ACTG1 and TLR3 are biomarkers for alcohol-associated hepatocellular carcinoma

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Abstract. Alcohol consumption is a risk factor for the development of hepatocellular carcinoma (HCC); however, the association between alcohol and HCC remains unknown. The present study aimed to identify key genes related to alcohol-associated HCC to improve the current understanding of the pathology of this disease. Alcohol-associated and non-alcohol-associated HCC samples in the GSE50579 dataset of the Gene Omnibus Database were analyzed to investigate altered gene expression. Integrated bioinformatics methods were employed to clarify the biological functions of the differentially expressed genes (DEGs), including Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and protein-protein interactions (PPIs). The present study reported that candidate biomarker micro(mi)RNAs via TargetScan Human 7.1. DEGs and their associated miRNAs (according to bioinformatics analysis) were validated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Additionally, 284 EGs from the GSE50579 dataset were revealed. In GO term analysis, DEGs were closely associated with the ‘regulation of nucleic acid metabolism’. KEGG pathway analysis indicated that the DEGs were tightly engaged in the ‘VEGF and VEGF receptor signaling network’, ‘proteoglycan syndecan-mediated signaling events’, ‘erbB receptor signaling’ and ‘β1 integrin cell surface interactions’.

According to the results of PPI and heat map analysis, the main hub genes were centrin 3 (CETN3), Toll-like receptor 3 (TLR3), receptor tyrosine-protein kinase (ERBB4), heat shock protein family member 8, actin β1 (ACTG1) and α-smooth muscle actin. It was demonstrated that the ACTG1, TLR3, miR‑6819‑3p and miRNA (miR)-6877-3P had undefined associations. Furthermore, RT-qPCR analysis revealed that miR‑6819‑3p and miR‑6877‑3p may enhance the expression levels of ACTG1 and inhibit the expression levels of TLR3 in alcohol-associated HCC tissues. TLR3 and ACTG1 were proposed as potential biomarkers of alcohol-associated HCC. Investigation into the regulatory functions of miR-6819-3p and miR-6877-3p may provide novel insights into the treatment of alcohol-associated HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of cancer to result in mortality, worldwide. It has been acknowledged that the primary carcinogenic factors of HCC include hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, alcohol and non-alcoholic steatohepatitis (1). Of these principal etiologies, HBV and HCV predominate in Asian cohorts, whereas European cohorts are associated with heterogeneous etiologies, including alcohol consumption, and viral hepatitis within cirrhotic and non-cirrhotic livers (2). The analysis of patients with chronic HCV infection, in addition to alcohol abuse and dependence, indicated that elevated proton pump activity increased the risk of hepatic decompensation and hepatocellular carcinoma (3). According to the study of non-alcoholic fatty liver disease (NAFLD) in Indian patients, 11 patients with a history of considerable alcohol consumption had at least one risk factor for NAFLD, including alcohol-associated HCC (4). Asian countries have been reported to have a high incidence of HBV infection. A previous study of the Indian population demonstrated a lower prevalence of HBV infection of unknown origin (4); however, the direct role of alcohol in the carcinogenesis of HCC, or the mechanism underlying alcohol-induced hepatotoxicity associated with alcohol metabolites, oxidative stress and iron metabolism, are yet to be elucidated (2,5). The administration of alcohol/diethyl...
nitrosamine/carbon tetrachloride in mice successfully induced an α-fetoprotein-secreting HCC model in adult male BALB/c mice. This research may improve current knowledge of the stages of occurrence and development associated with HCC (5). The underlying mechanism of the alcohol-induced development of liver diseases remains unknown. Recently, the epidemiological evidence regarding the association of alcohol and liver cancer was summarized; this publication demonstrated that it is crucial to identify all aspects associated with the effects of alcohol metabolism on alterations in hepatocyte metabolism, particularly neoplasia-associated alterations (6).

Microarray data analysis of the expression of different genes has provided powerful and feasible evidence for the clinical diagnosis and treatment of HCC. In the present study, the GSE50579 dataset was downloaded from the Gene Expression Omnibus database (GEO; http://www.ncbi.nlm.nih.gov/geo), which includes data regarding alcohol-associated HCC and HCC liver tissue samples; the differentially expressed genes (DEGs) were screened, and bioinformatics analysis was conducted for functional and pathway enrichment, and for the investigation of protein-protein interaction (PPI) networks. Statistical analysis and functional annotation revealed that actin γ1 (ACTG1; DFN20/26) and Toll-like receptor 3 (TLR3) may be biomarkers for alcohol-induced HCC. Notably, the present study reported that DEG-associated microRNAs (miRNAs) may be considered as potential targets in the treatment of alcohol-induced HCC. In addition, these biomarkers were assessed by reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) in samples collected from patients with HCC.

Materials and methods

**Ethics statement.** The present study was conducted with the approval of the Ethics Committee of the Affiliated Hospital of Qingdao University (Qingdao, China). Patients provided informed consent for the use of their tissue specimens. The mean age of 30 patients, including 18 males and 12 females, was 52.6±16.8 years. A total of 30 clinical tissue samples, including 12 from patients with alcohol-associated HCC, 12 from patients with non-alcohol-associated HCC tissues, and six from healthy individuals were obtained from the Department of Hepatobiliary Surgery of the Affiliated Hospital of Qingdao University between March 2017 and July 2017.

**Identification of differentially expressed genes.** The microarray data set GSE50579 (7) was downloaded from the GEO, in which 10 alcohol-associated HCC and 16 clinical HCC liver samples were analyzed. The platform employed for analysis was the Agilent-028004 SurePrint G3 Human GE 8x60K Microarray (Agilent Technologies, Inc., Santa Clara, CA, USA). The annotation file was also acquired; the original CEL format was converted into an expression matrix using a function to study RNA with an Affy package (8). Probes were mapped to genes according to the annotation file in R version 3.3.3 (9). Average expression levels were calculated for the probes corresponding to the same gene. DEGs between alcohol-associated HCC tissue samples and non-alcohol associated individuals
Bioinformatics analysis of DEGs. Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses (https://www.genome.jp/kegg) were performed for the DEGs using the Cluego APP in Cytoscape (version 3.5.1; www.cytoscape.org). A previous study (10) demonstrated that certain genes sharing the same pathway or similar biological functions and gene expression patterns exhibit the same pathological effects. Therefore, the construction of a gene co-expression network may help identify gene sets associated with specific pathways and biological processes. In the present study, Pearson correlation analysis of co-expression was performed with a coefficient >0.85 and P<0.05 as the criteria for investigation. In addition, PPIs were constructed using Cytoscape software. Hub genes were identified within the PPIs of DEGs, and the edge length between nodes from these hub genes was determined.

miRNA analysis of the DEGs. The present study investigated numerous genes, including, centrin 3 (CETN3), TLR3, erbB2 receptor tyrosine kinase 4 (ERBB4), heat shock protein family member 8 (HSPA8), ACTG1 and α-smooth muscle actin (ACTA2) on the TargetScan Human 7.1 website (www.targetscan.org/vert_71) to predict the associated miRNAs of these genes. A diagram of the miRNAs and their predicted target genes was produced using Fun Rich software (http://funrich.org; version 3.0). Scores ≥90 points and overlapping miRNA quantity ≥3 were set to obtain the desired miRNAs and target DEGs.

RNA extraction. Tissues were stored at -80˚C until use. Total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer’s instructions. The RNA solutions were stored at -80˚C. The RNA quality was determined by spectrophotometer.

RT-qPCR. RNA (1 µg) was reverse transcribed into cDNA using FastQuant RT kit (with gDNase) (Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer’s protocol. cDNA was amplified using SYBR Green Real Master mix (Tiangen Biotech Co., Ltd.), according to the manufacturer’s protocol. The PCR reaction was conducted at 95˚C for 15 min, followed by 40 cycles at 95˚C for 10 sec, 58˚C for 20 sec and 72˚C for 30 sec. RT-qPCR was performed on the ABI StepOnePlus™ thermocycler (Thermo Fisher Scientific, Inc.). U6 single nuclear (sn)RNA was employed as the loading control for micro (mi)RNA expression, and β-actin for the expression of genes; stem-loop RT primers were used for miRNAs, and oligo (dinucleotide) primers for genes. Primers are displayed in Table I. All reactions were repeated three times and the data were calculated by the comparative 2−ΔΔCq method (11).

Statistical analysis. All statistical analyses were performed using R software version 3.3 and GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, CA, USA). The data are presented as the mean ± standard deviation. Statistical significance was examined using Student’s t-test or one-way analysis of variance followed by Dunnett’s test. P<0.05 was considered to indicate a statistically significant difference. To identify the prognostic value of the differentially expressed coding genes, Kaplan-Meier survival analysis was conducted.

Results

Identification of DEGs. A total of 284 DEGs from the GSE50579 dataset were obtained, which included 68 upregulated and 216 downregulated DEGs. The top DEGs were presented as a heat map (Fig. 1).

Bioinformatics analysis of the DEGs. To further understand the functions of the DEGs, GO and KEGG pathway analyses were conducted. The GO analysis results indicated that the DEGs were involved in ‘immune response’ and ‘regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism’ (Fig. 2). In addition, KEGG pathway analysis revealed that the DEGs were closely associated with ‘VEGF and VEGFR signaling network,’ ‘proteoglycan syndecan-mediated signaling events’, ‘erbB receptor signaling network’ and ‘β1 integrin cell surface interactions’ (Fig. 3).

According to the PPI network in Fig. 4, including the upregulated (Fig. 4A) and downregulated PPI network analyses (Fig. 4B), seven hub genes were identified, including CETN3, TLR3, ERBB4, HSPA8, ACTG1, ACTA2 and interleukin-8. For consistency with the heat map, CETN3, TLR3, ERBB4, HSPA8, ACTG1 and ACTA2 were selected for further investigation.

A diagram was generated according to the results of TargetScan analysis of DEGs and their associated miRNAs (Fig. 5). In accordance with the criteria previously set, miRNA (miR)-6819-3P and miR-6877-3P, and their common target genes ACTG1 and TLR3, were selected for further analysis via RT-qPCR.

Validation of DEGs and miRNAs via RT-qPCR. The relative mRNA expression levels of the DEGs and selected miRNAs are presented in Fig. 6. The significantly upregulated mRNA expression levels of ACTG1 (Fig. 6A) were consistent with the bioinformatics analysis. Similarly, the results for TLR3 (Fig. 6B) were in line with the bioinformatics analysis, in which the gene was downregulated in alcohol-associated HCC tissues. It was also indicated that miR-6819 (Fig. 6C) and miR-6877 (Fig. 6D) were upregulated in alcohol-associated HCC compared with non-alcohol associated HCC. These results indicated that miR-6819 and miR-6877 may positively influence the ACTG1 gene, and exert a negative regulatory effect on TLR3.

Discussion

The role of chronic alcohol consumption in the induction and development of cancer is well known; heavy alcohol consumption has been reported to exhibit negative effects on human health, and there is increasing evidence to suggest that alcohol increases the risk of carcinogenesis (12).
The global HCC BRIDGE study reported that the most common risk factor in North America, Europe and Japan is HCV, and HBV in China, South Korea and Taiwan; together with estimates of alcohol consumption per capita (World Health Organization), alcohol has been reported as a strong risk factor for HCC. Additionally, the reported rate of alcohol abuse...
Figure 4. Protein–protein interaction network of huh genes obtained using Cytoscape software. The hub genes are ACTA2, HSPA8, IL-8, ACTG1, CETN3, TLR3 and ERBB4. (A) Network of upregulated protein–protein interactions. (B) Network of downregulated protein–protein interactions. Blue nodes represent hub genes. ACTA2, actin \( \alpha_2 \); HSPA8, heat shock protein family A (Hsp70) member 8; IL-8, interleukin 8; ACTG1, actin \( \gamma_1 \); CETN3, centrin 3; TLR3, Toll-like receptor 3; ERBB4, erb-b2 receptor tyrosine kinase 4.

Figure 5. Illustration of DEGs and their common regulatory miRNAs; generated according to the results of TargetScan analysis of DEGs and their associated miRNAs. DEG, differentially expressed genes; miRNA, microRNA; ACTA2, actin \( \alpha_2 \); HSPA8, heat shock protein family A (Hsp70) member 8; ACTG1, actin \( \gamma_1 \); CETN3, centrin 3; TLR3, Toll-like receptor 3; ERBB4, erb-b2 receptor tyrosine kinase 4.
in China, Taiwan and Japan was estimated to be 24, 18 and 2%, respectively (1). Studies of the alcohol-induced liver injury model revealed that hypothalamic β-endorphin neuron transplants may reduce liver weight and triglyceride accumulation; fewer pathological alterations occurred, including infiltration of inflammatory cells and steatosis of hepatocytes (13). In addition, investigation into the association between alcohol consumption and the risk of gastric cancer suggested that aldehyde dehydrogenase [ALDH2; National Center for Biotechnology Information (NCBI) accession no. rs671], rather than alcohol dehydrogenase 1B (ADH1B; NCBI accession no. rs1229984) and 1C (ADH1C; NCBI accession no. rs698), which modify acetaldehyde, may serve an important role in gastric cancer (14).

Xu et al (15) reported that alcohol may promote the metastasis of colorectal cancer cells by modulating the glycogen synthase kinase 3β/β-catenin/monocyte chemoattractant protein-1 signaling pathway. Furthermore, it has been demonstrated that an alcohol intake >30 g/day may be associated with an increased risk of colon cancer (12). Similarly, research into breast cancer suggested that the accumulation of alcohol at a sufficient level may induce cytochrome P450 2E1, which causes mutagenic DNA adducts (16). In the present study, DEGs were compared between alcohol-associated HCC (n=10) and HCC (n=16) tissues in the GSE50579 dataset; ACTG1 and TLR3 were selected among 284 DEGs as potential therapeutic biomarkers in alcohol-associated HCC, as determined by bioinformatics analysis.

The ACTG1 gene encodes actin γ1, which belongs to the actin family of six highly conserved proteins. These actin filaments are critical for the structure of the cytoskeleton and the shape of the cell, and as such have been reported to regulate cell motility, contraction and growth (17). Notably, researchers observed that within this Norwegian family, ACTG1 mutations were not frequently associated with hereditary hearing impairment. In addition, the p.V370A mutation may impair actin function, which was demonstrated by a yeast growth assay (17). K118N and E241K in ACTG1 have been considered as two novel mutations that cause an aberrant multi-vacuolar pattern in yeast and mammalian cells (19). According to whole exome sequencing data and massive parallel sequencing, p.M305T and p.E316k have been selected as nonsyndromic hearing loss variants (20,21). Moreover, ACTG1 is positively implicated in the growth and survival of mammals, as ACTG−/− animals were observed to be smaller in size and exhibited a delay in the development of the cardiac outflow tract (22). Utilizing targeted next generation sequencing analysis, another study reported that in Japanese families, ACTG1, nonsyndromic hearing impairment 5, POU class 4 homeobox 3, solute carrier family 26 member 5, SIX homeobox 1, myosin VIIA, cadherin related 23, protocadherin related 15 and Usher syndrome 2A may be candidate genes for early-childhood hearing loss (23).
ACTG1 has also been associated with other types of cancer. Studies have demonstrated that miR-888 may reduce the mRNA expression levels of all four adherens junction pathway-associated genes, including E-cadherin, ACTG1, receptor-type tyrosine-protein phosphatase T and cell division cycle 42 in MCF-7 cells (24). Additionally, ACTG1 has been reported to exhibit high expression levels in skin cancer tissue, where it may regulate A431 cell proliferation and migration via the Rho-associated protein kinase signaling pathway (25). ACTG1 has also been predicted to be a target gene of miR-145-5P in non-small cell lung cancer (26). Furthermore, ACTG1 and matrix metalloproteinase 14 (MMP14) were validated as targets of miR-10a in colorectal cancer cells; ectopic expression of ACTG1 and MMP14 may delay decreases in cell adhesion and anoikis resistance activity (27). It has also been reported that in HBx-induced HCC, ATCG1 was upregulated in human liver tissues (28).

TLR3 belongs to the Toll-like receptor family and is a receptor for double-stranded (ds)RNA (29); as such, TLR3 is reported to exhibit protective immunity during viral infection (30). TLR3 has also been reported in a number of cancer types and the TLR3 signaling pathway may serve an important role in cell homeostasis. BM-06, a 25-nucleotide dsRNA, may activate TLR3 to inhibit the proliferation and promote the apoptosis of HepG2.2.15 cells (29). TLR3-TIR-domain-containing adapter-inducing interferon-β (TRIF) signaling may contribute to myocardial inflammation and infarction when ischemia-reperfusion-associated extracellular RNA is released (31). In addition, TLR3 is positively associated with HBV antigen (HBsAg) in HepG2.2.15 cells, and may promote interstitial immunoreactive cell infiltration and HCC cell apoptosis (32). According to RNA-seq association analysis, TLR3 was proposed as an immune gene, particularly within BV-2 microglial cells (33). High expression levels of TLR3 are a distinctive feature of CD8+ dendritic cells, where activation of the receptor may induce an interferon (IFN)-dependent antiviral response in dendritic cell subtypes (34). It has been reported that in Chinese newborns with severe hepatitis, TLR3, TLR2, TLR4 and TLR9 may serve as potential therapeutic targets for treatment (35). Of note, TLR3 exhibits lower levels of expression in HCC when compared with adjacent tissues, and is positively associated with TRIF, nuclear factor-κB and IFN regulatory factor 3, which may inhibit HCC proliferation and promote HCC cell apoptosis (36). The expression levels of TLR3, TRIF and mitochondrial antiviral-signaling proteins were consistently decreased in chronic HCV-infected liver tissue, compared to that of than non-diseased liver tissue (37). Additionally, endogenous miR-155 can negatively regulate TLR3 expression and inhibit IFN-β production (38). Another study has reported that a TLR3 agonist may enhance the clinical efficacy of sorafenib in treating HCC (29,39). A TLR3 agonist may also inhibit angiogenesis and induce the apoptosis of human HCC cells (40). In addition, the cytoplasmic expression of TLR3 was positively associated with HBV infection (32,36). Compared with that of adjacent tissues, the expression of TLR3 is lower in HCC tissues and is correlated with longer durations of survival (36). The evidence that dsRNA induces the activation of TLR3 further strengthens the hypothesis that TLR3, as a cytotoxic agent, may contribute to developments in HCC therapy (41).

miRNAs engage in numerous cellular activities and affect important physiological functions. In the present study, ACTG1 and TLR3 were proposed to be targets of miR-6819-3P and miR-6877-3P. In addition, the expression levels of miR-6819-3P and miR-6877-3P were observed to be higher in alcohol-associated HCC compared with non-alcohol-associated HCC tissues; the upregulation of miR-6819-3P appeared to be higher compared with the upregulation of miR-6819-3P. Additionally, ACTG1 and TLR3 may regulate the VEGF and VEGFR signaling, proteoglycan syndecan-mediated signaling, erbB receptor signaling, and β1 integrin cell surface interactions. ACTG1 and TLR3 may also function in the immune response and nucleic acid metabolism. The results of the present study indicated that miR-6819-3P and miR-6877-3P enhanced the expression of ACTG1 and inhibited that of TLR3. Further investigation is required to determine the association between ACTG1, TLR3, miR-6819-3P and miR-6877-3P in alcohol-associated HCC tissues.

In conclusion, comprehensive bioinformatics analysis was performed to screen candidate genes for the treatment of alcohol-associated HCC; the physiological functions and regulatory mechanisms of TLR3 and ACTG1 require further study. To further investigate the effects of alcohol on HCC, molecular biological analysis in established HCC animal models may be advantageous; however, the findings of the present study may provide novel insight into the alcohol-associated etiopathogenesis of HCC and subsequently improve the clinical diagnosis of the disease.

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Availability of data and materials
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
JL and LM designed the experiments. BG, SL and ZT conducted the experiments. SL and ZT collected the clinical data and were responsible for ethics approval and consent of patients to participate in the study. BG and SL performed the bioinformatics and statistical analysis; JL and LM were responsible for the overall design and funding of the project.
The authors declare that they have no competing interests.

Informed consent was obtained from patients of the Affiliated Hospital of Qingdao University. Written consent for publication in the present study was approved by the Ethics Committee. All authors have and approved the final manuscript. JL, LM and BG prepared the figures and wrote the manuscript. The authors declare that they have no competing interests.

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JL, LM and BG prepared the figures and wrote the manuscript. The authors declare that they have no competing interests.

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