Establishment and verification of potential biomarkers for cholangiocarcinoma

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Abstract. Cholangiocarcinoma (CCA) is a malignancy arising from multiple locations along the biliary tree, which is still lacking effective diagnostic biomarkers. The present study aimed to provide a comprehensive differential gene expression profile for the disease. The differentially expressed genes (DEGs) for CCA were explored in-depth using a Gene Expression Omnibus (GEO) dataset, an internal cohort of clinical participants as well as in vitro experiments with the HUCCT1 cell line. Based on the GEO dataset, potential biomarker genes were proposed and the enriched biological processes as well as signaling pathways were further investigated. A protein-protein interaction network of target genes was established. In the clinical specimens, the functions of the primary candidate, FBJ murine osteosarcoma viral oncogene homolog B (FOSB), were evaluated by reverse transcription-quantitative (RT-q)PCR and western blot analysis. A Cell Counting Kit-8 (CCK-8) assay was used for a functional study on FOSB. The results indicated that, compared with non-tumor bile duct tissues, primary CCA samples had 676 differentially expressed genes, including 277 downregulated and 399 upregulated ones. Among these, HBD, FOSB, HBB, ITIH2, FCGBP, MT1JP, P1JR, SLC38A1, COL10A1 and MMP19 were determined to be the most significant DEGs. At the same time, upregulated genes were enriched in ‘vasculature development’ and ‘IL-17 signaling pathways’. Downregulated genes were enriched in ‘extracellular matrix progress’ and ‘glucuronate signaling pathway’. The patients with CCA displayed decreased levels of hemoglobin. Compared with paracancerous tissues, CCA cancerous tissues exhibited increased RNA and protein expression levels of FOSB according to RT-qPCR and western blot analysis, respectively. Furthermore, FOSB expression influenced the proliferation/viability of the CCA cell line HUCCT1. In conclusion, the present study suggested that the FOSB gene may serve as a primary biomarker candidate for CCA, providing a valuable reference for its clinical implementation.

Introduction

Cholangiocarcinoma (CCA) is an epithelial cell malignancy arising from multiple locations along the biliary tree with features of cholangiocyte differentiation; it is the second most common hepatic malignancy after hepatocellular carcinoma (HCC) (1). Over past decades, the mortality of CCA has gradually increased worldwide (2). The well-accepted classification of CCA is based on the anatomical location, referring to intrahepatic CCA (iCCA), perihilar CCA (pCCA) or distal CCA (dCCA) (3). iCCA refers to CCA located proximally to the second-degree bile ducts, while pCCA refers to CCA between the second-degree bile ducts and the insertion of the cystic duct, whereas dCCA pertains to CCA between the origin of the cystic duct and ampulla of Vater (4). Clinically, each subtype has been demonstrated to have a distinct epidemiology, prognosis and clinical management. According to a previous study, pCCA, dCCA and iCCA account for ~50, ~40 and ~10% of CCA cases, respectively, with certain rare cases of mixed CCA (5).

The worldwide incidence of CCA is not homogeneous due to the variable prevalence of risk factors. Similar to HCC, several studies have highlighted hepatitis B virus (HBV) and hepatitis C virus (HCV) as key factors for the development of CCA (6,7). Apart from HBV and HCV, there are still certain non-viral risk factors that may also induce CCA, such as diabetes, alcoholism and dyslipidemia (8,9). A combination of CT and MRI with magnetic resonance cholangiopancreatography is recommended for the diagnosis of CCA. Occasionally, the cancer antigen 19-9 (CA 19-9) is applied as a potential serum biomarker for CCA diagnosis, with CA 19-9 levels >1,000 U/ml indicating the presence of metastatic disease (10,11). However, the CA 19-9 is not specific
for CCA and the diagnostic rate is far from satisfactory. At present, resection is still the mainstay of effective therapy for CCA (12). At the same time, liver transplantation has been suggested as an alternative option for a subset of cirrhotic patients. However, the source of liver would be a major issue. Accumulating research has investigated the molecular pathogenesis of CCA. A study by Farshidfar et al (13) implied that isocitrate dehydrogenase, fibroblast growth factor receptor, encoding Family A receptors as well as biofilm-associated protein families were closely associated with the formation of CCA based on a comprehensive whole-exome and transcriptome sequencing analysis of a large cohort of patients with CCA. To date, several chemical therapeutic medicines targeting relevant signaling cascades (such as those regulating tumor cell proliferation, cell migration, survival maintenance as well as governance of cell fate) have been identified and confirmed in clinical trials (1). However, misclassification of CCA remains a critical concern for clinical administration. At the same time, based on the issues of therapeutic resistance and genetic heterogeneity of the disorder, there is still no curative therapy for CCA. If diagnosed at the early stage, patients with CCA have a 6-30% longer 5-year survival rate compared with those diagnosed at an advanced stage (14). Unfortunately, effective biomarkers for early diagnosis of CCA are out of scope at this moment due to the ‘silent’ clinical characteristics of this disorder (most patients of CCA are asymptomatic in the early stage), which makes it difficult to identify. All of these limitations restrict the sensitivity of cytological as well as other pathological diagnostic approaches in the clinic.

Encoded by the FOSB gene, the FBJ murine osteosarcoma viral oncogene homolog B (FOSB) protein shares structural similarities with the other members within the Fos family and is a key component of the activator protein-1 (AP-1) transcription factor. As a tumor-related gene enhancer element, AP-1 has been suggested to be closely associated with several types of cancer (15). The components of AP-1 complex have critical roles in cancer cell viability, proliferation and migration (16). Yet, the functions of FOSB in the development and progression of CCA remain to be fully elucidated.

Due to the high misdiagnosis rate as well as atypical symptoms of CCA in the early stage, current modalities for establishing a diagnosis of CCA are insufficient, so that the identification of effective biomarkers may be another beneficial future direction. In the present study, a systematically differentially expressed gene (DEG) profile for patients with CCA was generated. The underlying molecular mechanisms and external functions of potential target genes were also evaluated by functional experiments, which all together provided a valuable reference for future clinical manipulation.

Materials and methods

Data sources. In the present study, 3 independent data sources were utilized for DEG analysis of CCA: i) The open public data from the Gene Expression Omnibus (GEO) database; dataset no. GSE132305 (ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132305). This dataset included formalin-fixed, paraffin-embedded samples (182 primary CCA and 38 non-tumor bile duct tissues) from patients treated by surgical resection. The whole-genome expression profiles of the above samples were evaluated by the Affymetrix Human Genome U133 Plus 2.0 Array chip platform for differential gene analysis. ii) The second data source was the CCA cell line HUCC1, as previously reported (17), which was cultured for use in a cell proliferation assay. iii) As the third data source, clinical patients with CCA were recruited.

Differential gene expression analysis. The limma package in R was used for differentially expressed mRNA analysis, with an absolute value of the log-transformed differential expression multiple (Log2FC)>1 and P<0.05 as the selection criteria.

Functional enrichment analysis. The ‘clusterProfiler’ package in R was used for Gene Ontology (GO) enrichment analysis in the categories Biological Process, Molecular Function and Cellular Component and for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. P<0.05 was considered to indicate significant enrichment of the corresponding entries. The final results were visualized using the on-line tool from pathview (pathview.uncc.edu).

Protein-protein interaction (PPI) network construction. The Search Tool for the Retrieval of INteracting Genes and proteins (STRING) database (https://STRING-db.org/; version 11.0) was utilized to establish the key protein interactions (18). Cytoscape (cytoscape.org, version 3.7.2) was used to visualize the PPI network. At the same time, the cytoHubba plug-in in Cytoscape was utilized for target gene selection based on the algorithm method of maximum neighborhood component (19).

Cell transfection and Cell Counting Kit-8 (CCK-8) proliferation assay. The FOSB-low expression group (FOSB-LE) was obtained by transfection of FOSB-small interfering (si)RNA following the protocol of a previous study (20). FOSB-siRNA (5'-GCC AAC CAC AAT TCA CAC AAT 3') was designed and synthesized by Merck China. HUCC1 cells (Sunnecell Bio) were cultured in DEME (Thermo Fisher Scientific, Inc. cat. no. 11965118) at 37°C, 5% CO2 to obtain a final 60-80% confluence. Negative control siRNA (NC-siRNA, the NC siRNA scrambled sequence for siRNAs, Mercck; cat. no. SIC002) and FOSB-siRNA were transfected with Lipofectamine® (Thermo Fisher Scientific, Inc. cat. no. L3000015) according to the manufacturer’s protocol. The next day, the transfection complex medium was replaced with complete DMEM (Thermo Fisher Scientific, Inc. cat. no. 10565018). At the same time, FOSB overexpression (FOSB-OE) was achieved by transfection of 100 ng/ml FOSB-RNA plasmid (Hanbio, Inc. cat. no. 202103886). The empty vector was used as a negative control.

For the cell proliferation/viability assay, the HUCC1 cells were seeded and cultured with DMEM with 10% FBS+1% penicillin-streptomycin at 1x104 cells/well. The cultured cells with 100 ng/ml NC-siRNA or FOSB-siRNA or FOSB-RNA to reach a concentration of 2x105 cells/well for all groups and allowed to grow for another 24 h. The CCK-8 solution (10 µl; cat. no. HY-K0301; MedChemExpress) was added to the medium and cells were incubated for 2 h. The
absorbance at 450 nm was measured using a spectrophotometer and the final results were calculated. The experiments were repeated three times independently.

**Subjects and reverse transcription-quantitative PCR (RT-qPCR).** All patients with CCA in the present study were randomly selected from Tianjin Nankai Hospital (Tianjin, China) from January 2018 to January 2021 based on the following inclusion criteria: i) Age of 40-70 years; ii) confirmed by either CT or MRI measurement; iii) confirmed by pathology examination. At the same time, all individuals with chronic hepatobiliary disorder and/or the presence of a malignancy other than CCA were excluded. To eliminate inter-tumor error between different TNM stages of CCA, only those participants with TNM stage II CCA were selected for subsequent analysis. Based on these criteria, 12 patients with CCA were recruited, including 8 males and 4 females, and the average age was 62.32±5.86 years. The demographic and clinicopathological characteristics of patients are shown in Table S1.

The present study was officially approved by the ethics committee of Tianjin Nankai Hospital (Tianjin, China). The cancerous as well as paracancerous tissue samples of the clinical patients were processed for the gene/protein expression analyses (RT-qPCR and western blot analysis).

For RT-qPCR, the CCA cancerous and paracancerous tissues were dissected, snap-frozen and stored in liquid nitrogen. The total RNA was extracted using Rapid RNA extraction kit (Thermo Fisher Scientific, Inc.; cat. no. AM9775) according to the manufacturer's protocol. Subsequently, real-time PCR was performed with BrightGreen 2X qPCR MasterMix-Low ROX (Thermo Fisher Scientific, Inc.; cat. no. B21703). The RT-qPCR reaction system was set up as follows: 5 µl 2 X Master Mix (Thermo Fisher Scientific, Inc.; cat. no. 14001014), 0.5 µl 10 µmol/l PCR upstream and downstream specific primers, 2 µl cDNA template, with double-distilled water to 10 µl. PCR was carried out at 95°C for 10 min; 95°C for 10 sec, 60°C for 1 min, 40 cycles; 95°C for 15 sec, 60°C for 60 sec, 95°C for 15 sec. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method with GAPDH as the endogenous reference gene (21). The reaction was performed in an Applied Biosystems 7300 PULAS system (Thermo Fisher Scientific, Inc.). The primer sequences were FOSB as was follows: Forward, 5'-GCTGCA AGATCCCTTACGAAG-3' and reverse, 5'-ACGAAGAG AGTGACAGGTT-3' and GAPDH: Forward, 5'-GAACG ATATCCCTCACAAT-3' and reverse, 5'-GGCTGTGGTG CATACCTCTCCTG-3'.

**Western blot analysis.** The proteins of CCA cancerous and paracancerous tissues were extracted using RIPA Lysis Buffer (strong; cat. no. HY-K1001; MedChemExpress; 150-250 µl cold RIPA Lysis Buffer per 20 mg of tissue sample) and the total protein concentration was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc.). Subsequently, 50 µg protein of each sample was subjected to 0.1% SDS-PAGE and then transferred onto a PVDF membrane (cat. no. IFL00010; EMD Millipore). The membranes were blocked with 5% nonfat dry milk powder at 37°C for 1.5 h. The corresponding primary antibodies (1:10000 dilution) were added and then incubated at 4°C overnight. The membrane was washed extensively with PBS containing 0.1% Tween-20 and then incubated with horseradish peroxidase-labeled secondary antibodies (1:10,000) at room temperature for 1 h. After washing with PBS, the membrane was processed with ECL developer (cat. no. 6883P3; Univ-bio) and images were acquired with an ultrasensitive multifunctional imager. FOSB, GAPDH and secondary antibodies were purchased from Abcam (cat. no. ab252237, ab9485 and ab205718 respectively).

**Uniform Manifold Approximation and Projection (UMAP) algorithm analysis.** The analysis was performed as previously described (22) to convert mRNA information of high-dimensional data into two dimensional data. The analysis was conducted using online UMAP photography tool (cloudtutu. com/#/index).

**Statistical analysis.** All experiments were repeated 3 times independently. SAS version 19.0 (SAS Institute, Inc.) was used for data analysis. The continuous variables were tested for normality of distribution and Student's t-test was applied to identify significant differences between groups. For the non-continuous variables, the Wilcoxon rank-sum test was conducted to analyze differences between the groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Establishment of a differential gene expression profile for patients with CCA.** In the present study, the data from GSE132305 were first analyzed, based on the UMAP algorithm analysis. When dealing with large-sample genomic data, the UMAP may help reduce the dimension of the data to achieve data visualization and identify internal genetic relationships. At present, UMAP has been widely used in the study of population genetics. In the present study, it was demonstrated that the overall expression pattern between primary CCA and non-tumor bile duct was significantly different via the UMAP method (Fig. 1A).

The expression in the gene panel of 182 patients with primary CCA was compared with that of 38 non-tumor bile duct tissue with genes that had an absolute value of (Log2FC)>1 and P<0.05 considered DEGs. All of the DEGs are listed in Table SII. A negative value of LogFC indicated downregulation, while a positive value of LogFC suggested upregulation. Based on these criteria, 676 DEGs were identified in this CCA cohort, including 277 downregulated and 399 upregulated ones (Fig. 1B). All of the DEGs were further sorted based on the absolute value of Log2FC, and it was revealed that hemoglobin subunit alpha gene (HBA), hemoglobin subunit beta gene (HBB), ITIH2, FCGBP, MT1JP, PIJR, SLC38A1, COL10A1 and MMP19 were the most significant DEGs (with the largest absolute value of Log2FC, presented as Fig. 1C). Since two potential genes (HBB and HBD) represent the members of hemoglobin, the hemoglobin levels of 12 recruited patients with CCA were also evaluated. Both male and female patients displayed decreased levels of hemoglobin (Fig. 1D) (compared with normal ranges of hemoglobin levels: 120-160 g/l for males and 110-150 g/l for females).

**GO and KEGG enrichment analysis.** The GO enrichment analysis is able to determine the functions and biological
processes the gene products may be involved in. GO enrichment analysis may provide an understanding of the biological functions and metabolic pathways affected by differential gene enrichment, which is particularly important in mechanistic research. Metabolism processes were suggested to be closely associated with significantly up-regulated DEGs, suggesting these processes could be enhanced in CCA formation (including vasculature development; Fig. 2A). On the other hand, multiple metabolic processes were indicated to be connected with significantly down-regulated DEGs, indicating these processes should be inhibited in CCA formation (including extracellular matrix (ECM); Fig. 2B).

KEGG enrichment analysis comprises a collection of manually drawn pathway maps, which represent the current knowledge of the potential related molecular interaction, reaction and relation networks for DEGs. In the present study, the pathways associated with the 676 DEGs were investigated using the KEGG enrichment method. The upregulated genes supported by differentially expression analysis were categorized into distinct signaling cassettes (including IL-signaling pathway and mineral absorption; Fig. 2C). Downregulated genes were grouped into signaling cassettes (including glucuronate signaling pathway, protein digestion and absorption; Fig. 2D).

**PPI network construction.** The STRING database was used to construct a PPI network for the 676 DEGs and the gene interactions with a confidence score ≥0.4 were selected for visualization with Cytoscape software. Based on this criterion, 10 MCODEs (MCODE1-10) were identified as significant clustering modules (Fig. 3), among which MCODE1 and 2 were more obvious compared with the others. MCODE1 included HBB protein, which interacted with 3 differentially expressed candidate proteins (so that the degree of HBB=3). At the same time, MCODE2 included FOSB, which was associated with the most differentially expressed candidate proteins (score=7).

According to the DEG analysis, HBD, FOSB and HBB were suggested as potential biomarkers for CCA (Fig. 1C). Probably due to the abnormal expression of HBD and HBB, patients with CCA exhibited hemoglobin level changes (Fig. 1D). According to a literature search, the roles of FOSB in CCA progression have remained largely elusive. Of note, the PPI interaction network demonstrated that FOSB protein was
a central regulatory factor and is expected to have a pivotal role in the disorder. Accordingly, the present study further focused on the functions of FOSB to provide verification in independent experimental systems.

Investigation of the functions of FOSB in the formation of CCA. Based on the previous analysis, FOSB was suggested as a potential biomarker for CCA formation. Next, the present study sought to further evaluate the functions of

Figure 2. GO and KEGG enrichment analysis of DEGs in CCA. Metabolic processes associated with (A) up- and (B) downregulated DEGs for CCA. The entries with the largest number of DEGs were the top-ranking terms/pathways. Signaling pathways associated with (C) up- and (D) downregulated DEGs for CCA. The entries with the largest number of DEGs were the top-ranking terms/pathways. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEG, differentially expressed gene; CCA, cholangiocarcinoma.
**Discussion**

CCA represents a heterogeneous group of malignancies that probably occur at any location along the biliary tree of the human body. For the last decades, researchers have been trying to obtain breakthroughs in the discovery of early diagnostic biomarkers for CCA. However, the research progress is far from satisfactory. The well-accepted hypothesis of the
adenoma-dysplasia-carcinoma sequence observed in other cancers has not yet been fully approved in CCA, based on the fact that cells of varying origin may cause the disease (1). The present study first systematically analyzed the DEG profile for patients with CCA using an open public data source. Two primary target genes identified (HBD and HBB) belong to the hemoglobin gene family. Of note, iron-deficiency anemia is a relatively common presenting feature for multiple gastrointestinal malignancies. Ahmad et al (23) reported on a case of a Caucasian, 84-year-old female presenting with recurrent, severe iron-deficiency anemia, who was eventually diagnosed with intra-hepatic CCA. Since hemoglobin genes were also significantly differentially expressed in CCA in the present analysis, the hemoglobin levels were measured in the recruited patients. Compared with the normal range of hemoglobin levels (120-160 g/l for males and 110-150 g/l for females), patients with CCA displayed decreased level of hemoglobin. Hemoglobin is characterized as a key protein consisting of 2 α-like type and 2 β-like type chains. A variation on the globin genes results in an error in the production of the coded chains, which initiate hemoglobinopathies, such as thalassemia and sickle cell diseases (22). In the present case, elevated expression levels of HBB and HBD were observed, which is expected to lead to enhanced activity of hemoglobin. However, this was not the case. It was speculated that the decreased hemoglobin was either caused by other internal negative inhibitory signaling events or a consequence of unknown protective molecular mechanisms associated with CCA. Further investigation in this context is required. The function of hemoglobin is heterogeneous among individuals and anemia has been indicated to be closely associated with distinct diseases. To this end, the present study focused on another potential biomarker gene for CCA diagnosis, namely FOSB.

The AP-1 protein family consists of diverse factors, including FOSB, FOS-related antigen 1, FRA2, c-Jun and Jun-B, which have key roles in the transcriptional regulation of numerous genes related to cell proliferation, differentiation, migration and metastasis, as well as survival (20). To date, FOSB has been suggested to be involved in the progression of several types of cancer. For instance, FOSB was observed to be unmethylated in non-small cell lung cancer and identified as a novel predictive biomarker for NSCLC prognosis (24). At the same time, FOSB was indicated to be required for the migration and invasion of prostate cancer cells, which was dependent on the regulation of TGF-β1 (20). Another study reported that SERPINE1-FOSB fusion was identified as a consistent genetic alteration in pseudomyogenic hemangioendothelioma, suggesting FOSB as a useful diagnostic biomarker for the disease (25). In the present study, GO enrichment analysis indicated that ECM processes were markedly enriched in CCA. Accumulating evidence has demonstrated the connection between FOSB and ECM formation. An in vitro study supported that the DNA-binding activity of FOSB was enhanced after stretch stimulation of smooth muscle cells (26). FOSB silencing attenuated the expression of the profibrotic factors tenascin C and connective tissue growth factor, which all together indicated the role of FOSB as a mechanosensitive regulator of ECM production in smooth muscle (27). More interestingly, FOSB has also been implied to be a key regulator of matrix metalloproteinases (MMPs). For instance, one of the MMP members, MMP-9, is a central factor in several processes of tumor formation, such as angiogenesis, cell migration, as well as invasiveness based on the fact that it is able to almost fully degrade the basement membrane and ECM components. A study by Li et al (28) suggested that FOSB increased the expression of MMP-9 directly in MCF-7 breast cancer cells and overexpression of FOSB enhanced the cellular viability and decreased cell apoptosis. The present study supported these conclusions. Compared with paracancerous tissues, both RNA and protein expression levels of FOSB were increased in CCA cancerous tissues. Furthermore, FOSB also inhibited the proliferation of the CCA cell line HUCC7 according to the

![Figure 4. Investigations of the functions of FOSB in CCA.](image-url)

(A) Reverse transcription-quantitative PCR analysis of FOSB gene expression in cancerous and paracancerous tissues of 12 patients with CCA. (B) Western blot evaluation of FOSB protein in cancerous and paracancerous tissues of patients with CCA. (C) Impact of FOSB expression on the viability/proliferation of HUCCT1 cells determined by a Cell Counting Kit-8 assay. *P<0.05; **P<0.01. NC, negative control; FOSB-OE, FOSB-overexpression; FOSB-LE, FOSB low expression; FOSB, FBJ murine osteosarcoma viral oncogene homolog B; CCA, cholangiocarcinoma.)
CCK-8 assay results. Based on the PPI network established, FOSB potentially interacted with 7 differentially expressed targets, which are JUN, MYC, FOSL2, FOS, FOSL1, ATF3 and MAPK10. JUN, FOSL2, FOS, FOSL1, as well as ATF3 all belong to the AP-1 gene family, whereas FOSB is associated with them to a certain extent. AP-1 family elements are regulatory sequences within genomes, which direct the selective expression of particular genes in order to obtain their highly specialized forms and functions. Previously, AP-1 members were reported to be activated by the Ras/MAPK pathway in nearly all cell types (16). However, how the regulatory sequence elements associated with CCA remain elusive, providing a worthwhile direction for future study.

In summary, in this integrated study, the DEGs in patients with CCA were elucidated in-depth and several candidate genes (particularly FOSB) were proposed for early diagnosis. The present study provided a direction for the future clinical management of CCA.

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

Authors' contributions
SW collected data and contributed to the conception. LY performed the experiments, and analyzed and interpreted the data. XS analyzed and interpreted the data as well as supervised the work. BZ designed the experiments and wrote the manuscript. XS and LY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate
This study was officially approved by the ethics committee of Tianjin Nankai Hospital (Tianjin, China). Informed consent was obtained from all individual participants included in the study.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


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