Transcriptomic Analysis Reveals the Messenger RNAs Responsible for the Progression of Alcoholic Cirrhosis

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Alcohol-associated liver disease is the leading cause of chronic liver disease. We hypothesized that the expression of specific coding genes is critical for the progression of alcoholic cirrhosis (AC) from compensated to decompensated states. For the discovery phase, we performed RNA sequencing analysis of 16 peripheral blood RNA samples, 4 healthy controls (HCs) and 12 patients with AC. The DEGs from the discovery cohort were validated by quantitative polymerase chain reaction in a separate cohort of 17 HCs and 48 patients with AC (17 Child-Pugh A, 16 Child-Pugh B, and 15 Child-Pugh C). We observed that the numbers of differentially expressed messenger RNAs (mRNAs) were more pronounced with worsening disease severity. Pathway analysis for differentially expressed genes for patients with Child-Pugh A demonstrated genes involved innate immune responses; those in Child-Pugh B belonged to genes related to oxidation and alternative splicing; those in Child-Pugh C related to methylation, acetylation, and alternative splicing. We found significant differences in the expression of heme oxygenase 1 (HMOX1) and ribonucleoprotein, PTB binding 1 (RAVER1) in peripheral blood of those who died during the follow-up when compared to those who survived. Conclusion: Unique mRNAs that may implicate disease progression in patients with AC were identified by using a transcriptomic approach. Future studies to confirm our results are needed, and comprehensive mechanistic studies on the implications of these genes in AC pathogenesis and progression should be further explored. (Hepatology Communications 2022;6:1361-1372).

Excessive alcohol consumption is the leading cause of several medical conditions, including alcohol-associated liver disease (ALD).1-3 ALD comprises a spectrum of histopathological changes in patients with excessive alcohol consumption, ranging from alcoholic steatosis, steatohepatitis, advanced fibrosis, and cirrhosis.4,5 Alcoholic steatosis occurs in most if not all patients who consume alcohol excessively; however, progression of ALD to advanced stages, such as alcoholic cirrhosis (AC), only develops in 15%-20% of excessive drinkers. Patients with compensated cirrhosis generally do not have signs or symptoms. However, the disease can progress into the decompensated state when patients develop complications from portal hypertension, such as ascites or variceal bleeding.6 At this stage, the overall prognosis and survival are poor.7 The pathogenesis of alcohol-induced liver injury is complex, involving alterations of lipid metabolism, oxidative stress, and the inflammatory signaling pathway.8-10 Several genetic components involved in...
these pathways are associated with susceptibility to ALD. Single-nucleotide polymorphisms of patatin-like phospholipase domain-containing protein 3 (PNPLA3), transmembrane 6 superfamily member 2 (TM6SF2), and membrane-bound O-acyltransferase domain-containing 7 (MBOAT7) lead to an increased risk of AC among heavy drinkers. Furthermore, two studies using genome-wide association and exome
sequencing identified a new locus at Fas-associated factor family member 2 (FAF2) and hydroxysteroid 17-beta dehydrogenase 13 (HSD17B13) associated with a reduced risk of cirrhosis.\textsuperscript{(12,13)} While these studies uncover underlying risk factors for ALD development and progression, our understanding of the mechanisms and what triggers the ALD progression are lacking.

Several lines of evidence illustrate that coding and noncoding RNAs play a crucial role in ALD pathogenesis.\textsuperscript{(14-18)} A comprehensive RNA analysis may elucidate underlying mechanisms in gene expression and pathways leading to ALD and disease progression. Transcriptomic profiling is a comprehensive way of measuring gene expression allowing the detection of differentially expressed genes (DEGs) and identification of the unannotated polymerase II RNAs.\textsuperscript{(19)} We hypothesized that the expression of specific coding genes is critical for the progression of AC from compensated to decompensated states. To test this hypothesis, global RNA profiling from peripheral blood RNA was performed in a well-characterized cohort of healthy controls (HCs) and patients with AC with different disease severity ranging from compensated to decompensated states. We determined the association of the unique set of peripheral blood messenger RNA (mRNA) expression with that in the liver of patients with AC and performed gene set enrichment analysis (GSEA) and functional protein association networks (STRING) to further understand the function of DEGs during the progression from compensated to decompensated states in patients with AC. Lastly, we determined the prognostic significance of this unique mRNA signature on the survival outcomes in patients with AC.

Participants and Methods

STUDY DESIGN AND HUMAN SUBJECT COHORT

HCs were recruited from outpatient clinics at the Roudebush Veterans Administration Medical Center (VAMC), Indianapolis, IN. Patients with AC were those who attended liver clinics at Indiana University Hospital or Roudebush VAMC. These patients had a history of alcohol consumption averaging at least 80 g/day (for men) or 50 g/day (for women) for at least 10 years. This criterion is based on epidemiological evidence of the relationship between alcohol consumption and cirrhosis. The diagnosis of AC has been described.\textsuperscript{(12,14,20,21)} In brief, it was made using radiographic imaging, history of portal hypertension complications, or biopsy-proven cirrhosis, with the exclusion of other known causes of liver diseases, such as viral hepatitis B or C, autoimmune liver disease, hemochromatosis, or Wilson disease. The study was approved by the Institutional Review Board (IRB) at Indiana University–Purdue University Indianapolis (IUPUI) and Roudebush VAMC Research and Development Program. Written informed consent was obtained from each participant.

BASELINE DATA COLLECTION

Baseline demographics, clinical characteristics, and laboratory tests of the study cohort were obtained at the time of enrollment, as reported.\textsuperscript{(14)} Baseline Child-Pugh and Model for End-Stage Liver Disease (MELD) scores were calculated for patients with AC to further categorize them into compensated (Child-Pugh class A, AC1) and decompensated (Child-Pugh class B or C, relating to AC2 or AC3, respectively) liver diseases.

PERIPHERAL BLOOD COLLECTION AND RNA PROFILING

Peripheral blood was collected from venipuncture at enrollment into the PAXgene Blood RNA tube (Qiagen; catalog #762165). The sample was gently inverted and stored at −80°C within 2 hours of collection. Peripheral blood RNAs were extracted using a QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Blood RNAs from HCs (n = 4) and patients with AC (n = 12, with 4 patients for each Child-Pugh classification) were subjected to global transcriptomic profiling using the Arraystar human microarray, version 3.0 (Arraystar, Rockville, MD). The original raw data were uploaded to GitHub (https://github.com/yangjoe-iu/ALD_Blood-RNASeq). Quantitative polymerase chain reaction (qPCR) was used to validate the expression of mRNAs in a separate cohort of 17 HCs and 48 patients with AC. The baseline demographic and clinical characteristics are provided in Supporting Table S1. The primer’s sequences are provided in Supporting Table S2.
HUMAN LIVER SPECIMENS

We also collected de-identified human liver specimens from a different cohort of patients with AC for mRNA analysis. The collection was performed under the IRB-approved protocol at the IUPUI.

BIOINFORMATIC ANALYSIS

The raw intensities of samples were grouped and uploaded to the online software (https://www.metaboanalyst.ca/MetaboAnalyst/home.xhtml) for the statistical analysis using algorithms they provided. After the integrity check, the data were normalized by a pooled sample from HCs and logarithmically transformed. The partial least squares-discriminant analysis (PLS-DA) was performed using the plsr function provided by the R pls package. Classification and cross-validation were conducted using the caret package. Differentially expressed mRNAs were identified through an adjusted $P$ value of 0.05 and at least 2-fold differential gene expression to generate the volcano plot. The heat map and correlation analysis were generated using the default setting. Statistical analyses of differentially expressed mRNAs among HCs and patients with AC with different severities stratified by Child-Pugh classification were performed using one-way analysis of variance analysis (ANOVA).

GSEA was conducted using the GSEA software downloaded from https://www.gsea-msigdb.org/gsea/index.jsp. Raw intensities data were processed following the guidelines for RNA sequencing (RNA-Seq) data sets with GSEA.(23) Bubble plots were generated using the ggplot2 package in R. Enrichment plots and heat maps were provided from the GSEA software. Functional protein association networks were analyzed using STRING (https://string-db.org). Venn diagrams were generated using the Venn Diagram package in R. The list of unique differential gene expression from each group was submitted to DAVID (https://david.ncifcrf.gov) for the gene ontology terms and Kyoto Encyclopedia of Genes and Genomes pathway analysis. Bubble plots were generated by the R ggplot2 package.

STATISTICAL ANALYSIS

We used descriptive statistics, such as mean, SEM, and frequencies (percentages). Statistical analysis was performed using a Student $t$ test for unpaired data or ANOVA to determine the mean difference. We used survival analyses to determine the prognostic significance of the selected mRNA transcripts and the survival outcome in subjects with AC. The survival analysis was conducted to determine the significance of the variable of interest and time to mortality as calculated by the date of enrollment until the time of death. Patients who survived were censored. Cox regression models were used to determine the hazard ratio (HR) and its 95% confidence interval (CI). The cut-off $P$ value < 0.05 was considered statistically significant.

Results

TRANSCRIPTOMIC IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PERIPHERAL BLOOD mRNAs IN HCs AND PATIENTS WITH AC

To identify genes differentially expressed during the progression of AC, we first performed transcriptomic profiling of peripheral blood obtained from HCs and patients with AC. Arraystar Human Microarray, version 3.0, was used for the global profiling of approximately 26,000 human protein-coding transcripts. We first analyzed the profiling in 16 peripheral blood RNA samples, 4 HCs, and 12 patients with AC. PLS-DA illustrated two distinct clusters, suggesting the unique transcriptomic profiles in peripheral blood of patients with AC when compared to HCs (two-dimensional [2D] and 3D plots are shown in Supporting Fig. S1A,B). Using the cutoff of $P < 0.05$ and at least 2-fold differential gene expression, we identified 707 and 179 mRNAs that were up- and down-regulated, respectively, in patients with AC compared to HCs (Fig. 1A; Supporting Fig. S1C; Supporting Table S3). Genes with the most differential changes in their expression in AC compared to HCs are shown in Fig. 1A (bottom panel). Storkhead Box 1 (STOX1), sarcoglycan delta (SGCD), and cluster of differentiation 248 (CD248) were down-regulated, and major histocompatibility class, complex II, DR beta 1 (HLA-DRB1), peptidoglycan recognition protein 4 (PGLYRP4), and fibroblast growth factor 12 (FGF12) were up-regulated in patients with AC.

Next, we normalized the raw data with mean values and used the Ward algorithm to generate a heat map of the top 25 (Fig. 1B), top 1,000 (Supporting Fig.
S1E), and top 50 (Supporting Fig. S1F) DEGs based on the $P$ value. The correlation matrix also demonstrated two highly correlated gene groups with shared similar expression patterns according to the up- and down-regulated genes (Supporting Fig. S1D).

**GSEA FOR DEGs IN PATIENTS WITH AC**

GSEA was performed to gain an insight into the biological function of DEGs in patients with AC compared to those in HCs. Within the 50 enriched gene sets, 13 and 37 gene sets were up- and down-regulated, respectively, in patients with AC (data not shown). Among the up-regulated gene sets, three gene sets were significantly enriched at the family-wise error rate at $P < 0.05$, including genes related to angiogenesis ($P = 0.009$), oxidative phosphorylation ($P = 0.026$), and xenobiotic metabolism ($P = 0.038$) (Fig. 2A). The selected enrichment score plots and the top 20 up-regulated enriched genes are illustrated in Fig. 2C,E, respectively. Among the down-regulated gene sets, four gene sets were significantly enriched, including interferon-α (IFN-α) response ($P = 0.0001$), IFN-γ response ($P = 0.0001$), mitotic spindle ($P = 0.0001$), and G2M-checkpoint ($P = 0.0001$) (Fig. 2B). The selected enrichment score plots and top 20 down-regulated enriched genes are shown in Fig. 2D,F, respectively. Taken together, GSEA indicated the function of the up-regulated genes related to angiogenesis and metabolism while down-regulated genes were involved in IFN responses and cell-cycle pathways.

**DIFFERENTIALLY EXPRESSED GENES IN PATIENTS WITH AC STRATIFIED BY DISEASE SEVERITY**

We next explored the DEGs in peripheral blood of 12 patients with AC in our discovery cohort stratified by disease severity according to Child-Pugh
classification (4 patients for each Child-Pugh classification) compared to HCs (Fig. 3A). We observed that the number of differentially expressed peripheral blood mRNAs was more pronounced once disease severity worsened. Detailed analysis of these mRNAs in different Child-Pugh classes compared with HCs identified 142 and 164 mRNAs that were up- and down-regulated, respectively, in Child-Pugh class A patients compared to HCs, 581 up- and 270 down-regulated in Child-Pugh class B versus HCs, and 1,298 up- and 968 down-regulated in Child-Pugh class C versus HCs (Fig. 3B). PLS-DA demonstrated a unique gene expression profile in HCs and patients with AC with different severity (Fig. 3C). The top 25 most significantly DEGs among HCs and patients with AC with different Child-Pugh classification based on the fold change and P value are illustrated as a heat map (Fig. 3D) with the normalized reads of each gene in each group (Fig. 3E). HLA-DRB1 was up-regulated in patients with AC regardless of disease severity when compared to HCs. The results were consistent with those of Fig. 1B when we performed the analysis using samples from all patients with AC regardless of disease severity. The expression of ribosomal protein L41 (RPL41) and HLA-DRA progressively increased from compensated (Child-Pugh class A) to decompensated states (Child-Pugh class B and C) in patients with AC. The expression of papilin, proteoglycan like sulfated glycoprotein (PAPLN) was significantly up-regulated in patients with Child-Pugh class C compared to those in Child-Pugh class A and B (Fig. 3D,E). Expression of the genes pre-mRNA-splicing factor RNA binding motif protein 22 (RBM22), ribonucleoprotein, PTB binding 1 (RAVER1), nuclear receptor subfamily 3 group C member 2 (NR3C2), transformation/transcription domain-associated protein (TTPR4P), peroxisome proliferator-activated receptor gamma coactivator-related protein 1 (PPRC1), leucine-rich repeat-containing protein 37A3 (LRRC37A3), and translocated promoter region (TPR) was down-regulated during disease progression from Child-Pugh class A to class C (Fig. 3D,E).
To gain insight into pathways that may be involved during disease progression from compensated to decompensated states in patients with AC, we constructed a Venn diagram to determine the uniquely DEGs in peripheral blood for each Child-Pugh classification. We identified 104 DEGs in patients with AC regardless of disease severity (Fig. 4A). There were 98, 233, and 1,605 DEGs that were uniquely present in patients with AC with Child-Pugh class A, B, and C, respectively (Fig. 4A). Pathway analysis for DEGs specifically for patients with Child-Pugh class A demonstrated genes that are involved in inflammatory responses, including innate immunity and antiviral defense pathway (Fig. 4B). The DEGs in patients with Child-Pugh class B primarily belonged to pathways related to oxidation, hormone regulation, and alternative splicing (Fig. 4C). Lastly, those genes uniquely expressed in Child-Pugh class C were involved in isopeptide bond, ubiquitin conjugation, methylation, acetylation, phosphoprotein, and alternative splicing (Fig. 4D).

Validation of DEGs in peripheral blood was performed in a separate cohort of 17 HCs and 48 patients with AC (17 Child-Pugh class A, 16 class B, and 15 class C; Supporting Table S1). We selected genes based on up- and down-regulated genes in heat map analysis (Fig. 1D). In addition, we also performed network analysis using STRING software (https://string-db.org; Supporting Fig. S2), and genes highlighted in functional enrichments in the network were selected. In total, 24 genes were selected for validation using qPCR analysis. We first performed the analysis using pooled samples from HCs and patients with AC with different severity (Supporting Fig. S3) to identify genes that were differentially expressed among groups for subsequent studies using an individual sample (as shown in each box in Supporting Fig. S3). Ten genes (HLA-DRB1, HLA-DRA, heme oxygenase 1 [HMOX1], CD248, SGCD, Drosha ribonuclease III...
[DROSHA], polypyrimidine tract binding protein 1 [PTBP1], RAVER1, carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase [CAD], and NR3C2) were identified and validated in an individual sample (Fig. 5). There was a trend of increasing expression of HLA-DRA and HLA-DRB1 in patients with AC compared to HCs. However, the difference was not statistically significant. The expression of HMOX1 was progressively increased with worsening of AC from Child-Pugh class A to C. We also observed a significant reduction in the expression of CD248 and SGCD in patients with AC compared to HCs. Consistent with RNA-Seq results, DROSHA, PTBP1, CAD, RAVER1, and NR3C2 were significantly reduced in patients with AC with different severity (Fig. 5).

**COMPARISON OF DEGS IDENTIFIED IN PERIPHERAL BLOOD WITH THOSE IN THE LIVER OF PATIENTS WITH AC**

Once ingested, alcohol, through its ability to distribute through body fluid compartments, can cause cellular injury in several organ systems. As the primary organ responsible for alcohol metabolism, the liver is a major organ that is affected by alcohol. We next asked if the differentially expressed mRNAs identified from
peripheral blood in patients with AC (Figs. 1 and 5) have similar patterns in liver tissues. We examined the hepatic expression of each mRNA (Fig. 5) in liver tissues of another cohort of 8 HCs and 12 patients with AC. We observed a wide variability in the expression of each gene in the liver tissues of HCs and AC. Only expression of \textit{HMOX1} and \textit{PTBP1} was significantly reduced in the liver of patients with AC when compared to HCs (Fig. 6).

**PROGNOSTIC SIGNIFICANCE OF SELECTED mRNA TRANSCRIPTS AND MORTALITY OUTCOMES IN PATIENTS WITH AC**

During the median follow-up of 1.4 years, 14 patients died and 13 received a transplant among 48 patients with AC (Supporting Table S1). Baseline clinical characteristics and expression of selected mRNA transcripts for those who died and survived are shown in Supporting Table S4. Those who died had a significantly higher level of aspartate aminotransferase, total bilirubin, creatinine, MELD score, and \textit{HMOX1}. However, the baseline level of serum albumin and \textit{RAVER1} was significantly lower. Using the univariate COX proportional hazard model, we found that the expression of \textit{HMOX1} (HR, 6.54; 95% CI, 1.19-35.7; $P = 0.03$) and \textit{RAVER1} (HR, 0.006; 95% CI, 0-0.66; $P = 0.03$) in peripheral blood was associated with mortality during the follow-up (Supporting Table S5).

**Discussion**

Excessive alcohol consumption leads to adverse health outcomes affecting several organ systems. ALD represents a continuum of a disease process ranging from alcohol-induced hepatic steatosis, alcoholic hepatitis, advanced fibrosis, and cirrhosis.\(^{(1)}\) The mechanisms of alcohol-induced liver injury are complex, involving crosstalk between organ systems. Alteration of several metabolic pathways is observed with ALD, including dysregulation of immune responses, impairment in hepatic regeneration, and epigenetic regulation;\(^{(9,15,25)}\) and all of these pathological processes are controlled by regulatory gene networks.\(^{(16,26)}\) Hepatic steatosis develops in most, if not all, excessive alcohol drinkers; however, advanced liver disease, such as AC, only occurs in a subset of patients. Patients with AC, in general, have clinical features similar to those with other chronic liver diseases. Liver tests are nearly normal in the compensated state, while complications from portal hypertension, such as ascites, hepatic encephalopathy, and esophageal varices, are common in decompensated patients. Once decompensation occurs, overall prognosis and long-term survival are poor. Expression of coding genes is altered in multiple pathways in ALD pathogenesis and during the disease progression, leading us to hypothesize that the expression of specific coding genes is critical for the progression of AC from compensated to decompensated states.
Our results showed a unique peripheral blood RNA signature in patients with AC when compared to HCs. These differentially expressed mRNAs have distinct functions. We observed the genes related to angiogenesis as one of the top up-regulated gene sets. The formation of new vessels with the establishment of an abnormal angioarchitecture is a process related to progressive fibrogenesis and cirrhosis.\(^{(27)}\)

Additionally, several lines of evidence support a role for angiogenesis in the pathogenesis of portal hypertension.\(^{(27)}\) Excessive alcohol use leads to an impairment of mitochondrial respiration secondary to lipid peroxidation products and inflammatory cytokines.\(^{(28)}\) Together, they can affect mitochondrial permeability and thus impair oxidative phosphorylation,\(^{(28)}\) as reflected by the induction of the gene sets related to this pathway in patients with AC. The observation on the induction of xenobiotic gene sets in AC is not surprising as the liver is known for its essential role in the processing of xenobiotics. Alteration in the liver architecture secondary to cirrhosis leads to changes in the activity and level of drug-metabolizing enzymes, such as the cytochrome P450 gene family and glucuronidation capacity\(^{(21,29)}\). A previous report demonstrated that peripheral blood mononuclear cells from patients with AC exhibited down-regulated IFN-stimulated gene expression, both constitutively and after an acute stimulus,\(^{(30)}\) findings that are similar to our study. Lastly, \(\alpha\)-tubulin is a major target for modification by highly reactive ethanol metabolites and reactive oxygen species.\(^{(31)}\) Alcohol also impairs the replication of normal hepatocytes at both the G1/S, and the G2/M transitions of the cell cycle.\(^{(32)}\)

These previous mechanistic studies likely underlie the observation in the alteration of the mitotic spindle and G2M-checkpoint gene sets in our patients with AC.

We carefully analyzed the function of the coding genes to gain a better insight into disease progression from compensated and decompensated states. As the severity of the disease worsens, we observed a higher
quantity of DEGs. We identified 10 genes from peripheral blood based on the fold-change, network analysis, and functional enrichments for validation; eight genes (HMOX1, CD248, SGCID, DROSHA, PTBP1, RAVER1, CAD, and NR3C2) are likely involved in disease progression from Child-Pugh class A to class C. Of these, we found (i) significant differences in the expression of HMOX1 and RAVER1 in peripheral blood of those who died during the follow-up when compared to those who survived and (ii) the expression level of these genes was associated with mortality in univariate COX analyses.

The HMOX1 gene encodes for enzyme heme oxygenase; its function is to catalyze the reaction that degrades the heme group contained in hemoglobin, myoglobin, and cytochrome p450. HMOX1 is ubiquitously expressed; its expression can be induced by pathogen-associated molecular patterns (PAMPs) and oxidative stress, two common mechanisms in the pathogenesis of ALD. Lipopolysaccharides (LPS) from gram-negative bacteria are representative of PAMP molecules. We previously reported a continuing increase in serum LPS level during disease progression from Child-Pugh class A to C in patients with AC (35); this is a plausible mechanism underlying our observation of an increase in HMOX1 expression among patients with AC, as shown in the current study (Fig. 5). Moreover, induction of HMOX1 may also represent an adaptive response against oxidative damage that is worsened during disease progression. (36)

RAVER1, together with PTB, plays an important role in alternative splicing. (37) This is a process of selecting different combinations of splice sites within a messenger RNA precursor during RNA processing to produce variably spliced mRNAs. (38) We found the expression of RAVER1 progressively decreased with worsening disease severity. How RAVER1 and alterations in the RNA splicing process affect disease progression and mortality in patients with AC is not known and deserves further studies.

In the compensated state, alteration of the genes in peripheral blood primarily belongs to inflammatory responses and the innate immunity pathway. However, when the disease is shifted toward a decompensated state, we observed alterations of DEGs related to epigenetic regulation. MicroRNAs (miRNAs), as epigenetic modulators, affect the protein levels of the target mRNAs without modifying the gene sequence. (39) We recently reported changes in a unique miRNA signature among patients with alcoholic hepatitis. (40) DROSHA, part of a multiprotein complex, is an upstream pathway of miRNA generation; it mediates the nuclear processing of the primary miRNAs into pre-miRNA. (41) Mechanistic studies to determine the functional role of DROSHA in AC pathogenesis should be further explored.

We acknowledge several shortcomings of our study. First, the differential gene expression in peripheral blood may not reflect that in the liver, which is more aligned with the disease process in AC. To address this issue, we also examined the hepatic expression of each mRNA in the liver tissues of patients with AC. As expected, differential expression of genes in peripheral blood is not in the same direction as that in the liver. Second, because our analysis is performed in whole blood, it limits our ability to further explore the distribution of cell types that contribute to the differential expression we observed. Third, due to the nature of the study, we did not explore the underlying mechanism of how these genes are implicated in the pathogenesis of AC. Fourth, we did not capture data on the timing of last alcohol consumption before our transcriptomic analysis. Alteration in the gene expression and the potential contribution of lipid peroxidation products on oxidative dysfunction could depend on the duration of abstinence. Lastly, our sample size is relatively small, a future study to validate our results is needed.

In summary, we used a transcriptomic approach to identify unique mRNAs that may implicate disease progression in patients with AC. Future studies to confirm our results are needed, and comprehensive mechanistic studies on the implications of these genes in AC pathogenesis and progression should be further explored.

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Supporting Information
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