor expression and the consequent alterations in transcriptional silencing play an important role in the regulation of differentiation (25,26). However, the detailed functions of AAK1 and TBL1XR1 in ALD remain unknown. These 79 regulated genes play numerous biological functions, including immune response, activity of cancer gene, inflammatory mediated response, apoptosis process, cell cycle, glutathione metabolism, metabolism of xenobiotics by cytochrome P45H, drug metabolism, proteolysis, and fatty acid elongation in mitochondria. Therefore, the downstream genes regulated by microRNAs participate in numerous complex biological mechanisms in the process of ALD. If the activities of node genes and key microRNAs were inhibited, or their regulatory pathways among other genes in the network were inactivated, the whole regulatory network would be paralyzed, and the occurrence and development of ALD would then be influenced.

We previously examined microRNA expression in different stages in nonalcoholic fatty liver using rat models, and gained certain insight (27). However, we need to verify the conclusions and the detailed functions of these node genes and regulatory mechanism of the key microRNAs in a large number of samples. Real-time qPCR was used to confirm the expression of these genes. Although there is some deficiency in our experiment design, data analysis and writing in our study, we believe these data provide a theoretical basis for further studies on the pathogenesis and gene therapy of ALD.

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