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ORIGINAL RESEARCH

Exploration and Validation of Key Genes and Immune Infiltration in Alcoholic Hepatitis

Yingna Mei[1], Yitao Zheng[2], Xingfen Zhang[1], Ting Hu[1], Xiaoqing Zheng[1], Cui Zhang[3], Qinzhi Deng[1]

[1]Department of Liver Disease, Ningbo No.2 Hospital, Ningbo, People's Republic of China; [2]Department of Pancreatic and Gastrointestinal Surgery Division, Ningbo No. 2 Hospital, Ningbo, People's Republic of China; [3]Department of Cardiology, Renmin Hospital of Wuhan University Cardiovascular Research Institute, Wuhan University; Hubei Key Laboratory of Cardiology, Wuhan, 430060, People's Republic of China

Correspondence: Qinzhi Deng, Department of Liver Disease, Ningbo No.2 Hospital, Ningbo, People's Republic of China, Email dqznbey@126.com; Cui Zhang, Department of Cardiology, Renmin Hospital of Wuhan University, Wuhan, People's Republic of China, Email cuizhang2005@126.com

Objective: Apart from alcohol abstinence and glucocorticoids, there is still no effective treatment to improve alcoholic hepatitis, and the specific mechanism of its pathogenesis is still unclear.

Methods: We screened the differential genes in GEO alcoholic hepatitis database by differential analysis and screened the eQTL genes that have causal relationship with alcoholic hepatitis by Mendelian randomization analysis. The intersection of differential genes and eQTL genes was used to obtain candidate genes. The candidate genes were then screened out by machine learning, and their expression was further verified in clinical patients and mice with alcoholic hepatitis. Based on key genes, pathway analysis via single-gene GSEA analysis and immune microenvironment analysis via ssGSEA analysis were conducted to explore the relationship between key genes and immune microenvironment. Finally, regulatory relationship between key genes and immune cells was explored based on cell experiments.

Results: Based on the GEO Alcoholic hepatitis database (GSE28619 and GSE142530) and Mendelian randomization of eQTL genes, we obtained 17 candidate genes. We then obtained two key genes (CXCL8 and CTNNA1) through lasso and random forest tree algorithms. CXCL8 and CTNNA1 were highly expressed in the alcoholic hepatitis group, which were verified in clinical patients and mice. Through single-gene GSEA analysis, two key genes were identified to be enriched in the antigen presentation pathway. At the same time, the alcoholic hepatitis group had obvious immune infiltration disorder, and two key genes were correlated with immune environment via correlation analysis. B cells and NKT cells exhibited the highest correlation with key genes. In alcoholic hepatitis mice, liver infiltration of B cells and NKT cells was verified. Through cell experiments, ethanol exposure increased CTNNA1 and CXCL8 expression in NKT and B cells, enhancing inflammatory cytokine release and suppressing IgG production, respectively. Silencing CXCL8 and CTNNA1 reversed these effects.

Conclusion: These results suggested that CXCL8 and CTNNA1 were potential biomarkers for alcoholic hepatitis, and might be new targets for the treatment of alcoholic hepatitis.

Keywords: alcoholic hepatitis, immune environment, Mendelian randomization analysis, machine learning, CXCL8, CTNNA1

Introduction

Alcoholic hepatitis (AH) is a clinical syndrome marked by jaundice and severely liver dysfunction, caused by chronic excessive alcohol intake.[1] Its high morbidity and mortality have caused a huge burden to countries around the world.[2] Although alcoholic liver disease has relatively fixed diagnostic criteria, including the amount and duration of alcohol consumption,[3] it was sometimes difficult to assess and intervene early based on individual drinking patterns, genetic predisposition and other factors.[4]

Liver biopsy is an important diagnostic method for AH. However, liver biopsy is an invasive method that might lead to acute bleeding complications. Therefore, searching for non-invasive diagnostic biomarkers to assist in the assessment of alcoholic liver disease is particularly important. Mendelian randomization is an epidemiological tool that establishes causal reasoning between exposure and outcome by using genetic variation as an instrumental variable (IVs).[5] This approach reduces the impact of confounding factors that often influence traditional epidemiological studies, ruling out any possibility of reverse causality.[6]

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Besides, the treatment of alcoholic hepatitis is limited. Cortisol, as the main treatment, can only increase short-term survival, and 40% of patients could not respond to glucocorticoids.[7] Therefore, the search for new treatments is urgent. Prior research indicated that liver alcohol metabolism activated ROS, triggered inflammatory factor release, and disrupted the immune microenvironment,[8,9] such as CXCL8 was confirmed to be highly expressed in the liver of AH patients to promote inflammatory cell recruitment.[10] The released inflammatory factors recruited a variety of immune cells in liver, such as NK cells, T cells, and Kupffer cells. Activated Kupffer cells could activate the NF-kB pathway and further release pro-inflammatory factors such as IL-1β and TNF-α.[11] NK cells could promote apoptosis by producing interferon gamma (IFN-γ) and directly killing hepatic stellate cells.[12] These innate immune cells also recruited circulating immune cells to aggregate in liver, further contributing to liver cell damage. However, the regulatory mechanism of immune microenvironment disorders caused by alcohol consumption remains unclear.

Based on alcoholic hepatitis GEO database, combined with eQTL and GWAS database, this study explored the key genes and immune microenvironment of alcoholic hepatitis. At the same time, the relevant verification was carried out in human cohort, animal and cell experiments (Figure 1). Its potential biomarkers and related regulatory mechanisms may help provide new therapeutic directions.

Materials and Methods

Data Acquisition

The GSE28619 (15 AH vs 7 control), GSE142530 (13 AH vs 16 control) and GSE155907 (5 AH vs 4 control) datasets were downloaded from the Gene Expression Omnibus database, and sequenced using the GPL570, GLP11154 and GPL21290 platforms. GSE155907 was used as the validation cohort. Gene expression profiles were normalized using the "normalizeBetweenArrays" function in the "limma" package of R software (4.2.1). The batch effect between two datasets (GSE28619 andGSE142530) was corrected using the "ComBat" function from the "sva" R package (4.2.1).[13] The differentially expressed genes were extracted by "limma" package and visualized through heat and volcano maps (adj.p<0.05, $|\log_2 FC|>2$).

To further identify important genes causally associated with alcoholic hepatitis, we collected GWAS datasets on eQTL and AH from the GWAS Catalog website (https://gwas.mrcieu.ac.uk/). A total of 19942 eQTL datasets[14] were collected as exposure variable and AH dataset (finn-b-ALCOLIVER, 1416 cases vs 217376 controls) as outcome variable.

Mendelian Randomization Analysis

We choose single nucleotide polymorphisms (SNPS) significantly associated with the exposure data as instrumental variables (IVs) ($P < 5\times10^{-8}$) and removed SNPS with linkage imbalances (kb=10000, r^2=0.001).[15] To eliminate weak instrumental variables, the F statistic should exceed 10.[16] To investigate the causal relationship between eQTL and alcoholic hepatitis, several methods were employed, including inverse variance weighted (IVW),[17] Mendelian randomization-Egger (MR-Egger),[18] weighted median (WM),[19] simple mode,[20] and weighted model,[21] with IVW being the primary analysis method. A p-value less than 0.05 suggested a possible causal link. The stability of MR results was evaluated by heterogeneity, pleiotropy and one sensitive line remaining analysis.[15] The results were presented in scatter plots and forest plots. MR analyses were conducted using the "TwoSampleMR" package in R (version 4.2.1).

Key Genes Obtained for Alcoholic Hepatitis

The genes that exhibit a causal relationship with alcoholic hepatitis, as determined through Mendelian randomization analysis, were intersected with the differential genes identified in the GEO database. This intersection yielded 17 significant genes, which were visually represented using Wayne and forest diagrams.

In order to obtain the key genes that may play an important role in alcoholic hepatitis, we applied lasso algorithm and random forest model based on 17 significant genes. The LASSO algorithm (family = "binomial", alpha=1, nfolds = 10) was performed by "glmnet" package in R (version 4.2.1).[22] The random forest model (ntree = 500, importance=TRUE) was performed by "randomForest" package in R (version 4.2.1).[23] The genes extracted by the two machine learning methods were intersected to obtain two key genes. The expression levels of key genes were demonstrated by violin maps.

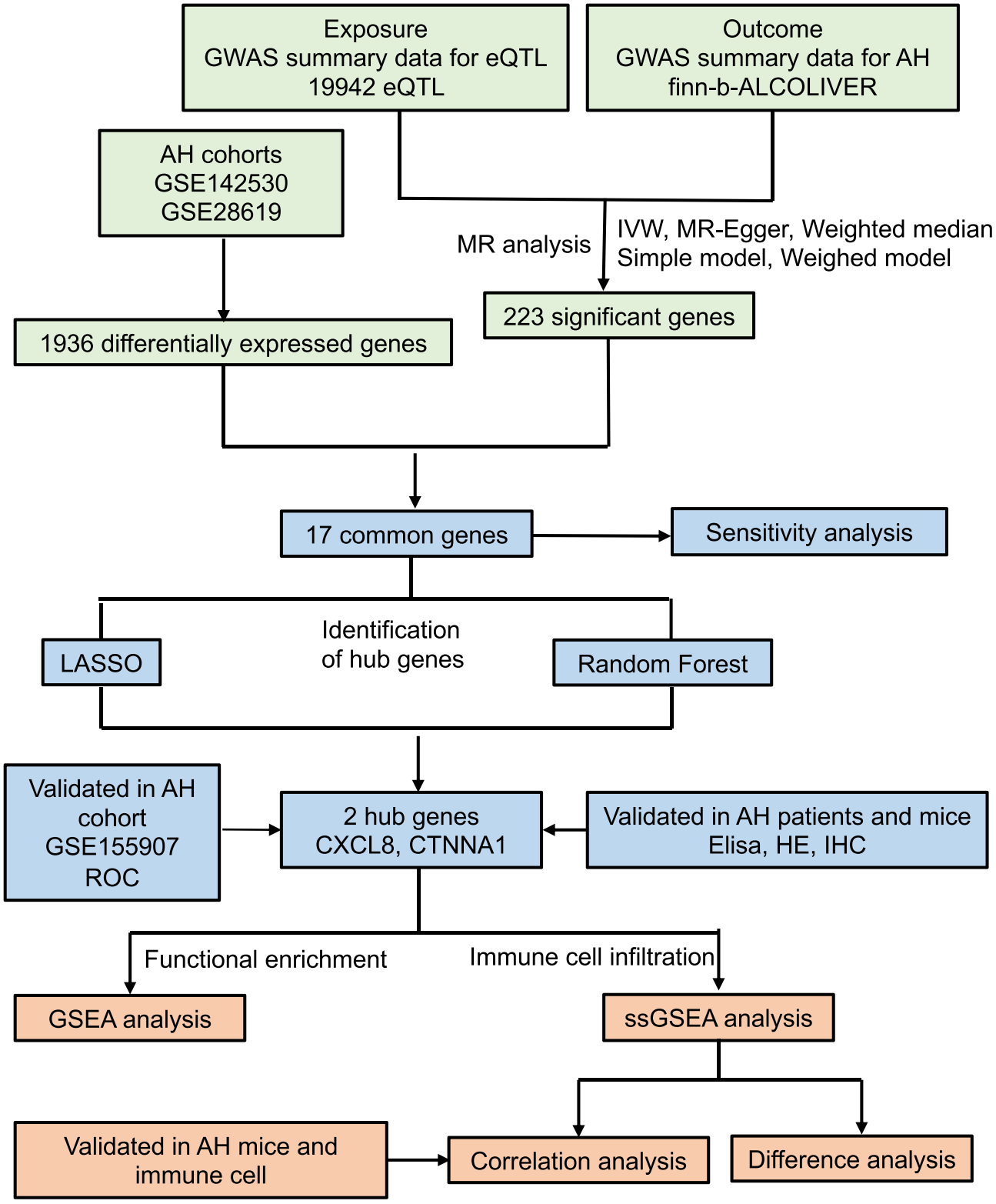

Figure 1 Overview of research design. Based on GEO database and GWAS database, we selected 17 common genes and screened out two key genes (CXCL8 and CTNNA1) through machine learning. Its expression was validated in clinical patients and model mice. Based on two key genes, single gene GSEA analysis and ssGSEA analysis were performed to investigate the correlation between key genes (CXCL8 and CTNNA1) and immune microenvironment. Finally, we conducted cell experiments to explore the regulatory relationship between key genes and immune cells.

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The "pROC" package[24] was utilized for receiver operating characteristic (ROC) analysis to evaluate the efficacy of key genes in differentiating AH from normal samples.

Patient Samples

Patient blood samples were collected from 13 alcoholic hepatitis patients and 13 normal physical examination patients in the Ningbo No. 2 hospital. Control patients were physical examination patients with no known disease and had not taken antibiotics or immunosuppressive drugs during the first two months of enrollment. The alcoholic hepatitis patients' inclusion criteria were: 1) age over 18 and under 65; 2) a history of alcohol consumption (>40g of alcohol per day for women and >60g for men for 6 months or more; 3) 50 IU/L <ALT, AST <400 IU/L, and the AST/ALT ratio was >1.5; 4) bilirubin concentration greater than 3 mg/dL^3; 5) 12≤MELD<20.[25]

Exclusion criteria were as follows: 1) patients with other types of liver diseases such as viral hepatitis, autoimmune hepatitis and drug-induced hepatitis; 2) fatty liver disease caused by other causes; 3) patients with cardiovascular and cerebrovascular diseases; 4) patients with tumor disease; 5) patients with other inflammatory diseases; 6) patients with abnormal thyroid function; 7) patients with abnormal functions of important organs; 8) patients with other metabolic diseases; 9) patients who have used immunosuppressants in the past 2 months; 10) women get pregnant.

Establishing Alcoholic Hepatitis Mice Model

Twelve 8-week-old SPF male C57BL/6J mice were kept at the animal center. Twelve mice were divided into control group (n=6) and AH group (n=6) by random number method. The AH group was fed alcohol liquid feed (Lieber-DeCarli diet)[26] (Dyets, Lieber-5kg), while the control group was fed Lieber-DeCarli control feed (Dyets, Lieber-C-5kg). Each mouse freely consumed the liquid feed for 3 weeks. Nine hours before sacrifice, AH mice were given alcohol intragastric administration (5 g/kg), while control mice were given the same volume and calories of maltose dextrin solution. Finally, all the mice were euthanized under isoflurane anesthesia. Blood was taken from eyeballs, which were then centrifuged to obtain serum. Serum levels of liver function indexes (ALT, AST, TBil and Alb) were measured using the test kit (BIOBASE, 10427010H,10323011H; Cchuili C120; RAYTO S03043) according to the manufacturer's instructions. Liver tissue samples were taken for follow-up experiments.

Hematoxylin-Eosin (HE) Staining

The liver tissues were fixed with 4% paraformaldehyde, cut into 4μm paraffin-embedded tissue sections (Leica). The sections were baked in an oven at 60 °C for about 2 hours, then placed in xylene and 100%,95%,80%, 70% ethanol for gradient dewaxing, respectively, and then stained with hematoxylin and eosin. Finally, the sections were placed in 70%, 80%, 95%, and absolute ethanol for gradient dehydration and observed under a microscopy (OLYMPUS).

Immunohistochemical and Immunofluorescence Staining

Liver tissues were fixed with 4% paraformaldehyde solution, paraffin embedded and sectioned. The immunohistochemical staining procedure followed established works.[27] The primary antibodies were CXCL2 (1:200) (proteintech, 83758-1-RR) and CTNNA1(1:100) (proteintech, 12831-1-AP). After DAB color development and restaining of nuclei, the sections were observed under a microscope, and image J was used for statistics.

For immunofluorescence staining, the primary antibodies contained CD3 (1:1000) (proteintech, 81324-1-RR), NKR-P1C (1:500) (Abcam, ab289542) and CD20 (1:1000) (Abcam, ab64088). Image J software was used to quantify the number of NKT and B cells under 400x magnification.

Analysis of Critical Genes Pathways

To investigate the potential regulatory pathways of critical genes, we performed single-gene GSEA analysis. Adjusted p values <0.05 for significant differences. The first 5 up-regulated and down-regulated pathways of key gene enrichment were shown, respectively.

Comparison of the Immune Microenvironment Between AH and Normal Groups

The "GSVA" package was utilized to conduct single-sample gene-set enrichment analysis (ssGSEA) for evaluating immune infiltration in AH and control samples. A violin plot was used to visualize the expression of immune cells in the two groups. Using Pearson correlation analysis test the relationship between the key genes and immune microenvironment. Boxplots showed high key genes expression and low-expression group immune cell infiltration.

Cell Culture and Cell Intervention

Human NKT cell line was obtained from Renmin Hospital of Wuhan University, and human B lymphocyte line (GM12878) was purchased from FUHENG BIOLOGY (FH1258). Two cell lines were cultured in 1640 medium supplemented with 10% fetal bovine serum, kept in the incubator at 37°C with 5% CO_2.

Before intervention, the cells were spread into six-well plates. When the cells had grown to 70% to 80%, they were replaced with medium without fetal bovine serum. Then, 100mM of EtOH was treated for 24h.[28] After that, cells were transfected with siRNAs (siCXCL8 and siCTNNA1) (General Biol (Anhui) Co., Ltd), cultured for 24h. Finally, NKT cells and B cells were extracted with protein and cell supernatant for subsequent experiments. The sequence of siRNA primers was shown in Table 1.

Elisa for CXCL8, CTNNA1 and IgG

The patients' blood upper serum and cell supernatant were applied for ELISA detection. CXCL8, CTNNA1 and IgG ELISA kits (Bioswamp HM10222, Bioswamp HM12272, Bioswamp HM10134) were used to calculate the serum CXCL8 and CTNNA1 concentrations of patients and IgG levels of cell supernatant according to the generated standard curves.

Western Blot

Protein extracts were obtained by lysing the cells, followed by grinding and centrifugation. Protein concentrations were quantified using the BCA Protein Assay Kit (Servicebio). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. The membranes were blocked and incubated overnight at 4°C with primary antibodies against CXCL8 (proteintech, 27095-1-AP), CTNNA1 (proteintech, 12831-1-AP), IL-1β (proteintech, 16808-1-AP), TNF-α (proteintech, 17590-1-AP), and β-actin (proteintech, 66009-1-Ig) in a suitable diluent.

The next day, membranes were incubated with appropriate HRP-conjugated secondary antibodies at room temperature for 2 hours. Protein bands were visualized using enhanced chemiluminescence, and band intensity was quantified using ImageJ software.

Statistical Analysis

The majority of the statistical analyses were conducted using R software version (4.2.1). The *t*-test was used to animal and cell experiments. $P < 0.05$ was considered statistical significance.

Result

Screening Candidate Genes for Alcoholic Hepatitis

We downloaded two AH GEO datasets (GSE142530 and GSE28619), comprising a total of 28 AH samples and 23 normal samples. The results showed that the treated samples were evenly distributed after the de-batch effect (Figure 2A). Subsequently, we identified 1936 differential genes through differential analysis, which were visualized by the heat map and volcanic map (Figure 2B and C).

Table 1 Sequences of siRNAs

Primer	5′-Sense-3′	5′-Antisense-3′
CXCL8-siRNA	GCAUAAAGACAUACUCCAATT	UUGGAGUAUGUCUUUAUGCTT
CTNNA1-siRNA	CCUCAGAGAUGGACAACUATT	UAGUUGUCCAUCUCUGAGGTT

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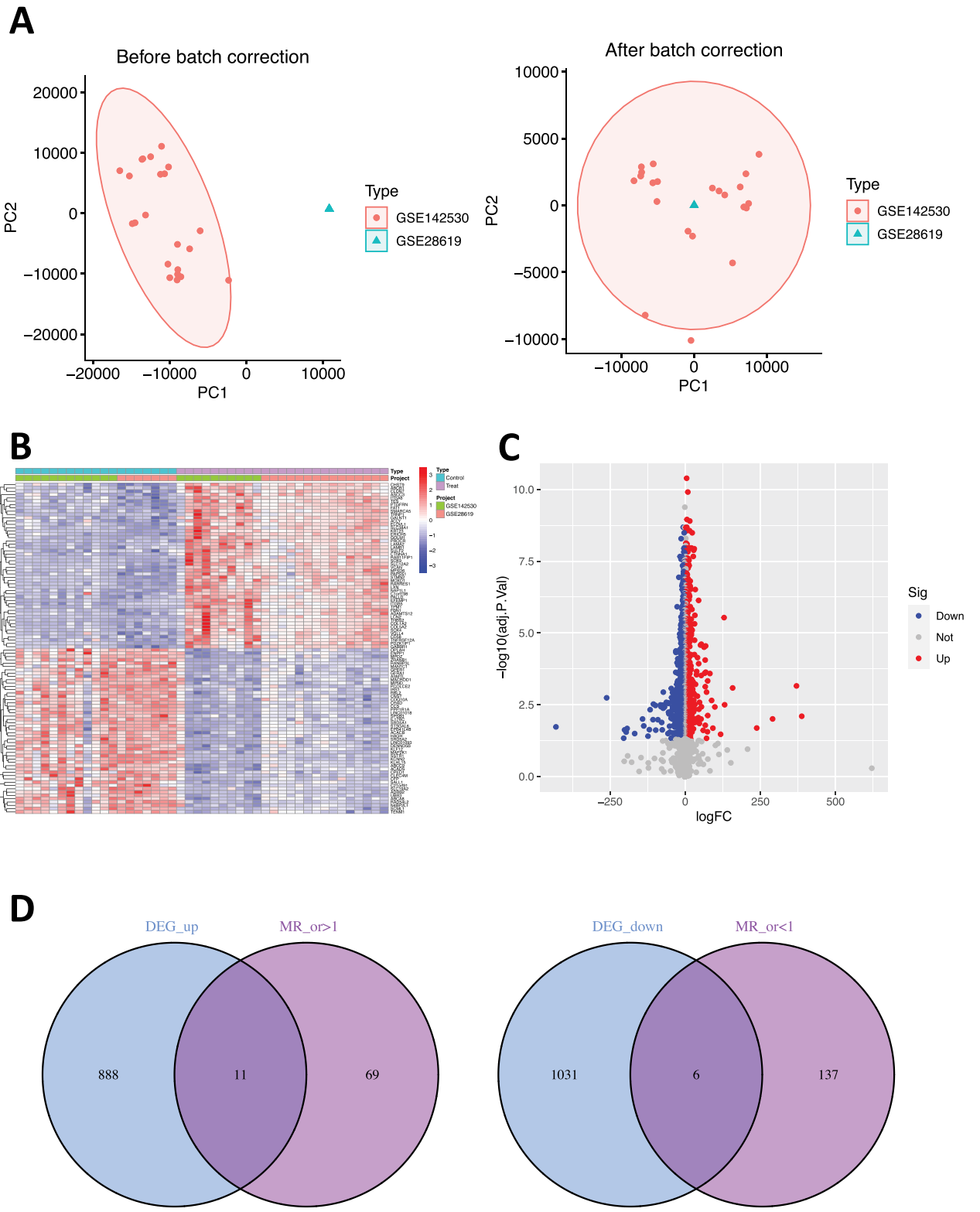

Figure 2 Screening candidate genes for alcoholic hepatitis. (**A**) The datasets before and after batch correction were visualized by principal component map. (**B** and **C**) Differentially expressed genes screened out from AH datasets were visualized by heatmap and volcano plot. (**D**) Venn diagram depicting the intersection of differentially expressed genes and eQTL genes.

Next, we performed Mendelian randomization analysis on eQTL data and alcoholic liver disease, and selected 223 candidate targets with possible causal relationship with alcoholic liver disease (IVW $p < 0.05$, pleiotropic analysis $p > 0.05$). The obtained candidate targets were intersected with the differential genes in GEO database, and 17 candidate genes were obtained, containing ABI1, ACSL1, ACTN4, CHST15, CTNNA1, CXCL8, CYSTM1, DBNDD1, DNASE1L3, EPHX1, IGFBP7, PIK3R1, PRKCSH, RDH5, TNFAIP2, TNFSF10 and TRIM8 (Figures 2D and 3). IVW analysis showed that ABI1, ACTN4, CHST15, CTNNA1, CXCL8, CYSTM1, DBNDD1, IGFBP7, PRKCSH, TNFAIP2 and TRIM8 were positively correlated with the risk of alcoholic hepatitis. ACSL1, DNASE1L3, EPHX1, PIK3R1, RDH5 and TNFSF10 were negatively

exposure	nsnp	method	pval	OR(95% CI)
ABI1	3	Weighted median	**0.014**	1.497 (1.087 to 2.062)
	3	Inverse variance weighted	**0.015**	1.460 (1.077 to 1.979)
ACSL1	4	Weighted median	**0.002**	0.426 (0.250 to 0.726)
	4	Inverse variance weighted	**0.037**	0.582 (0.350 to 0.967)
ACTN4	3	Weighted median	**0.015**	1.666 (1.102 to 2.519)
	3	Inverse variance weighted	**0.013**	1.664 (1.113 to 2.489)
CHST15	4	Weighted median	0.113	1.330 (0.935 to 1.891)
	4	Inverse variance weighted	**0.024**	1.435 (1.049 to 1.963)
CTNNA1	7	Weighted median	**0.048**	1.155 (1.001 to 1.332)
	7	Inverse variance weighted	**0.015**	1.165 (1.030 to 1.317)
CXCL8	9	Weighted median	0.108	1.145 (0.971 to 1.350)
	9	Inverse variance weighted	**0.035**	1.183 (1.012 to 1.382)
CYSTM1	4	Weighted median	**0.048**	1.388 (1.003 to 1.922)
	4	Inverse variance weighted	**0.030**	1.386 (1.032 to 1.860)
DBNDD1	4	Weighted median	0.067	1.196 (0.988 to 1.449)
	4	Inverse variance weighted	**0.033**	1.216 (1.016 to 1.456)
DNASE1L3	4	Weighted median	**0.006**	0.744 (0.604 to 0.917)
	4	Inverse variance weighted	**0.032**	0.772 (0.609 to 0.978)
EPHX1	3	Weighted median	**0.040**	0.684 (0.476 to 0.983)
	3	Inverse variance weighted	**0.027**	0.699 (0.508 to 0.961)
IGFBP7	6	Weighted median	0.132	1.178 (0.952 to 1.457)
	6	Inverse variance weighted	**0.028**	1.219 (1.021 to 1.454)
PIK3R1	4	Weighted median	0.074	0.728 (0.513 to 1.032)
	4	Inverse variance weighted	**0.041**	0.720 (0.526 to 0.986)
PRKCSH	3	Weighted median	**0.017**	1.536 (1.078 to 2.188)
	3	Inverse variance weighted	**0.004**	1.620 (1.167 to 2.250)
RDH5	3	Weighted median	0.064	0.846 (0.708 to 1.010)
	3	Inverse variance weighted	0.050	0.838 (0.702 to 1.000)
TNFAIP2	4	Weighted median	**0.003**	1.552 (1.159 to 2.080)
	4	Inverse variance weighted	**<0.001**	1.597 (1.231 to 2.072)
TNFSF10	4	Weighted median	**0.022**	0.725 (0.551 to 0.954)
	4	Inverse variance weighted	**0.036**	0.764 (0.594 to 0.983)
TRIM8	5	Weighted median	**0.038**	1.252 (1.012 to 1.547)
	5	Inverse variance weighted	**0.020**	1.260 (1.037 to 1.530)

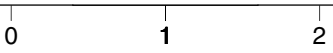

Figure 3 MR forest plot of candidate genes. MR displayed the relationship between the 17 candidate genes and alcoholic hepatitis. IVW < 0.05 was considered statistically significant.

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associated with the risk of alcoholic hepatitis (Figure 3). At the same time, the heterogeneity test and pleiotropy test of these genes were not statistically significant, which verified the robustness of our findings (Table 2).

Identify Key Genes of Alcoholic Hepatitis via Machine Learning

Based on 17 candidate genes, we applied LASSO and random forest algorithms to identify key genes. Through the lasso analysis, we obtained 5 genes (Figure 4A and B). At the same time, through the random forest model, we ranked the importance of genes and selected the top 5 genes (Figure 4C and D). Then, taking the intersection of the two machine learning analysis results, we obtained two key genes, CTNNA1 and CXCL8 (Figure 4E). Two violin graphs showed that the expression of two key genes was significantly increased in alcoholic hepatitis samples compared to normal samples (Figure 5A and B, $p < 0.001$). Meanwhile, the line graph reflected the expression levels of CTNNA1 and CXCL8 in each sample (Figure 5C). Similarly, the OR values of two key genes were both >1 in the results of Mendelian randomization analysis, suggesting that the high expression of these two key genes may promote the progression of alcoholic hepatitis. Furthermore, the ROC curves based on the training cohort and validation cohort both showed that the two key genes had good accuracy in predicting AH (Figure 5D–F).

Key Genes Verified in Alcoholic Hepatitis Patients and Mice

We applied the Elisa assay to evaluate CXCL8 and CTNNA1 levels in AH patients. The results showed that the expression of CXCL8 and CTNNA1 was increased in AH patients compared to healthy people (Figure 6A, $p<0.001$).

In addition, we constructed AH mice model. The transaminase (ALT and AST) and total bilirubin levels in model group were up-regulated than that in control group (Figure 6B, $p<0.05$, $p<0.001$), while the expression level of albumin had no significant difference between two groups (Figure 6B). HE staining showed increased steatosis and Mallory bodies in hepatocytes of model group (Figure 6C). These results indicated that the alcoholic hepatitis mice model has been successfully constructed. CXCL8 was not expressed in mice. It has been reported that CXCL2 in mice could replace CXCL8 in humans.[29] Thus, we applied immunohistochemical staining to evaluated the expression of CXCL2 and CTNNA1 in mice liver. CTNNA1 and CXCL2 levels were increased in AH mice compared to control mice (Figure 6D and E, $p<0.05$, $p<0.001$). These data validated the two key gene expression patterns in AH.

Table 2 Heterogeneity and Pleiotropy Test

Exposures	Heterogeneity Test						Pleiotropy Test		
	MR-Egger			Inverse Variance-Weighted			MR-Egger		
	Q	Q_df	Q_P	Q	Q_df	Q_P	Intercept	SE	p
ABI1	0.16	1	0.69	0.25	2	0.88	−0.01	0.04	0.82
ACSL1	1.42	2	0.49	4.70	3	0.20	0.17	0.09	0.21
ACTN4	2.04	1	0.15	2.49	2	0.29	0.04	0.08	0.72
CHST15	2.72	2	0.26	3.01	3	0.39	0.03	0.06	0.69
CTNNA1	2.16	5	0.83	2.21	6	0.90	0.01	0.06	0.84
CXCL8	9.99	7	0.19	10.00	8	0.27	−0.002	0.03	0.95
CYSTM1	1.11	2	0.58	1.11	3	0.78	−0.001	0.06	0.99
DBNDD1	1.06	2	0.59	1.48	3	0.69	0.03	0.05	0.58
DNASE1L3	3.37	2	0.19	4.18	3	0.24	0.03	0.05	0.56
EPHX1	0.002	1	0.97	1.34	2	0.51	0.12	0.10	0.45
IGFBP7	2.62	4	0.62	3.24	5	0.66	0.03	0.04	0.47
PIK3R1	1.34	2	0.51	2.63	3	0.45	0.05	0.05	0.37
PRKCSH	0.68	1	0.41	1.30	2	0.52	0.04	0.06	0.58
RDH5	1.03	1	0.31	1.03	2	0.60	0.0008	0.06	0.99
TNFAIP2	2.20	2	0.33	2.26	3	0.52	−0.01	0.05	0.84
TNFSF10	0.38	2	0.83	2.26	3	0.52	0.07	0.05	0.30
TRIM8	1.86	3	0.60	1.86	4	0.76	0.003	0.04	0.94

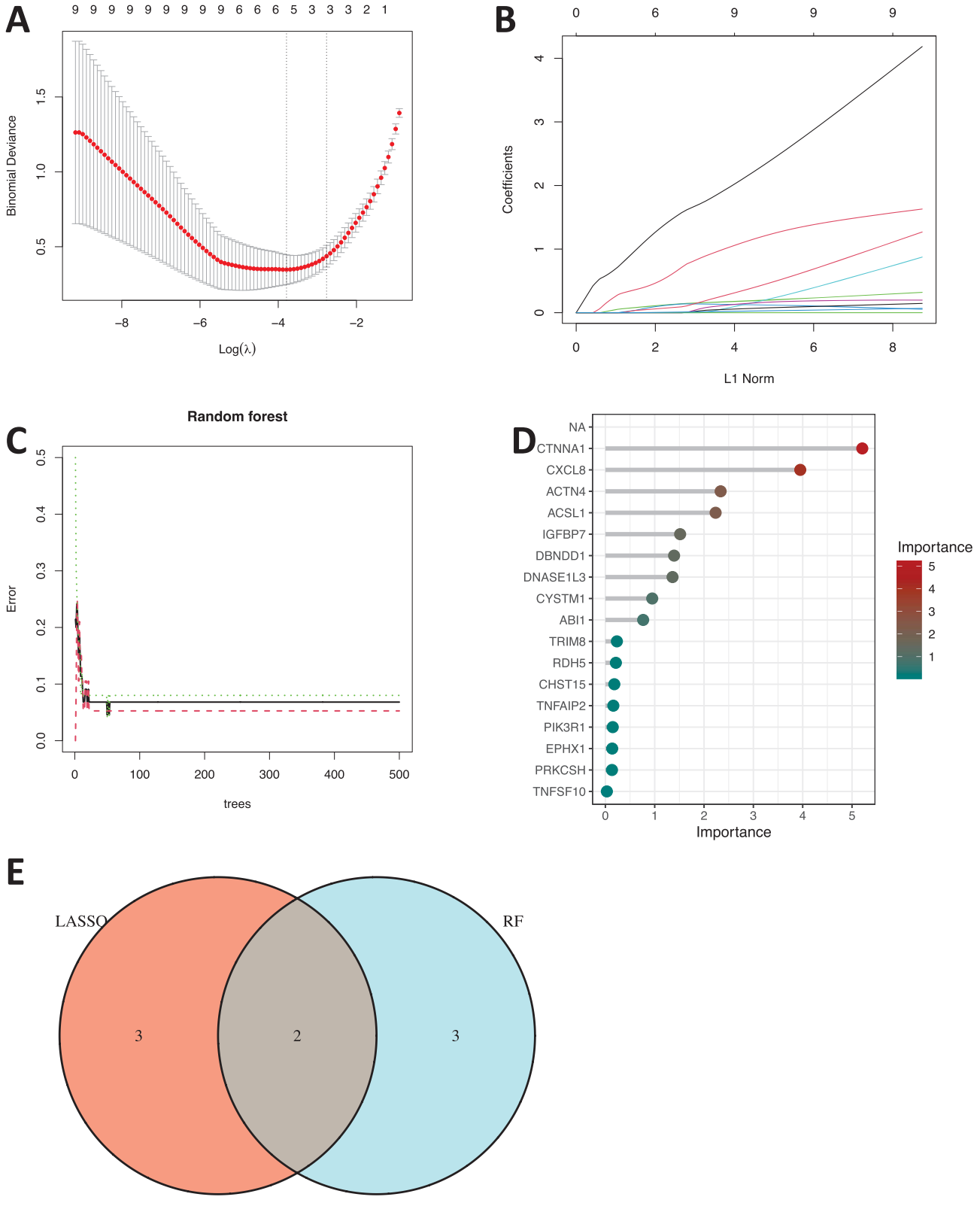

Figure 4 Identify key genes of alcoholic hepatitis via machine learning. (**A** and **B**) Five genes were identified as the biomarkers with the lowest binominal deviation via LASSO regression. (**C** and **D**) Based on random forest algorithm, biomarkers were selected according to importance score. In order of importance, the top five are CTNNA1, CXCL8, ACTN4, ACSL1 and IGFBP7. (**E**) The biomarkers obtained by the two machine learning methods were intersected to obtain two key genes (CXCL8 and CTNNA1), which were visualized by Wayne diagram.

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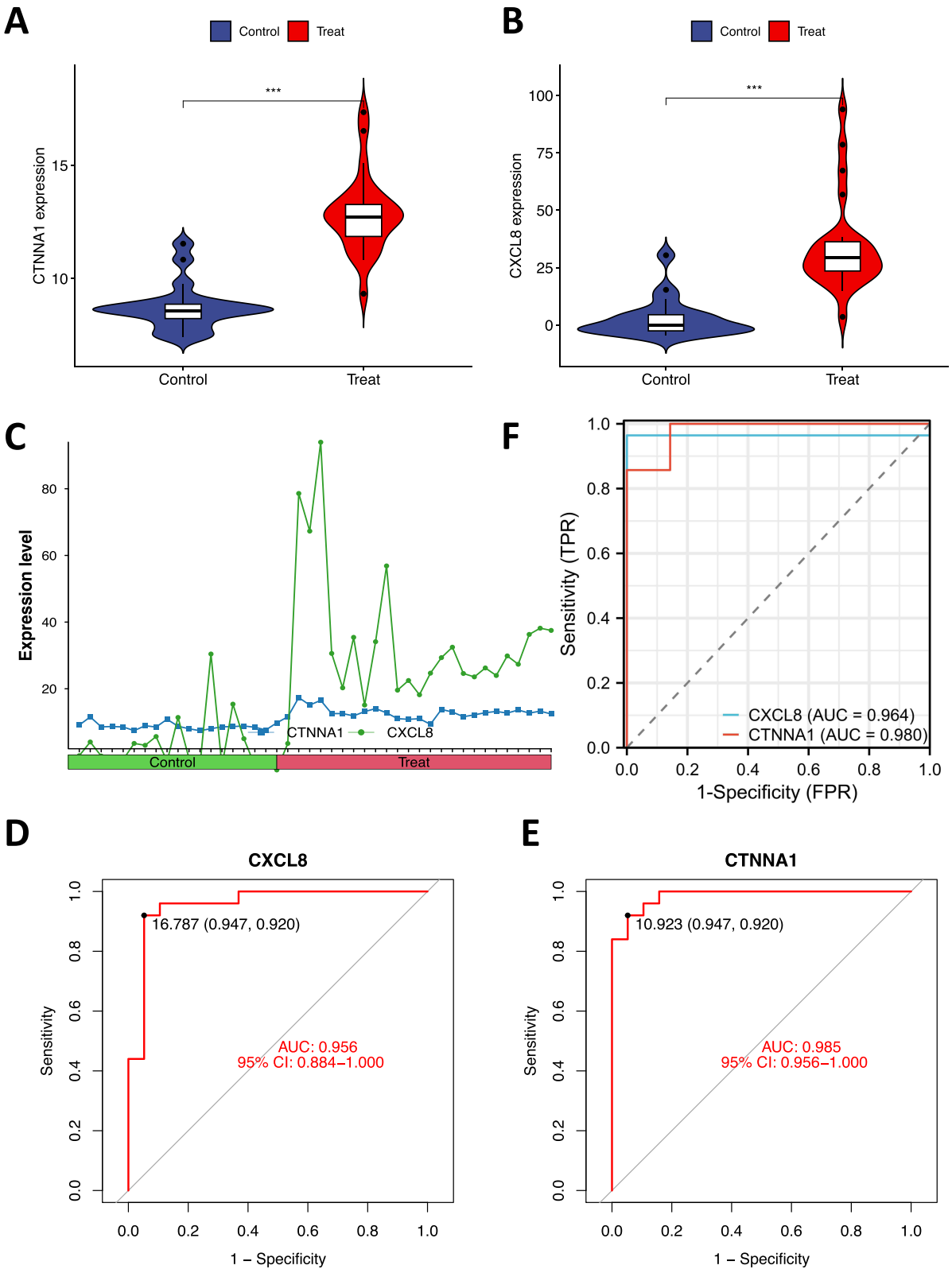

Figure 5 Expression patterns of CXCL8 and CTNNA1. (**A** and **B**) The expression of CTNNA1 and CXCL8 was visualized by violin graphs. (**C**) The CTNNA1 and CXCL8 levels of each sample were visualized by line chart. (**D** and **E**) The ROC curve displayed the predictive ability of key genes in alcoholic hepatitis datasets in the train set. (**F**) The ROC curve displayed the predictive ability of key genes in alcoholic hepatitis datasets in the validation set. ***p<0.001 was considered statistically significant.

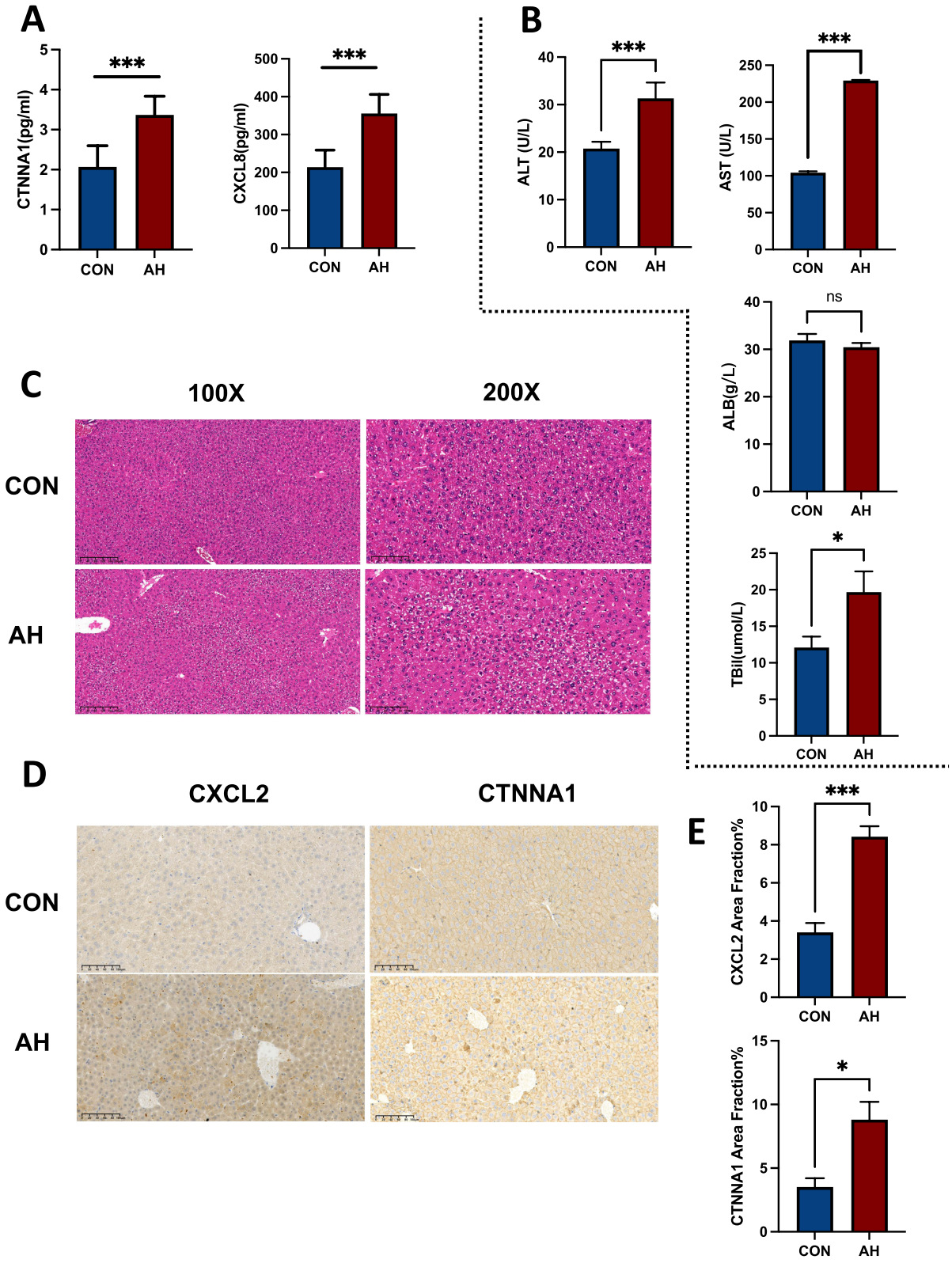

Figure 6 Key genes verified in alcoholic hepatitis patients and mice. (**A**) The levels of CTNNA1 and CXCL8 of clinical AH patients and control people. (**B**) The levels of aminotransferase (ALT and AST), total bilirubin and albumin of AH mice and control mice. (**C**) HE staining was used to observe the pathological changes in the liver of AH mice (100X and 400X). (**D** and **E**) Representative images and quantification of CXCL2 and CTNNA1 positive immuno-histochemical staining in the liver of AH mice and control mice (scale bar:100um). *p<0.05, ***p<0.001 was considered statistically significant.

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Pathway Analysis of Crucial Genes

To investigate the underlying pathways involved in these two key genes, we performed single-gene GSEA analysis. The results showed that CTNNA1 and CXCL8 were both highly enriched in antigen processing and presentation (Figure 7A and B). Meanwhile, two key genes were both lowly enriched in drug metabolism cytochrome p450, metabolism of xenobiotics by cytochrome, PPAR signaling pathway and retinol metabolism (Figure 7C and D). Antigen processing and presentation were closely linked with immune response. It indicated that CTNNA1 and CXCL8 might play an important role in the immune environment of AH patients. Drug metabolism cytochrome p450 and metabolism of xenobiotics by cytochrome were related to alcohol metabolism.[30] This suggested, to some extent, that the up-regulation of CXCL8 and CTNNA1 might damage the alcohol metabolic pathway and further aggravate liver injury.

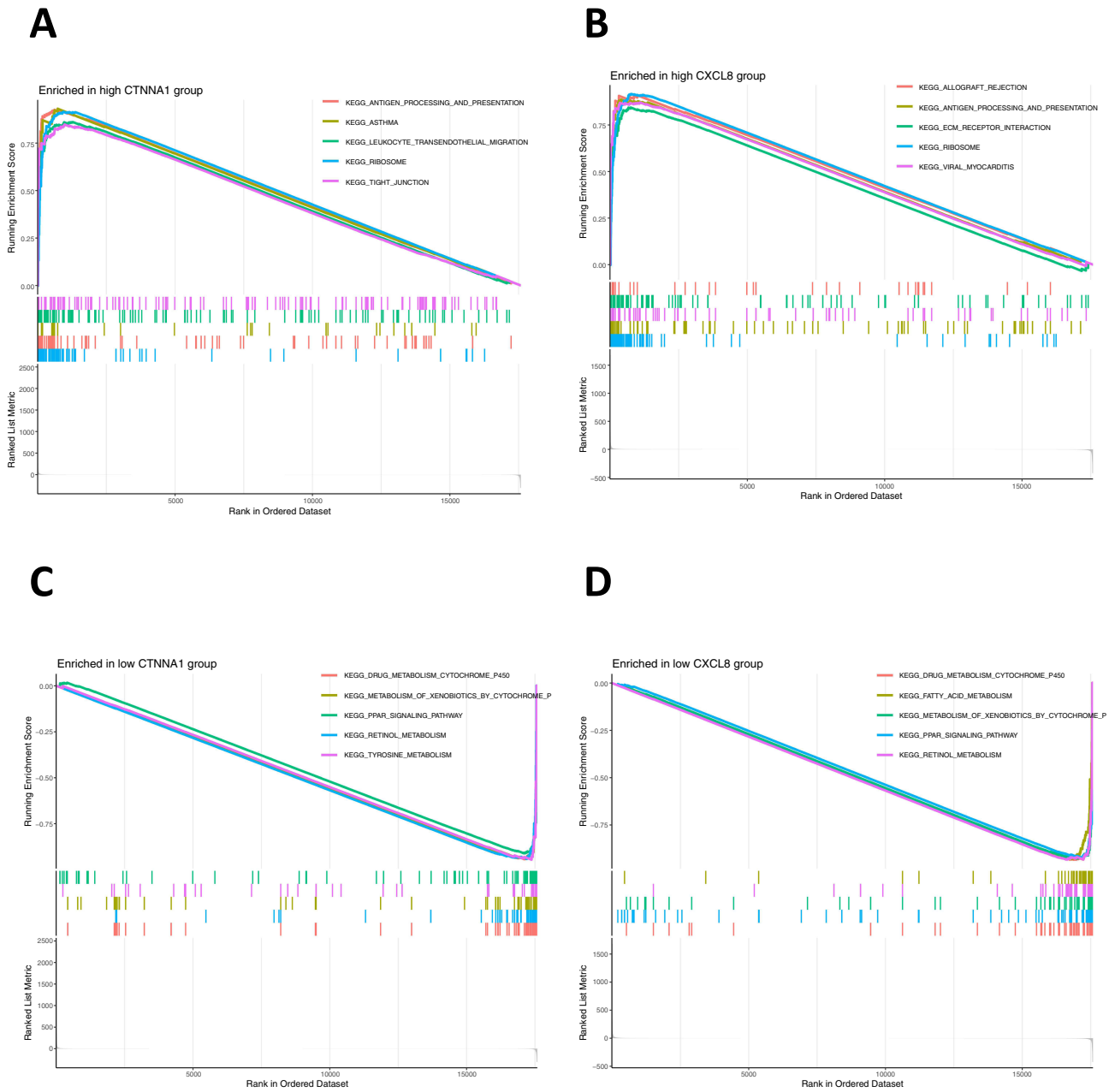

Figure 7 Pathway analysis of key genes. (**A** and **B**) Top five pathways positively associated with CTNNA1 and CXCL8. (**C** and **D**) Top five pathways negatively associated with CTNNA1 and CXCL8.

Comparison of the Immune Microenvironment Between AH and Normal Groups

We utilized the ssGSEA method to examine immune infiltration in AH and control groups. The results showed that activated dendritic cell, CD56dim natural killer cell, gamma delta T cell, immature dendritic cell, MDSC, natural killer T cell, natural killer cell, plasmacytoid dendritic cell, regulatory T cell, and type 1 T helper cell were higher enriched in AH samples than normal samples (Figure 8A, p<0.05, p<0.01, p<0.001). Eosinophil, type 2 T helper cell, memory B cell and central memory CD4 T cell were lower enriched in AH samples than normal samples (Figure 8A, p<0.05, p<0.001).

In addition, the correlation analysis showed that NK T cells, type 17 T helper cell, mast cell which were highly expressed in AH group were positively associated with key genes, while memory B cell was negatively associated (Figure 8B, p<0.05, p<0.01, p<0.001). We examined the variation in immune cell infiltration levels between high and low

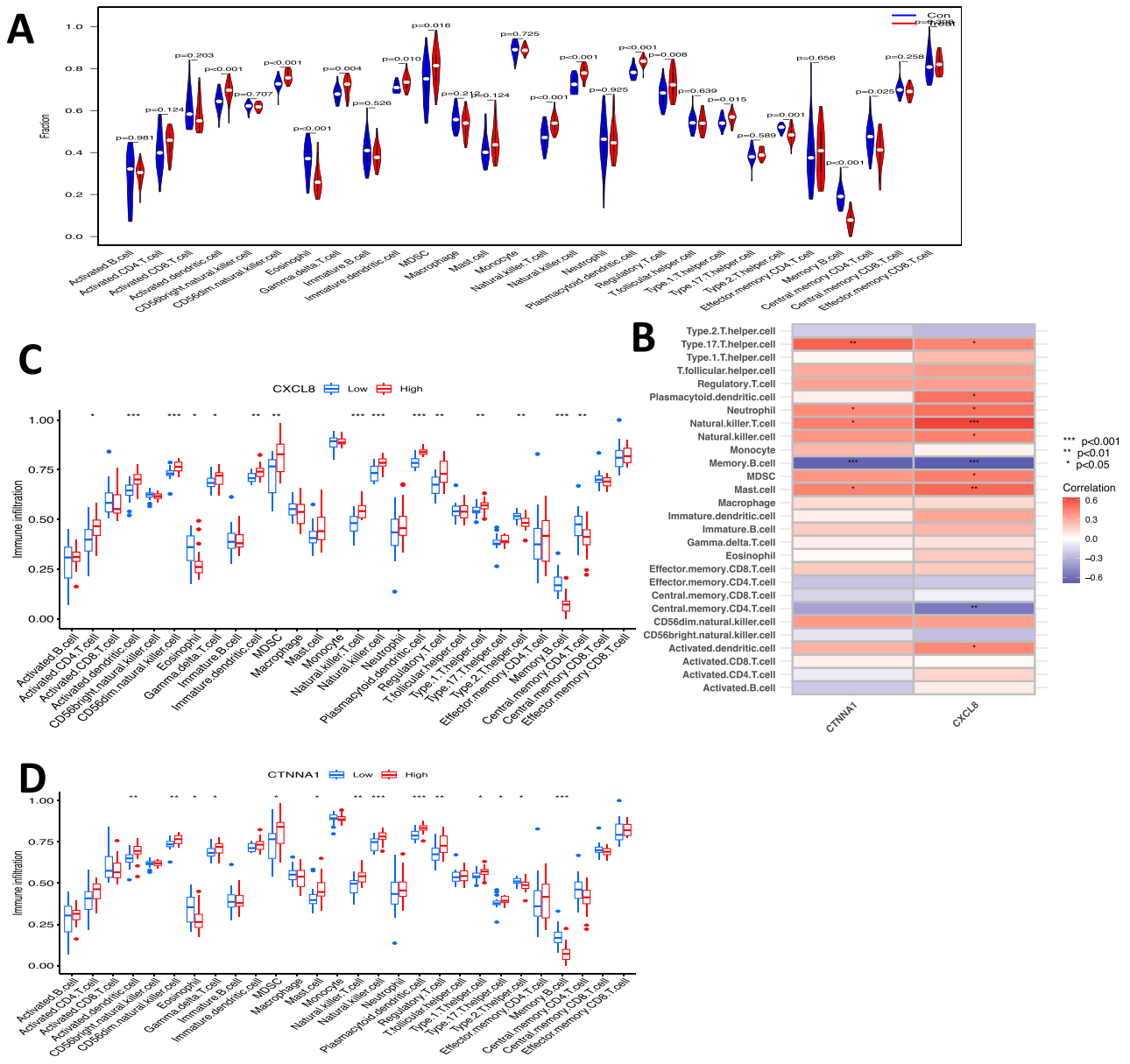

Figure 8 Analysis of immune environment in AH and normal samples. (**A**) The immune infiltration in AH samples and control samples visualized by violin plots. (**B**) The correlation of two key genes (CXCL8 and CTNNA1) and immune infiltration via Pearson correlation analysis. (**C** and **D**) The abundance of immune cells in the high and low expression groups of key genes (CXCL8 and CTNNA1) were visualized by boxplots. *p<0.05, **p<0.01, ***p<0.001 was considered statistically significant.

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expression of CTNNA1 and CXCL8, finding results that aligned with the correlation analysis (Figure 8C and D). The elevated expression of key genes might participate in an abnormal immune response in AH patients.

NKT Cells and B Cells in AH Mice

NKT cells and memory B cells were the most correlated with two key genes. Due to the difficulty in specifically detecting memory B cells in liver by immunofluorescence staining, and given that memory B cells constitute a subset of total B cells, we evaluated the expression of NKT cells (CD3 and NKR-P1C markers)[31] and B cells (CD20 marker) in the liver of AH mice by immunofluorescence staining. The results showed that NKT cells were significantly activated in AH mice compared to the control group (Figure 9A and C, $p<0.01$), while B cells were inhibited (Figure 9B and D, $P<0.001$). It was consistent with the analysis of immune infiltration described above.

Exploration of the Relationship Between Key Genes and Immune Cells

To explore whether CXCL8 and CTNNA1 were involved in alcoholic hepatitis by regulating B cells and NKT cells, we conducted gene silencing and EtOH intervention on NKT cells and B cells respectively. Following EtOH stimulation, the expression levels of CXCL8 and CTNNA1 were significantly upregulated in NKT cells compared to the control group (Figure 10A, B, E and F, $p<0.05$), and inflammatory factors (IL-1β and TNF-α) levels in NKT cells were significantly upregulated (Figure 10A, B, E and F, $p<0.05$, $p<0.01$). After subsequent silencing the CXCL8 expression, the IL-1β and TNF-α levels were significantly downregulated compared with the EtOH group (Figure 10A and E, $p<0.05$, $p<0.01$). Similarly, compared with the EtOH group, the levels of IL-1β and TNF-α in NKT cells intervened by EtOH and siCTNNA1 were significantly downregulated (Figure 10B and F, $p<0.05$).

In B cells, EtOH intervention resulted in a significant increase in CXCL8 and CTNNA1 expression compared to controls (Figure 10C, D, G and H, $p<0.05$), accompanied by a reduction in IgG secretion (Figure 10I and J, $p<0.05$). However, silencing either CXCL8 or CTNNA1 significantly restored IgG levels relative to the EtOH group (Figure 10I and J, $p<0.05$). These findings indicated that CXCL8 and CTNNA1 might promote inflammation and impair humoral immunity under alcoholic conditions by modulating the functions of NKT cells and B cells.

Discussion

In this study, based on the AH dataset and mice and cell experiments, we found the following key points: (1) two key genes (CTNNA1 and CXCL8) of AH were selected by Mendelian randomization analysis and machine learning, which were both up-regulated in AH patients and AH mice. (2) CXCL8 and CTNNA1 had good predictive value for alcoholic hepatitis. (3) CXCL8 and CTNNA1 might be involved in immune response and alcoholic metabolism via ssGSEA analysis. (4) Key genes might have correlation with the immune environment of AH patients. (4) NKT cells were activated in AH mice, while B cells were inhibited in AH mice. (5) The expressions of CXCL8 and CTNNA1 in NKT cells and B cells increased after EtOH intervention. (6) Silencing CXCL8 or CTNNA1 significantly reduced IL-1β and TNF-α levels in EtOH-stimulated NKT cells and restored IgG production in B cells. The upregulation of key genes might be involved in the immune disorder of alcoholic hepatitis and the progression of the disease.

Alcoholic hepatitis is a form of hepatitis resulting from alcohol abuse. Severe cases can lead to refractory liver failure with hyperbilirubinemia and impaired coagulation function.[32] Additionally, cessation of alcohol consumption does not halt disease progression in certain patients.[2] Despite being the standard treatment, glucocorticoids have limited efficacy and are associated with potential adverse effects such as infection and gastrointestinal bleeding.[33] Therefore, novel therapeutic approaches are needed to enhance disease management of alcoholic hepatitis patients. In this study, we applied Mendelian randomization analysis and machine learning to screen out two key genes (CTNNA1 and CXCL8) as AH biomarkers. CXCL8 and CTNNA1 were up-regulated in AH samples compared to control samples. Previous studies have shown that CXCL8 expression in alcoholic hepatitis patients was significantly higher than that in normal groups.[34,35] As a pro-inflammatory factor, CXCL8 up-regulated expression can further promote the recruitment of neutrophils, release a variety of inflammatory factors, and mediate monocytes/macrophages to promote the progression of liver fibrosis.[35] Few studies have been conducted on CTNNA1 in hepatitis. CTNNA1 is involved in many physiological activities such as adhesion synthesis and signal transduction.[36] It has been reported that CTNNA1 could inhibit

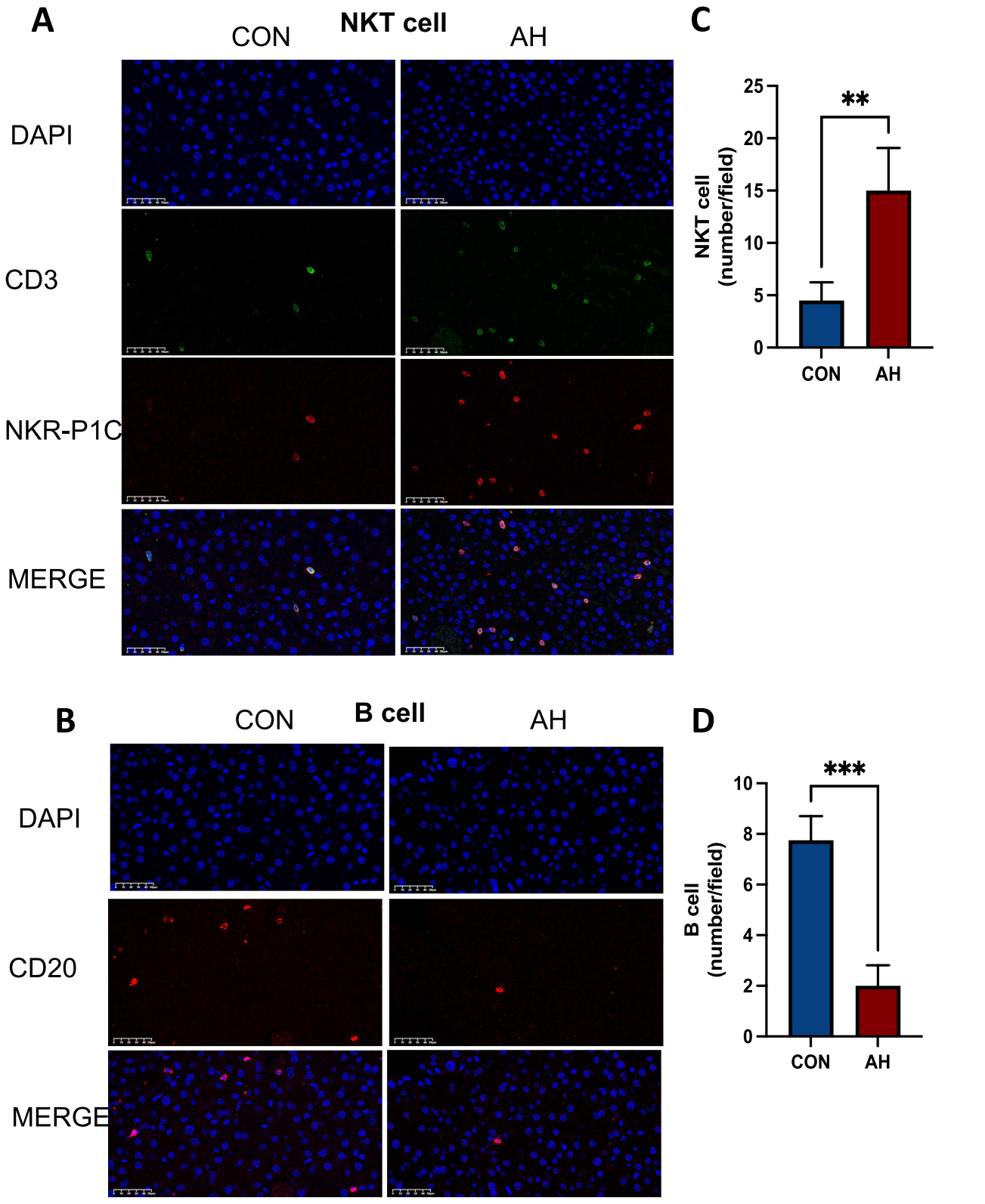

Figure 9 The infiltration of NKT cells and B cells in AH mice and control mice. (**A**) Representative images of NKT cells in the liver of AH mice and control mice via immune-fluorescence staining (scale bar: 50um). NKR-P1C (red) was stained with TYR-570, CD3 (green) was stained with TYR-520 and the nucleus (blue) was stained with DAPI. (**B**) Representative images of B cells in the liver of AH mice and control mice via immune-fluorescence staining (scale bar: 50um). CD20 (red) was stained with TYR-570 and the nucleus (blue) was stained with DAPI. (**C** and **D**) Quantification of NKT cells and B cells in images of AH mice and control mice. **p<0.01, ***p<0.001 was considered statistically significant.

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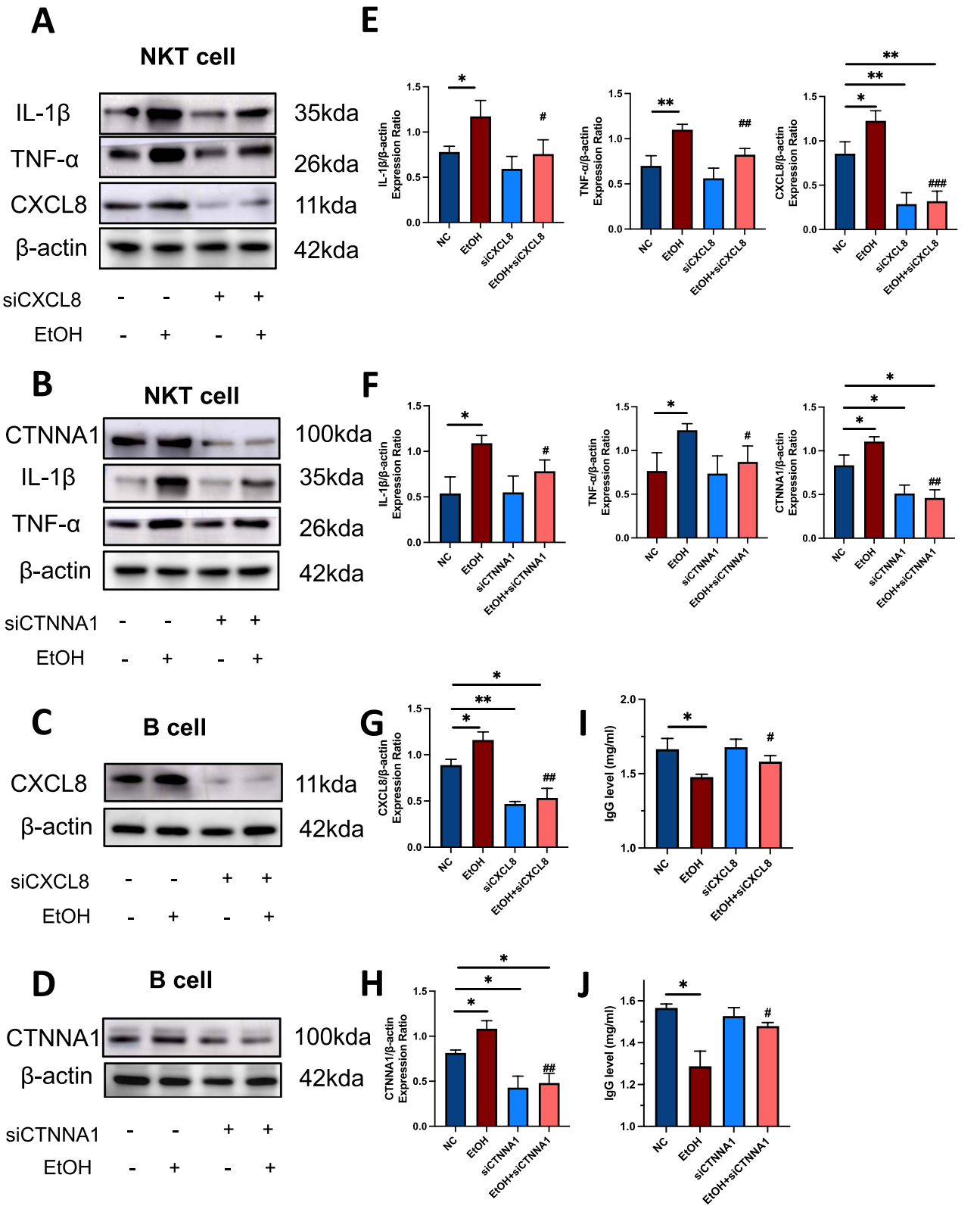

Figure 10 Exploration the relationship between key genes and immune cells. (**A** and **E**) Representative immunoblots and quantitative analysis of IL-1β, TNF-α, CXCL8 and β-actin proteins in the NC group, EtOH group, siCXCL8 group and EtOH+siCXCL8 group of NKT cells. (**B** and **F**) Representative immunoblots and quantitative analysis of IL-1β, TNF-α, CTNNA1 and β-actin proteins in the NC group, EtOH group, siCTNNA1 group and EtOH+siCTNNA1 group of NKT cells. (**C** and **G**) Representative immunoblots and quantitative analysis of CXCL8 and β-actin proteins in the NC group, EtOH group, siCXCL8 group and EtOH+siCXCL8 group of B cells. (**D** and **H**) Representative immunoblots and quantitative analysis of CTNNA1 and β-actin proteins in the NC group, EtOH group, siCTNNA1 group and EtOH+siCTNNA1 group of B cells. (**I**) The IgG level in NC group, EtOH group, siCXCL8 group and EtOH+siCXCL8 group of B cells. (**J**) The IgG level in NC group, EtOH group, siCTNNA1 group and EtOH+siCTNNA1 group of B cells. *p<0.05, **p<0.01 vs NC. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs EtOH.

cell proliferation and migration, promoting cell apoptosis.[37] These reports indicated that two key genes might play a vital role in regulating the progression of liver inflammation. Meanwhile, we verified two key genes in AH patients and mice groups. We found that CXCL8 and CTNNA1 levels were significantly increased in AH patients and AH mice. In addition, the ROC curves constructed based on key genes showed good accuracy in predicting the risk of AH. These data indicated that the two key genes might be significant variables of AH development.

Next, we explored the potential pathway key genes involved in. Using ssGSEA analysis, we found that both CXCL8 and CTNNA1 were highly enriched in antigen processing and presentation pathway and lowly enriched in drug metabolism cytochrome p450, metabolism of xenobiotics by cytochrome, PPAR signaling pathway and retinol metabolism. Antigen processing and presentation were closely related to immune recognition, immune response and immune regulation.[38] Immune response was involved in the development of alcoholic hepatitis. It has been reported that liver macrophages (M1 and M2 phenotypes) associated with antigen processing and presentation were significantly increased during the progression of alcoholic liver disease.[39,40] At the same time, the proteasome function of macrophages and dendritic cells is disrupted in alcoholic hepatitis, which interferes with cell-mediated adaptive immunity and thus alters alloantigen presentation.[41] Therefore, CXCL8 and CTNNA1 might participate in AH progression by regulating immunity. In addition, liver cell damage in alcoholic hepatitis could lead to impaired drug metabolism pathways and lipid metabolism pathways.[41] This was also relatively consistent with our results.

Studies show that alcoholic hepatitis patients in a heightened state of immune disorders, characterized by immune activation and dysfunction and failure.[42–44] In our study, the results showed that dendritic cells, NK cells, NK T cells and some other types of T cells were high enriched in AH samples. NK cells and NK T cells have been reported been significantly increased in alcoholic hepatitis patients and alcohol-treated mice, secreting pro-inflammatory factors and pro-apoptotic factors (such as TNF-α, IL-4, FasL), promoting liver damage.[40,45–47] Dendritic cells, as antigen presenting cells, played a key role in inducing innate and adaptive immunity. Dendritic cells could interact with many immune cells such as NKT cells.[48] The high expression of dendritic cells may further activate the subsequent immune response. We also found that Eosinophil, type 2 T helper cell, memory B cell, and central memory CD4 T cell were lowly enriched in AH samples. Previous studies have shown that peripheral CD4+ T cell depletion in patients with alcoholic hepatitis may reduce the suppression of the immune response and further promote immune overactivation, leading to liver cell damage and liver fibrosis.[49,50] It has also been suggested that patients with alcoholic liver disease have a significant reduction in B cells, which might be related to galectin-9 expressed on Kupffer cells, and ultimately impair the adaptive immune response.[51] These data suggested that the liver immune microenvironment in AH patients was significantly disturbed, which not only overactivated the immune system to secrete a large number of inflammatory and apoptotic factors but also had impaired immune function, which greatly increases the probability of infection.

In order to explore whether key genes are related to immune infiltration disorder, we conducted correlation analysis. The results showed that CTNNA1 and CXCL8 were both positively related to NK T cell, mast cell and Type 17 T helper cell, both negatively related to memory B cell. Among them, NKT cells and memory B cells have strong correlation with key genes. Based on the fact that memory B cells are very difficult to obtain in vitro and belong to a subtype of B cells, we conducted staining of NKT cells and total B cells in the liver of mice. Our results showed that NKT cells were increased in AH mice, while B cells were decreased in AH mice. This was consistent with the results of our bioinformatics analysis. Meanwhile, the abundance of most immune cells in the group with high CXCL8 expression was significantly higher than that in the group with low CXCL8 expression, as well as CTNNA1 groups. CXCL8 as a proinflammatory factor, not only had a strong ability to recruit neutrophils but also recruited NK cells and a variety of lymphocytes.[52,53] Most research on CTNNA1 has focused on its relationship with tumors. Previous studies indicated that CTNNA1 can bind to CTNNB1 to activate t cell factor (TCF)/lymphocyte enhancer factor (LEF) transcription.[54] In addition, CTNNA1 has been reported to be involved in the regulation of inflammatory factor release in macrophages.[55] These results suggested that CNNA1 and CXCL8 may mediate immune infiltration to some extent. To further explore whether CXCL8 and CTNNA1 directly regulate NKT cells and B cells and the mechanism in alcoholic hepatitis, we conducted in vitro experiments. We treated EtOH-NKT cells and EtOH-B cells with siCXCL8 and siCTNNA1. The results showed that after silencing CXCL8 or CTNNA1, the expression of inflammatory factors (IL-1β and TNF-α) in NKT cells decreased, and the expression of IgG in B cells was upregulated. This result further suggested that CXCL8 and CTNNA1 might play an important role in immune dysregulation in alcoholic hepatitis by regulating NKT cells and B cells.

Overall, we found that CXCL8 and CTNNA1 were closely related to the immune microenvironment of alcoholic hepatitis, but the specific mechanism still awaits further study. Targeting CXCL8 and CTNNA1 may be a new direction in the treatment of alcoholic hepatitis.

This study also had certain limitations. Firstly, CXCL8 was not expressed in mice, and using CXCL2 as a substitute may not fully imitate the function of human CXCL8. Secondly, although gene silencing provides functional insights, further mechanism studies are needed, including downstream pathway analysis and in vivo knockout experiments, to confirm causal relationships. Thirdly, the memory B cells obtained from bioinformatics analysis were expanded to total B cells in vitro. Subsequently, memory B cells will be further obtained through primary cell culture and sorting to further explore the mechanism.

Conclusion

CXCL8 and CTNNA1 were identified as potential biomarkers for alcoholic hepatitis. CXCL8 and CTNNA1 might be involved in the immune infiltration disorder of alcoholic hepatitis. Targeting key genes may provide new therapeutic directions in the future. The specific regulatory mechanism awaits further study.

Statement of Ethics

The clinical project has been approved by Ethics Committee of Ningbo No. 2 hospital (MR-33-24-028857). The study complied with the Declaration of Helsinki. Informed consents have been obtained from the study participants. The animal protocol was approved by the Ethics Committee of Ningbo NO.2 hospital (GX-2024-XM-1098), in compliance with GB/T35892-2018《Guidelines for Ethical Review of Experimental Animal Welfare》.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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