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Original Article

Potential targeting pathways of microRNA-376c in human cholangiocarcinoma: an analysis based on mRNA microarray and bioinformatics

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Abstract: Cholangiocarcinoma, with high morbidity, poses a threat to human life and health obviously. Meanwhile, microRNA-376c, an emerging non-coding RNA, has been reported to play vital roles in various cancers. The objective of the present study was to unravel the potential targeting pathways of microRNA-376c in human cholangiocarcinoma based on data from an mRNA microarray and bioinformatical analysis. The Agilent mRNA microarray GSE47186 was obtained from Gene Expression Omnibus, which included the differently expressed genes post pre-miR-376c-transfection in cholangiocarcinoma HuCCT1 cells. The down-regulated genes by pre-miR-376c-transfection were first gathered. Then, we collected the potential target genes via in silico prediction and merged them with the above down-regulated genes. We further carried out bioinformatical analysis with regard to the potential target genes of microRNA-376c, including GO functional enrichment analysis, KEGG pathway analysis and protein-protein interaction (PPI) network analysis. The microarray GSE47186 provided 1309 down-regulated genes and in silico prediction yielded 5370 genes. Eventually, a total of 403 genes were regarded as most possible potential targets of microRNA-376c in human cholangiocarcinoma. In terms of KEGG analysis, 10 pathways were found to be significantly enriched, the top three of which were FoxO signaling pathway, MAPK signaling pathway and Chronic myeloid leukemia. PPI network showed that GRB2, TRIO, FYN could be hub target genes of microRNA-376c in cholangiocarcinoma. MicroRNA-376c may play vital parts in cholangiocarcinoma via targeting several pivotal signaling pathways. However, the exact molecular mechanism and clinical role of microRNA-376c in cholangiocarcinoma needs further investigation.

Keywords: Cholangiocarcinoma, MicroRNA-376c, target genes, GO analysis, KEGG pathway analysis

Introduction

Cholangiocarcinoma (CC) is the cancer originated from epithelium of bile duct, classified into hilar cholangiocarcinoma, midpiece cholangiocarcinoma and hypomere cholangiocarcinoma, of which hilar cholangiocarcinoma comprised the highest morbidity, and its age of onset concentrated in the 50-70 year-old. Cholangiocarcinoma can also occur in intrahepatic bile duct, which is a relatively scarce tumor which merely accounts for less than 10% of primary hepatic carcinoma [1-3]. On account of innovation of new diagnostic techniques, the detection rate of cholangiocarcinoma increased in recent years. While radical excision is still a puzzle in terms of cholangiocarcinoma, worse still prognosis remains unoptimistic [4, 5].

MicroRNA (miRNA), a potential research focus, has received wide attention recently [6-10]. Researches on the relationship of cholangiocarcinoma and some miRNAs have been carried out, and some miRNAs gain the potential to be applied in the clinical setting of cholangiocarcinoma, including diagnosis and prognosis prediction [11-13]. However, the role and mechanism of microRNA-376c in cholangiocarcinoma has seldom been reported. To the best of our knowledge, only Kawahigashi et al reported that microRNA-376c was downregulated in an intrahepatic cholangiocarcinoma (ICC) cell line (HuCCT1), as compared to that of a normal intrahepatic biliary epithelial cell line (HIBEpiC) [14]. From the same group, Iwaki et al [15] further verified that miR-376c markedly sup-

Table 1. Reported target genes of microRNA-376c through published articles

Target genes	Verifying method	Disease	Reference
GRB2, MMP, IL1B	RT-PCR and western blotting	Cholangiocarcinoma	Iwaki, J [15]
AGO1, AGO2	qRT-PCR	Idiopathic pulmonary fibrosis	Oak, S. R [16]
UGT2B15, UGT2B17	qRT-PCR	Prostate Cancer	Margaillan, G [17] Wijayakumara, D. D [18]
HIF-1	qRT-PCR	Glioblastoma	Agrawal, R [19]
NFAT5	qRT-PCR	Tuberculosis	Zhang, H [20]
ALK7	RT-PCR and western blotting	Ovarian cancer	Ye, G [21]
ALK5, ALK7	qRT-PCR	Preeclampsia	Fu, G [22]
TGFA	qPCR and western blotting	Osteosarcoma	Jin, Y [23]
IGF1R	qRT-PCR	Melanoma	Zehavi, L [24]

Table 2. Gene Ontology (GO) analysis of target genes of microRNA-376c in cholangiocarcinoma

Category	Term	Count	*P* Value
Biological process	GO: 0030036~actin cytoskeleton organization	14	4.88E-6
Biological process	GO: 0098609~cell-cell adhesion	17	2.99E-4
Biological process	GO: 0008286~insulin receptor signaling pathway	9	3.03E-4
Biological process	GO: 0006915~apoptotic process	26	6.46E-4
Biological process	GO: 0043065~positive regulation of apoptotic process	17	1.35E-3
Biological process	GO: 0006950~response to stress	7	2.18E-3
Biological process	GO: 0006468~protein phosphorylation	21	2.35E-3
Biological process	GO: 0023014~signal transduction by protein phosphorylation	6	2.50E-3
Biological process	GO: 0007165~signal transduction	40	4.26E-3
Biological process	GO: 0010613~positive regulation of cardiac muscle hypertrophy	4	6.43E-3
Cellular component	GO: 0005737~cytoplasm	161	7.64E-9
Cellular component	GO: 0005829~cytosol	110	6.87E-7
Cellular component	GO: 0005925~focal adhesion	23	2.80E-5
Cellular component	GO: 0005913~cell-cell adherens junction	20	4.75E-5
Cellular component	GO: 0005654~nucleoplasm	85	1.85E-4
Cellular component	GO: 0005634~nucleus	144	4.04E-4
Cellular component	GO: 0030496~midbody	10	1.56E-3
Cellular component	GO: 0015629~actin cytoskeleton	13	2.22E-3
Cellular component	GO: 0005856~cytoskeleton	18	2.41E-3
Cellular component	GO: 0031234~extrinsic component of plasma membrane	7	2.82E-3
Molecular function	GO: 0005515~protein binding	188	1.03E-8
Molecular function	GO: 0098641~cadherin binding involved in cell-cell adhesion	20	2.23E-5
Molecular function	GO: 0050699~WW domain binding	5	0.44E-2
Molecular function	GO: 0003779~actin binding	14	0.89E-2

pressed cell migration in cholangiocarcinoma HuCCT1 cells and growth factor receptor-bound protein 2 (GRB2) could be a direct target of microRNA-376c in cholangiocarcinoma. However, since a single microRNA can be targeted by numerous genes and thus influences various signaling pathways, we in the current study attempted to unravel the possible targeting pathways of microRNA-376c in human cholangiocarcinoma based on high-throughput mRNA microarray data and *in silico* target gene prediction.

Materials and methods

Relevant mRNA microarray searching

To collect the relevant mRNA microarray data which was performed post transfection of microRNA-376c mimic or microRNA-376c inhibi-

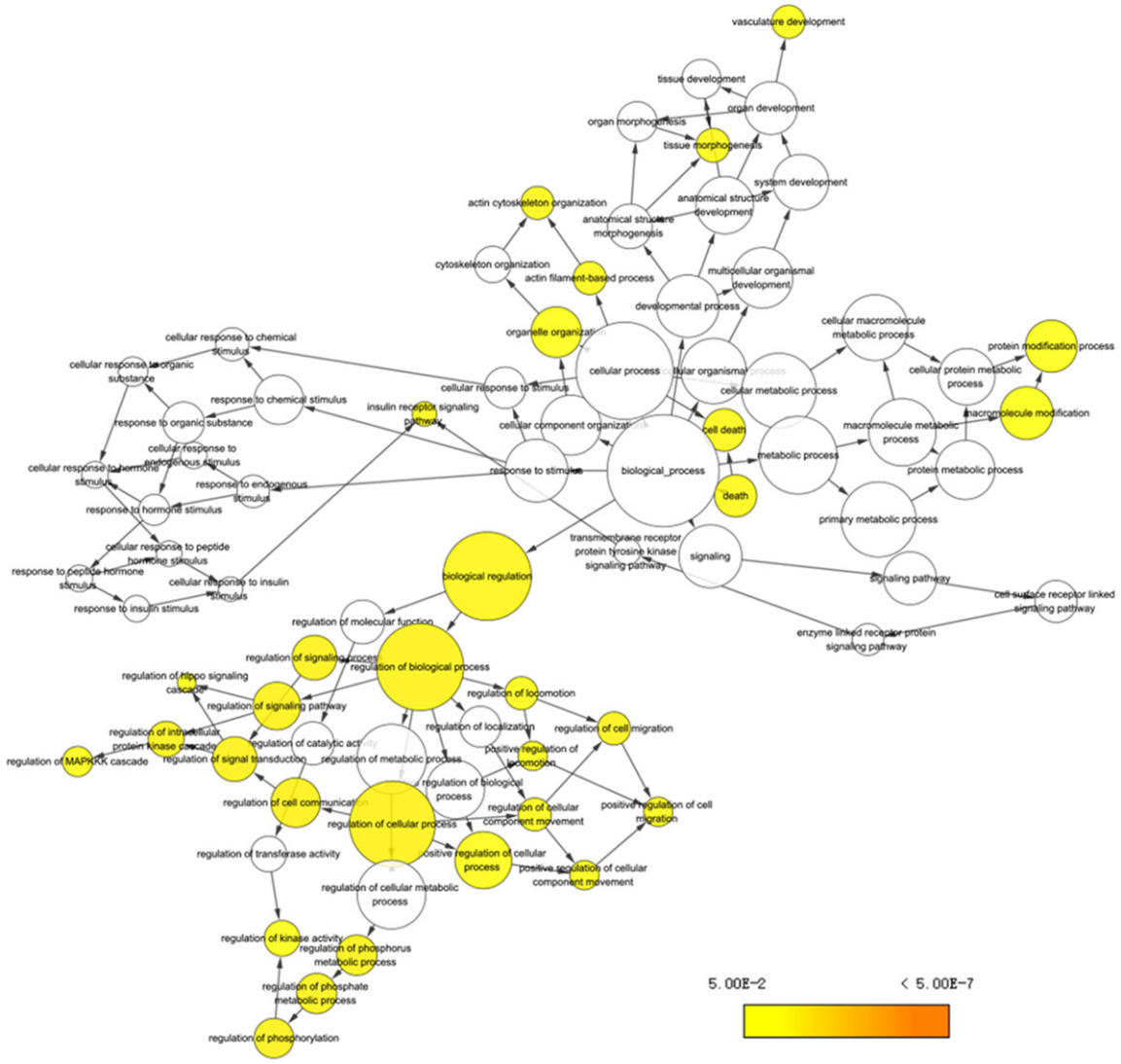

Figure 1. The target gene network of microRNA-376c in cholangiocarcinoma in biological processes. Gene network analysis with the potential target genes of microRNA-376c of biological processes (BP) was drawn by Cytoscape 3.4.0. The circles delegate different terms of BP. The relationships among terms are represented by arrows. The significance level of 0.05 was steered for the current Direct Acyclic Graph (DAG).

tor in cholangiocarcinoma, we performed the comprehensive searching both in public datasets for microarray and literatures. Such keywords were taken in searching in Gene Expression Omnibus (GEO, http://www.idf.org/global-guideline-type-2-diabetes-2012) and ArrayExpress (www.ebi.ac.uk/arrayexpress): (miR-376c OR miRNA-376c OR microRNA-376c OR miR376c OR miRNA376c OR microRNA376c OR "miR 376c" OR "miRNA 376c" OR "microRNA 376c" OR miR-376c-3p OR miRNA-376c-3p OR microRNA-376c-3p) AND (malignan* OR cancer OR tumor OR tumour OR neoplas* OR carcinoma OR adenocarcinoma) AND (bile duct OR cholangio* OR biliary tract OR klatskin). Since microRNA-368 and microRNA-376c are the previous names of microRNA-376c-3p (http://www.idf.org/global-guideline-type-2-diabetes-2012), they were also considered in the strategy of microarray collection. As for the searching in literatures, the following criteria were applied: (MicroRNA OR miRNA OR "Micro RNA" OR "Small Temporal RNA" OR "non-coding RNA" OR ncRNA OR "small RNA") AND (malignan* OR cancer OR tumor OR tumour OR neoplas* OR carcinoma OR adenocarcinoma) AND

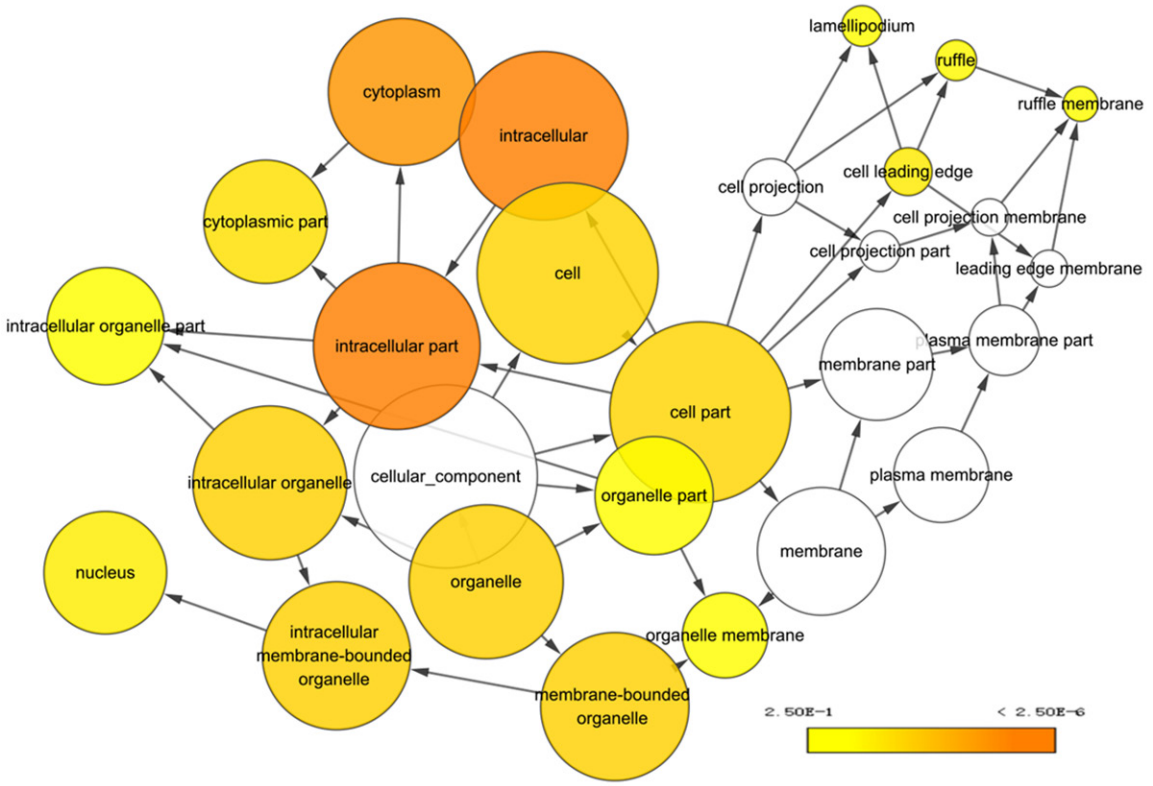

Figure 2. The target genes network of microRNA-376c in cholangiocarcinoma in cellular component. Gene network analysis with the potential target genes of microRNA-376c of cellular component (CC) was drawn by Cytoscape 3.4.0. The circles delegate different terms of CC. The relationships among terms are represented by arrows. The significance level of 0.25 was selected for the current Direct Acyclic Graph (DAG).

(bile duct OR cholangio* OR biliary tract OR klatskin). Except PubMed, searching was also performed in Web of Science, EMBASE, Science Direct, Wiley Online Library, Ovid, Cochrane Central Register of Controlled Trials, LILACS and Google Scholar, as well as Chinese literatures collection from CNKI, WanFang, ChongQing VIP, China Biology Medicine disc. The microarray data included in the current study were updated up to January 10th, 2017.

Calculation of the differently expressed genes influenced by microRNA-376c

The signals from the included microarray data were normalized to the 75th percentile signal intensity. Subsequently, the differently expressed genes were calculated with a fold-change (FC) more than two with GeneSpring GX software (ver. 11.5; Agilent). The potential target genes of microRNA-376c were collected from the down-regulated ones if microRNA-376c was over-expressed *in vitro* and vice versa, due to the inverse relationship between a miRNA and its targets.

Identification of predicted targets of microRNA-376c

Initially, all Pubmed, Web of Science, EMBASE, Science Direct, Wiley Online Library, Ovid, Cochrane Central Register of Controlled Trials, LILACS and Google Scholar, CNKI, WanFang, ChongQing VIP and China Biology Medicine disc were applied to gather the validated target genes of microRNA-376c reported or confirmed through qPCR, Western blot or Reporter assay in the published articles. In addition, a search was conducted for the prediction of the potential target genes of microRNA-376c with 12 established target prediction programs online, including DIANA-microTv4.0, DIANA-microT-CDS, miRanda-rel2010, mirBridge, miRDB4.0, miRmap, miRNAMap, PicTar2, PITA, RNA22v2, RNAhybrid2.1 and Targetscan6.2. Subsequently, we calculated the frequency the predicted genes occurred in above online tools. Target genes were further considered only when predicted by at least three out of 12 target genes-predicting online tools.

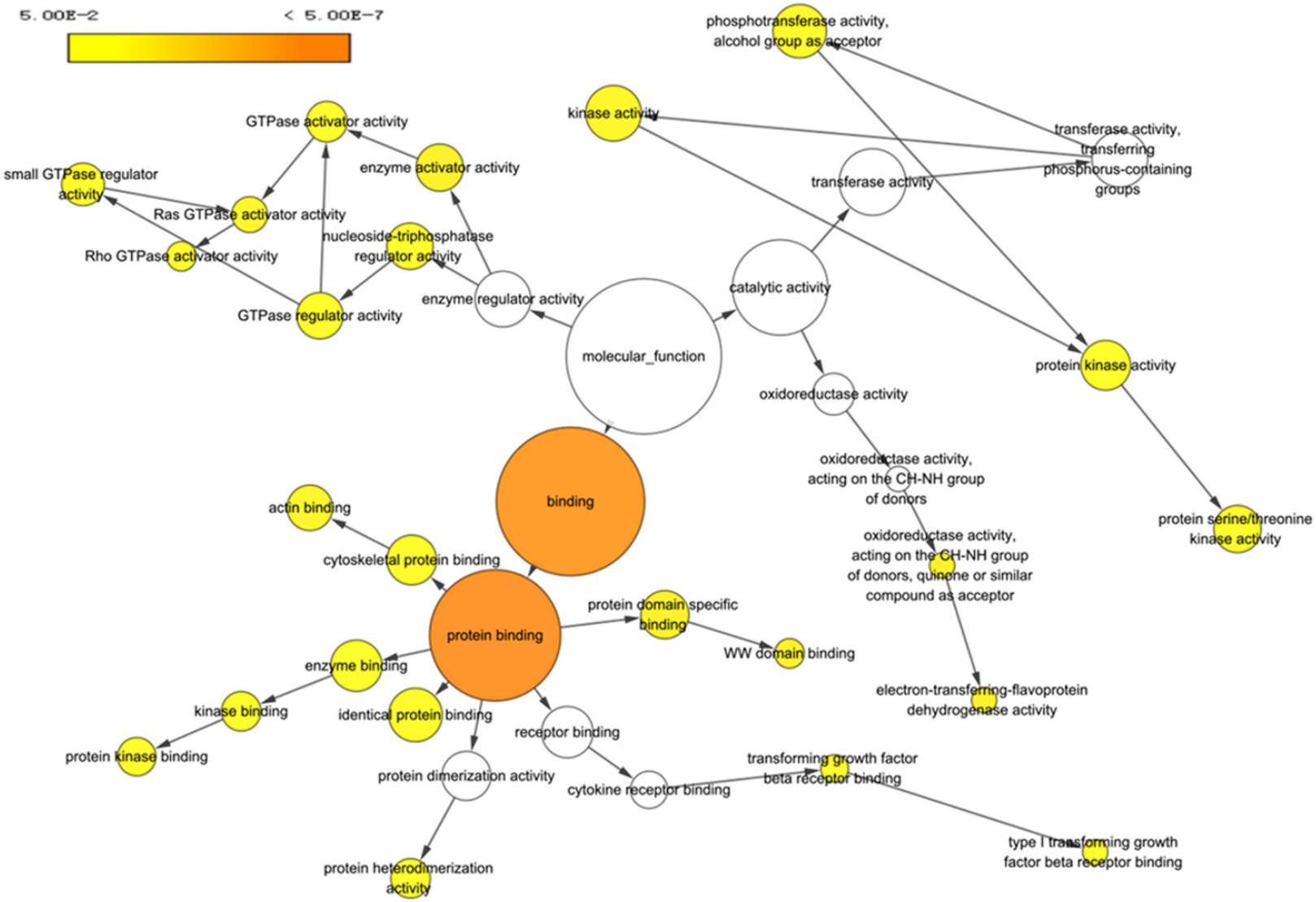

Figure 3. The target genes network of microRNA-376c in cholangiocarcinoma in molecular function. Gene network analysis with the potential target genes of microRNA-376c of molecular function (MF) was drawn by Cytoscape 3.4.0. The circles delegate different terms of MF. The relationships among terms are represented by arrows. The significance level of 0.05 was chosen for the current Direct Acyclic Graph (DAG).

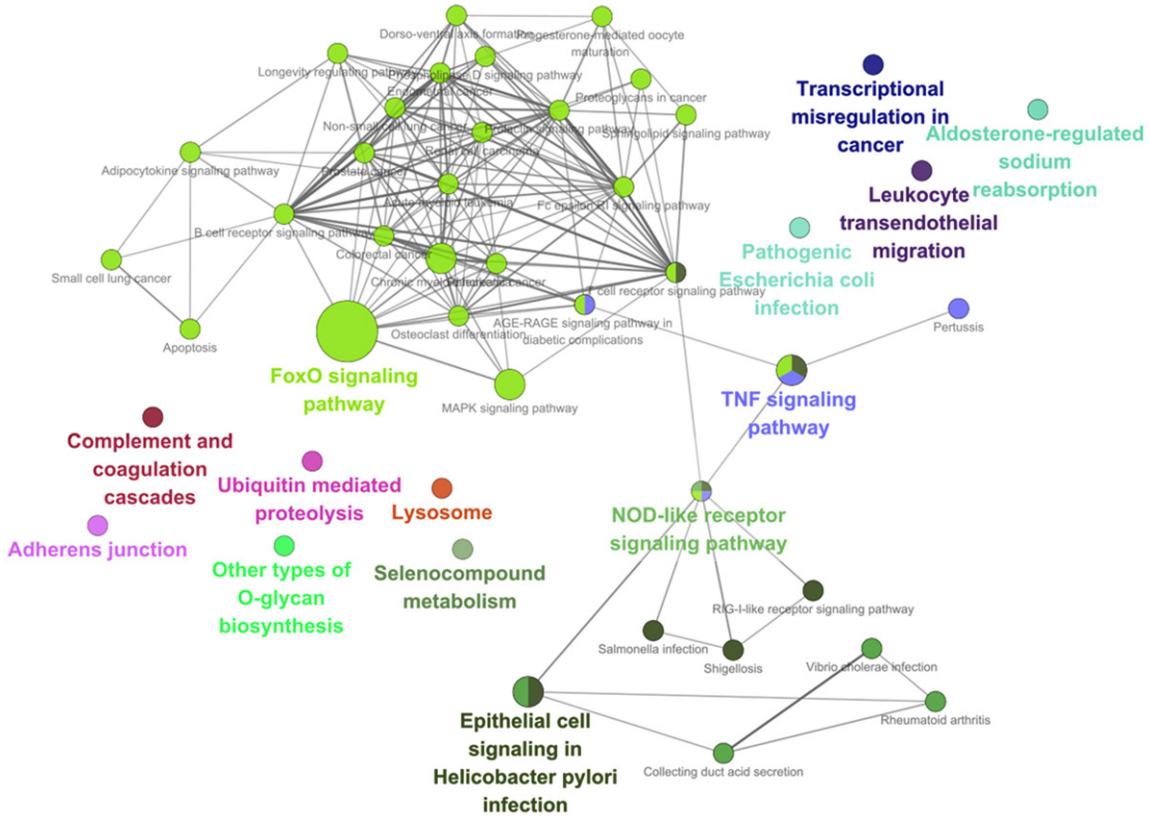

Figure 4. Pathway analysis in KEGG term of targets of microRNA-376c in cholangiocarcinoma. Gene network analysis with the potential target genes of microRNA-376c of KEGG was visualized by ClueGo of Cytoscape 3.4.0. Each color represents one pathway term. The related terms which share similar associated genes was multicolor.

Table 3. KEGG pathways of potential target genes of microRNA-376c in cholangiocarcinoma

Category	Term	Count	*P* Value
KEGG_PATHWAY	hsa04068: FoxO signaling pathway	12	3.35E-4
KEGG_PATHWAY	hsa04010: MAPK signaling pathway	15	0.30E-2
KEGG_PATHWAY	hsa05220: Chronic myeloid leukemia	7	0.72E-2
KEGG_PATHWAY	hsa05212: Pancreatic cancer	6	0.19E-1
KEGG_PATHWAY	hsa05211: Renal cell carcinoma	6	0.19E-1
KEGG_PATHWAY	hsa05205: Proteoglycans in cancer	11	0.21E-1
KEGG_PATHWAY	hsa05120: Epithelial cell signaling	6	0.21E-1
KEGG_PATHWAY	hsa04668: TNF signaling pathway	7	0.40E-1
KEGG_PATHWAY	hsa05221: Acute myeloid leukemia	5	0.44E-1
KEGG_PATHWAY	hsa04120: Ubiquitin mediated proteolysis	8	0.45E-1

Bioinformatical analysis for signaling pathways and hub genes

The genes appeared in both microarray data and prediction were regarded as prospective targets of microRNA-376c in cholangiocarcinoma. The Gene ontology (GO) functional enrichment analysis was exerted to explore the molecular bioinformatical role of the underlying target genes in three aspects (biological processes, cellular component and molecular function) with the utilization of DAVID (https://david.ncifcrf.gov/). Besides, pathway analysis was carried out to obtain the genomic pathway enrichment information of the potential target genes of microRNA-376c by the Kynto Encyclopedia of Genes and Genomes (KEGG) pathways database with a significant statistical level with *P*-value less than 0.001. Then, we performed network analysis adopting Bingo to reveal GO annotation information hierarchically and represented it with a visualized hierarchical map of networks by Cytoscape. Protein-protein interac-

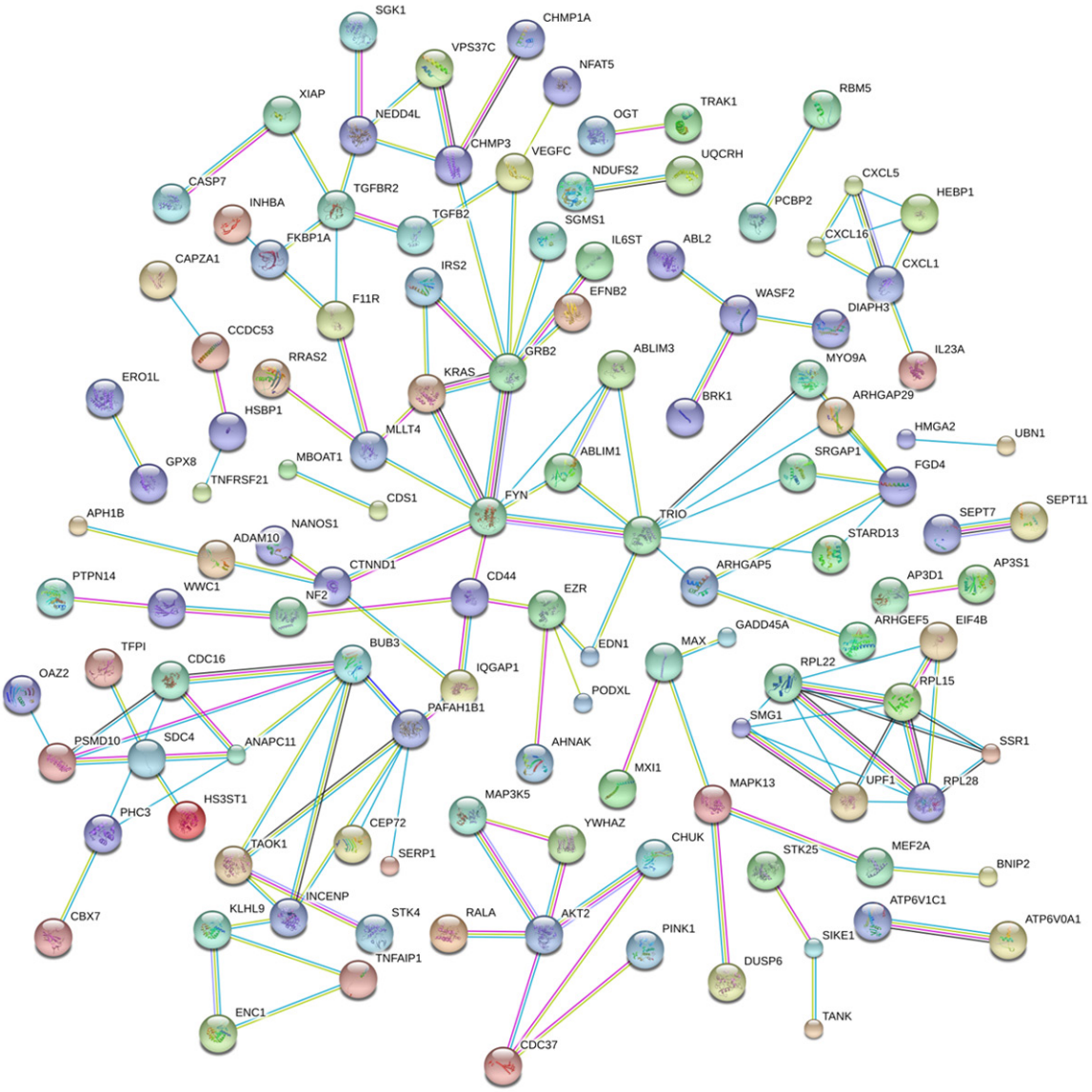

Figure 5. Protein-protein interaction (PPI) network of the potential mRNAs targeted by microRNA-376c in cholangiocarcinoma. Network nodes represent genes, and edges represent protein-protein associations. We set 0.9 as interaction score to show the network.

tion (PPI) was further conducted to investigate the hub genes among all potential targets of microRNA-376c.

Results

Collection of the potential targets of microRNA-376c in cholangiocarcinoma

Eventually, only one microarray (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE47186) met the inclusion criteria and coincidentally, the only one paper achieved from literature searching was performed by the same group [15]. Thus, the original data from mRNA microarray GSE47186 was downloaded. Since miR-376c-overexpressing HuCCT1 cells was used to perform the microarray analysis, only down-regulated genes (n=1309) were collected. 5370 potential target genes were obtained using 12 target prediction programs. Those genes appearing for at least three times from different programs were picked up. Finally, 403 genes were further employed with bioinformatical function analysis. Simultaneously, 14 verified target genes of microRNA-376c were assembled from the published literatures

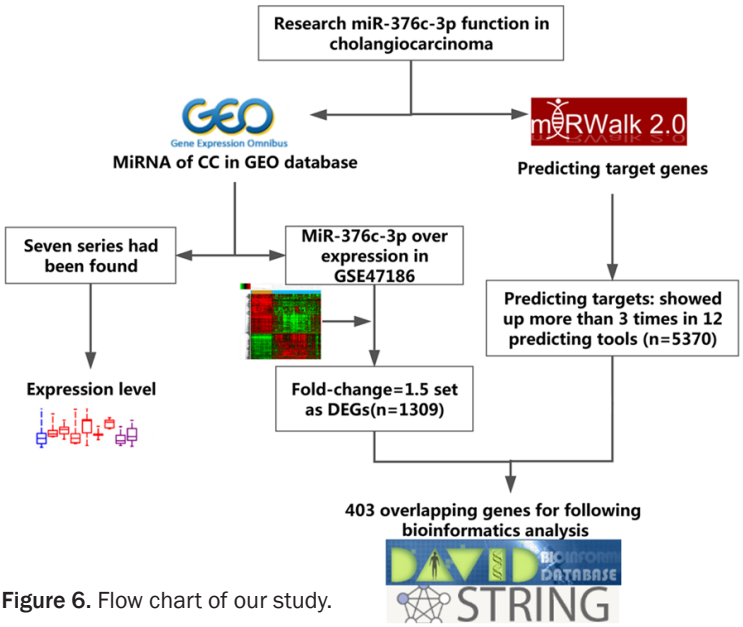

Figure 6. Flow chart of our study.

(**Table 1**). GRB2 and NFAT5 were overlapped between 403 and 14 genes.

Functional enrichment analysis

The results of GO were shown in **Table 2**, including the enriched pathways in biological process, cellular component and molecular function. The most significant pathways of each term were actin cytoskeleton organization, cytoplasm and protein binding (**Figures 1-3**). KEGG pathway analysis was further conducted to identify biological pathways of the microRNA376c in which conserved targets were involved (**Figure 4**). The most remarkable involved in pathways were FoxO signaling pathway, MAPK signaling pathway, Chronic myeloid leukemia, Pancreatic cancer, Renal cell carcinoma, Proteoglycans in cancer, Epithelial cell signaling in Helicobacter pylori infection, TNF signaling pathway, Acute myeloid leukemia and Ubiquitin mediated proteolysis ($P<0.05$, **Table 3**). To further understand the biological function of miR-376c-3p, the core genes among the 403 target genes of PPI network was performed by STRING 10.0 (**Figure 5**) and GRB2, TRIO, FYN, BUB3, RAFAH1B1, RPL15 were regarded as hub target genes for microRNA-376c in cholangiocarcinoma. The flow chart of our study was shown in **Figure 6**.

Discussion

In the current study, we re-calculated the differentially expressed genes after pre-microRNA-376c-transfection in cholangiocarcinoma HuCCT1 cells from GEO microarray data GSE47186. These down-regulated genes by microRNA-376c were further combined with the predicted targets. Signaling pathway analyses revealed that several pivotal pathways could be related to microRNA-376c in cholangiocarcinoma. Finally, GRB2, TRIO, FYN, BUB3, RAFAH1B1, RPL15 could be the hub genes among all prospective targets (**Figure 5**). This study provides new insights based on microRNAs for the research of cholangiocarcinoma.

Cholangiocarcinoma is generally diagnosed at advanced period on account of the poor deficiency of early clinical symptoms or responsible biomarkers. In spite of obvious developments in diagnostic methods, it still remains an aggressive cancer which sounds scarce [25-27]. Therefore, reliable diagnostic biomarkers, including microRNAs, are a pressing need. Several microRNAs have been investigated in cholangiocarcinoma by far. For example, microRNA-148a, microRNA-152 and microRNA-494 could modulate cell proliferation. MicroRNA-29b, microRNA-205 and microRNA-221 were contributed to the sensitivity to Gemcitabine for cholangiocarcinoma cells. MicroRNA-21 played an essential part in the tumor cell growth in cholangiocarcinoma [28-30]. Among these microRNAs in cholangiocarcinoma, microRNA-376c was reported to function as a tumor inhibitor targeting GRB2 in cholangiocarcinoma HuCCT1 cells and could control the cell motility of cholangiocarcinoma HuCCT1 cells. However, except GRB2, many other targets could exist for microRNA-376c in cholangiocarcinoma. The current study was performed to unravel the possible target genes and signaling pathways microRNA-376c in cholangiocarcinoma with the combinatorial genes from both microarray and in silico prediction. The bioinformatics analysis revealed that microRNA-376c could impact the biological processes, cellular component and molecular function of cholangiocarcinoma via targeting different signaling pathways. More importantly, several key pathways of KEGG were identified

for microRNA-376c in cholangiocarcinoma, including FoxO signaling pathway, MAPK signaling pathway, chronic myeloid leukemia, and TNF signaling pathway. Among these pathways, FoxO signaling pathway is involved in the regulation of the cell cycle, apoptosis and metabolism [31, 32]. Furthermore, among all members in these pathways, Fox03a acts as an antitumor gene in hepatocellular carcinoma, which can induce the expression of pro-apoptotic genes, or interfere with signaling cascades generally distinct in hepatocellular carcinoma such as Wnt/β-catenin, PI3K/AKT/mTOR or MAPKs pathways [33]. However, the real targets of microRNA-376c in cholangiocarcinoma needs further studied.

Besides the biological function and prospective mechanism of microRNA-376c, we are also interested in its clinical value. By far, only Kawahigashi et al documented that microRNA-376c was downregulated in an ICC cell line (HuCCT1), as compared to that of a normal intrahepatic biliary epithelial cell line (HIBEpiC) [14] and no clinical evaluation has been performed. We then attempted to perform a meta-analysis to reveal the clinical significance of microRNA-376c in cholangiocarcinoma; however, the insufficient data limited this plan. We further searched all available microRNA microarrays detecting the level of microRNA-376c in clinical cholangiocarcinoma tissues from GEO and ArrayExpress, and seven eligible datasets, including GSE57555 (Japan), GSE60978 (Norway), GSE53870 (China), GSE59856 (Japan), GSE53992 (USA), GSE47764 (China) and GSE32957 (USA) could be involved and evaluated. In total, 241 cholangiocarcinoma patients and 197 healthy people were involved in the seven datasets. However, no significant relevance of microRNA-376c in the diagnosis of cholangiocarcinoma could be achieved, probably due to the small sample size and limited approaches of microarray (data not shown). The clinical role of microRNA-376c in cholangiocarcinoma needs to be evaluated with larger sample size and other methods, such as real time RT-qPCR or fluoresce in situ hybridization (FISH).

Collectively, the prospective molecular mechanisms of microRNA-376c in cholangiocarcinoma were assessed with in silico approaches, which provide novel insights into the significance of the target genes in the tumorigenesis of cholangiocarcinoma. However, larger cohorts of patients and in-depth functional experiments are still expected to unravel the clinical role and precise mechanisms of microRNA-376c in cholangiocarcinoma.

Acknowledgements

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Disclosure of conflict of interest

None.

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