

Original Article

A six-microRNA set as prognostic indicators for bile duct cancer

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Abstract: MicroRNAs (miRNAs) play important roles in cancer progression by altering transcriptional control. The purpose of this study is to identify and explore specific miRNAs as prognostic and predictive biomarkers for bile duct cancer (BDC) by analyzing Next-generation data. miRNA expression profiles and corresponding clinical information of BDC samples were extracted from The Cancer Genome Atlas (TCGA). The differentially expressed miRNAs were determined by SAMR package in R software. Target genes of those miRNAs were predicted by Targetscan. Functional enrichment analysis and hypergeometric test analysis of target genes were performed. Then, diagnosis accuracy of miRNAs was judged by ROC Curves analysis. Total 120 differentially expressed miRNAs were obtained, of which six important miRNAs were selected and predicted as prognosis and predicting biomarkers in BDC. Besides, functional analysis showed that both enriched pathways were significantly related with ion binding, which might involve in the carcinogenesis of BDC. Moreover, top 3 important pathways sharing the most influence were noted. Our results demonstrated that hsa-miR-483-5p, hsa-miR-675, hsa-miR-139-3p, hsa-miR-598, hsa-miR-625 and hsa-miR-187 could serve as prognostic and predictive markers for survival of BDC patients and could potentially be provided as targets for future therapy.

Keywords: Bile duct cancer, microRNA, target gene, functional enrichment, pathway analysis

Introduction

Currently, bile duct cancer (BDC) is the second most common primary hepatobiliary cancer, with an increasing incidence rate and become a potential threat to human health worldwide [1, 2]. The investigation of BDC associated genes for early detection or therapeutic targeting could potentially improve the survival rates. Although in recent years, considerable efforts have been done to investigate the miRNAs and genes involved in BDC pathogenesis and progress [3], the precise pathogenesis and biomarkers involved in the development of BDC also needs further elucidation.

MicroRNAs (miRNAs or miRs) are small and non-coding small RNAs endogenous molecules, and mainly in the un-translated region of mRNA, involving in post-transcriptional gene regulation [4]. Notably, miRNAs play roles in several physiological activities with important regulatory functions [5], especially in progress of cancer. Actually, miRNAs have been showed to be the

potential implication targets for cancer diagnosis and prognosis [6, 7]. The possible relationship between miRNAs and oncogenesis is explored by next-generation sequencing tumor versus normal tissues in recent reports [8]. For example, it have been demonstrated that miRNAs, such as miRNA-21 [9], miR-141 and miR-200b [10], to be involved in the malignant transformation of cholangiocytes and contributed to the different clinical biofeatures of cholangiocarcinoma [8]. The development of genome-wide sequencing and bioinformatics facilitate the discovery of such prognostic and predictive indicators. Moreover, The Cancer Genome Atlas (TCGA), which launched in 2005 by the National Institute of Health (NIH), create a comprehensive "atlas" of cancer genomic profiles. Therefore, we utilized The Cancer Genome Atlas (TCGA) as a publicly available dataset to identify differentially expressed miRNAs, hoping to explore functions that miRNAs play in the development and progression of BDC.

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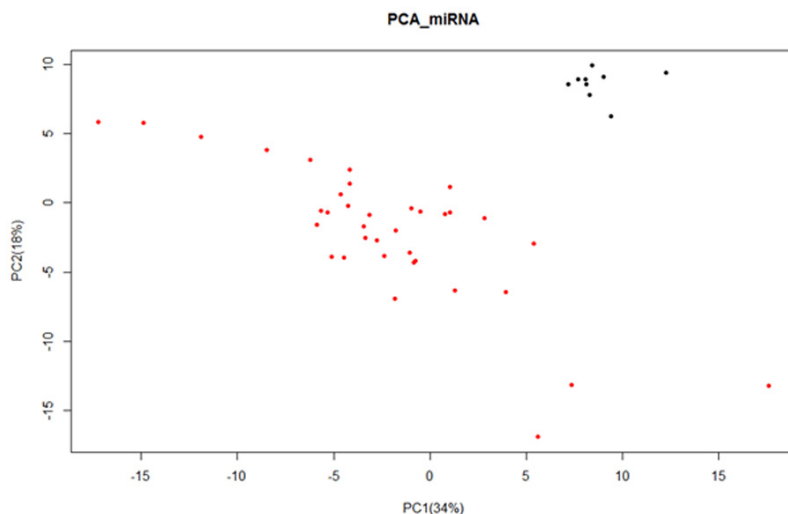


Figure 1. Principal component analysis of the expression of miRNA. The abscissa axis represents that the first principal component occupies 34%, while the longitudinal axis represents that the second principal component occupies 18%. Red spots show specimens of bile duct cancer, while black spots show normal specimens.

Ordinarily, TCGA was utilized as a reference to increase the available sample size, compare RNA sequencing between normal and BDC samples, as well differentially expressed genes and gene networks. Thus, this novel bioinformatics pipeline could help us identify and filter out potential artifacts due to the small sample size. Accordingly, differentially expressed miRNAs were credible for further study.

In our study, we tried to identify differentially expressed miRNAs as prognostic and predictive biomarkers for BDC. Additionally, both enriched GO terms and KEGG pathways were analyzed for further understanding of the functions in which these miRNAs participated. Finally, the predicted miRNAs will have high potential to play critical roles in the progression of BDC and could potentially provide as targets for future therapy.

Materials and methods

Source data and primary analysis

To yield the detection error, three separate batches were analyzed from TCGA dataset (<https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp>) with total 45 specimens, incorporating 9 normal specimens of bile ducts and 36 specimens of BDC. Sequencing of the dataset was based on Illumina HiSeq platform [11].

The dataset acquired above contained 1046 noted miRNA expression data. Expression data of each sample from three batches were standardized, and the standardization between different samples was performed by Limma package of R software [12]. As a result, the differences between experiments of different samples were revoked. Only those miRNAs overlapping for two or three times among three batches were selected.

Screening of differentially expressed miRNAs

Differentially expressed miRNAs between normal and cancer specimens of bile

ducts were screened by SAMR (significance analysis of microarrays) [13] package in R software, under the condition of FC (fold change) was 2 and FDR (false discovery rate) less than 0.05. Then cluster analysis was performed to guarantee those miRNAs could classify normal and BDC specimens well. After that, principal component analysis was utilized to observe the contradistinction between those two kinds of specimens.

Analysis about target genes of differentially expressed miRNAs

The putative targets of differentially expressed miRNAs were acquired from Mirtarbase [14] database. Functional Gene Ontology (GO) biological processes terms, as well as KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways of the putative targets of candidate miRNAs were performed basing on DAVID (database for annotation visualization and integrated discovery) [15]. The DAVID could calculate out the *P*-value of functional enrichment and *P*-value after Benjamini correction. Putative targets of miRNAs were predicted by TargetScan Human 6.2 software [16]. Then GO terms and KEGG analysis were utilized to obtain potential medical target point. The differences were considered statistically significant for *P*-value < 0.05.

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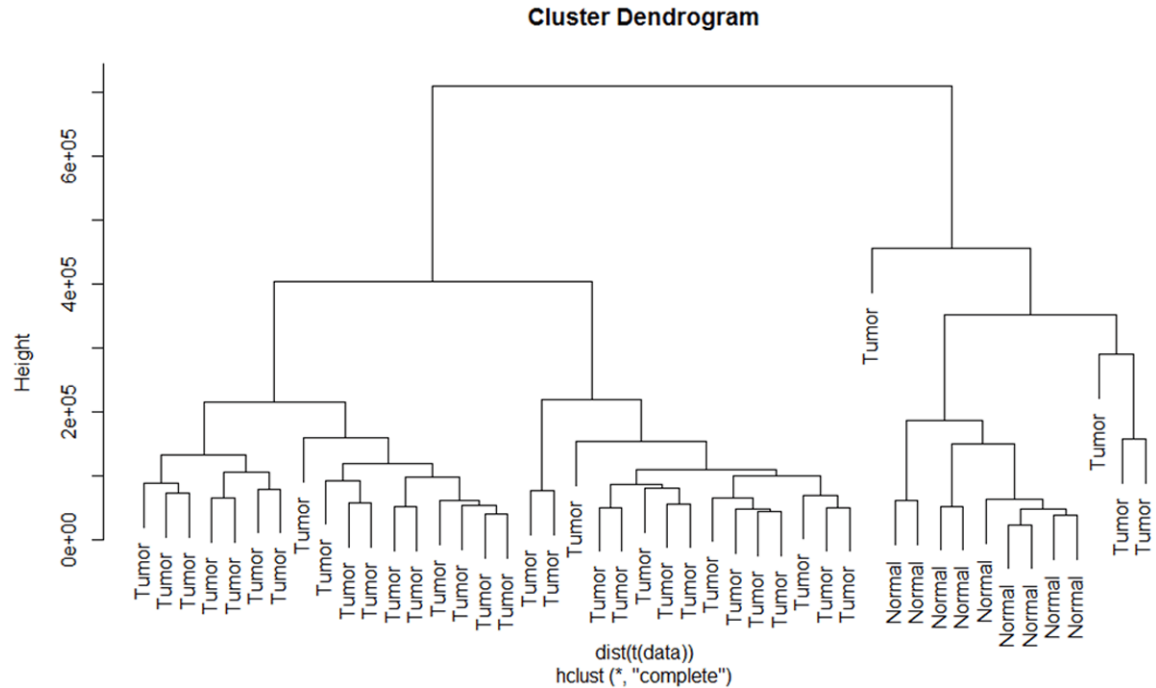


Figure 2. Dendrogram of differentially expressed miRNAs by cluster analysis. The longitudinal axis represents the difference degree between specimens, the cluster tree shows specimens share similar expression volume will gather together. Normal specimens are scattered at the right cluster.

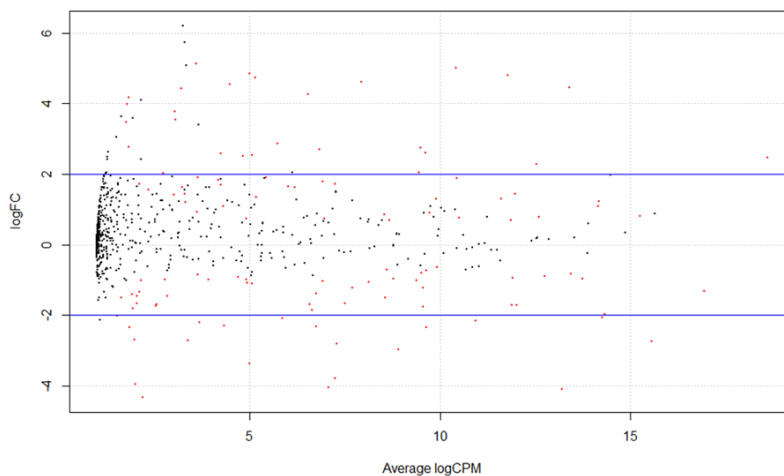


Figure 3. Difference of miRNA expression analysis. Black spot shows normal expression miRNA, Red spot shows different expression miRNA, red spot above the figure was up-regulated miRNAs while red spot below the figure shows down-regulated miRNA.

Diagnosis accuracy of important miRNA as biological marker of BDC

We could obtain important miRNAs, which were predicted as biological markers, after above performance. Next, the diagnosis accuracy of important miRNAs as biological markers of BDC was judged by ROC curves. ROC curves were analyzed by MedCalc software [18], and AUC (area under curve) which was an index of the diagnosis accuracy was calculated by the analysis.

Results

Differently expressed miRNAs in BDC

Total 1041 differentially expressed miRNAs were acquired after principal component analysis and cluster analysis. There was a significant difference between those two kinds of specimens, because normal specimens were scattered in right upper, while the cancer specimens in left lower (**Figure 1**). In addition, nor-

Functional network of differently expressed miRNA

The topological properties of validated targets of interested miRNAs protein network were analyzed and modularized by network analyzer in Cytoscape software [17], then top 3 modules with P -value of significant less than $1.0e^{-05}$ were functional analyzed.

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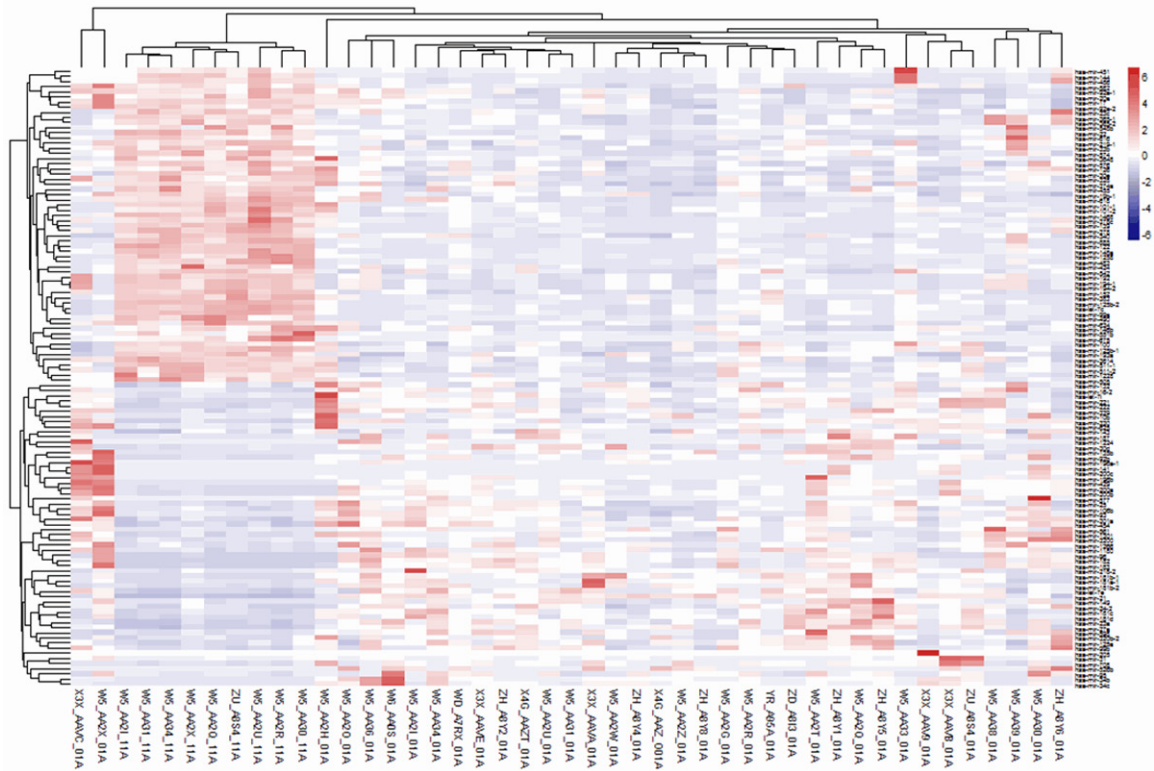


Figure 4. Cluster analysis of differentially expressed miRNA. The below abscissa axis represents specimen. The right longitudinal axis represents miRNA, and the left longitudinal axis represents cluster of miRNA. Red shows up-regulated miRNAs while green shows down-regulated miRNAs. There are two clusters of those specimens clustering: one is normal specimens of bile ducts and the other one is bile duct cancer specimens. Besides, there are two clusters of miRNA expression clustering: one is down-regulated miRNAs of bile duct cancer specimens and the other one is up-regulated miRNAs of bile duct cancer specimens.

mal specimens were scattered at the right cluster while cancer specimens at the left cluster in **Figure 2**, which means miRNA expression was different between normal specimens and BDC specimens.

Analysis of differentially expressed miRNA

Total 120 differentially expressed miRNAs between normal and BDC specimens were obtained after screening by SAMR [13] package. There were 59 up-regulated miRNAs and 61 down-regulated miRNAs accounting for quartiles 49.2% and 50.8% of differentially expressed miRNAs, respectively (**Figures 3 and 4**).

Analysis about target genes of differentially expressed miRNAs

Total 4371 down-regulated target genes and 3810 up-regulated target genes were predicted by Targetscan software. In GO biological pro-

cesses of putative targets of important miRNAs, 9 up-regulated and 11 down-regulated differentially expressed miRNAs were significantly enriched. Likewise, analysis of KEGG pathway showed differentially expressed miRNAs enriched in 4 up-regulated and 4 down-regulated pathways. Furthermore, the most significantly down-regulated GO terms and KEGG pathways were cation binding and Jak-STAT (the Janus kinase-signal transducer and activator of transcription) signaling pathway, respectively. The most significantly up-regulated GO terms and KEGG pathways were ion binding and graft-versus-host disease, respectively (**Figure 5**).

Interaction network and functional analysis of interested miRNAs and target genes in BDC

We calculated the important topological parameters, such as node degree distribution, shortest path length distribution, closeness centrality and the topological degree (**Figure 6**), then found that the degree distribution of the inter-

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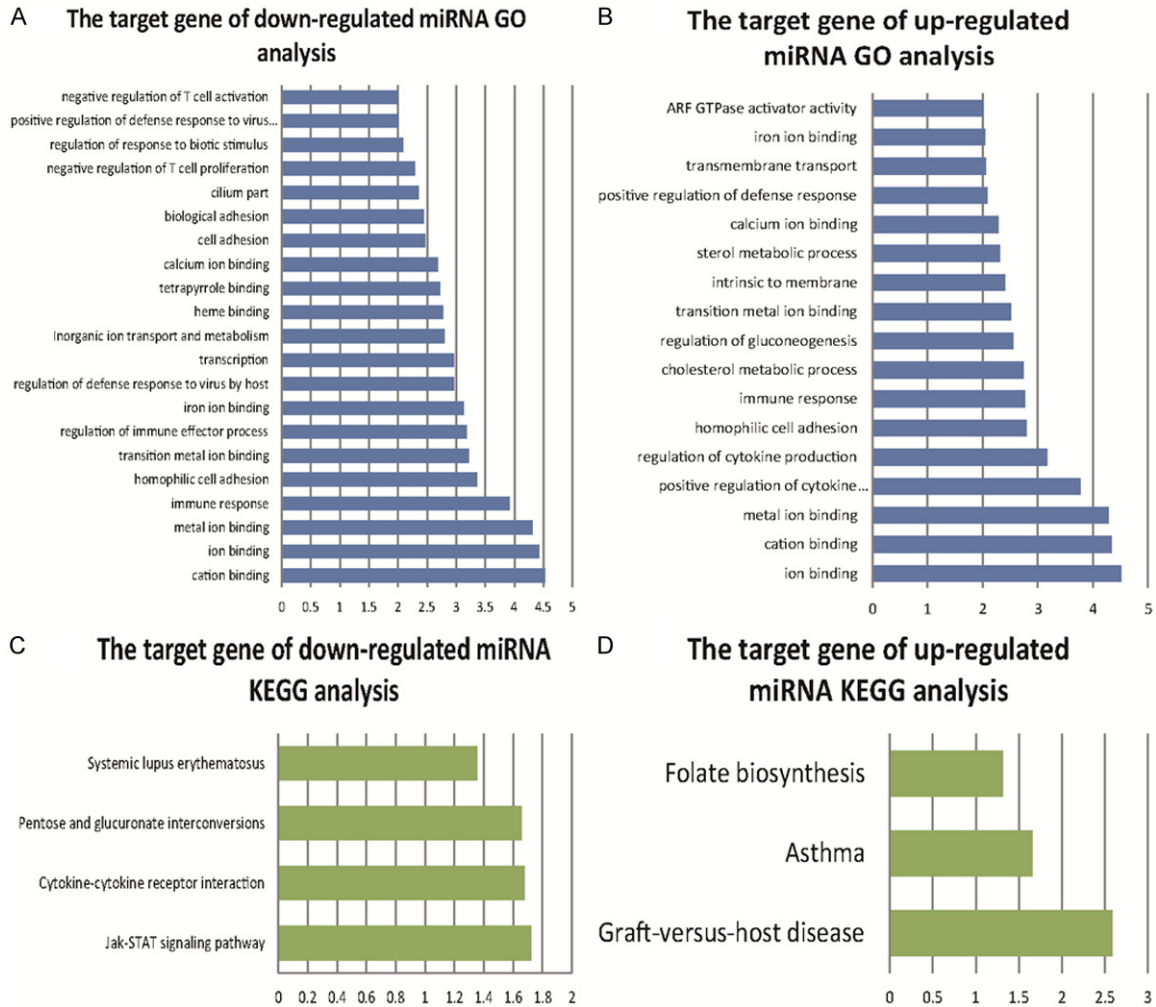


Figure 5. The GO terms and enriched KEGG pathways of target genes of differentially expressed miRNA. (A and B) are GO terms of target genes of differentially expressed miRNA, while (C and D) are KEGG pathways. The abscissa axis represents the level of discrepancy between different target genes expression. The longitudinal axis represents functional annotations. The closer the correlation between target genes and annotations is the higher level of the difference.

action network of differentially expressed miRNAs and target genes followed the Power-law of network, and had small-world network characteristics (short average shortest path and large average clustering coefficient).

Moreover, as shown in **Figure 7**, every miRNA has conglomerate target genes, and miRNAs can co-function on the same target gene. Top 3 miRNAs of down-regulated miRNAs and up-regulated miRNAs on node degree were acquired after screening and functional analysis, respectively. As a result, there were 173 target genes in hsa-miR-483-5p, 157 target genes in hsa-miR-675 and 140 target genes in hsa-miR-139-3p, among those 3 down-regulated miRNAs. Meanwhile, there were 104 target genes in

hsa-miR-598, 97 in hsa-miR-625 and 94 in hsa-miR-187, among those 3 up-regulated miRNAs.

Likewise, KEGG analysis was utilized to annotate target genes. As a result, top 3 important pathways, such as nuclear receptors in lipid metabolism and toxicity, phosphatidylinositol signaling system and endocytosis, were nuclear receptors in lipid metabolism and toxicity, phosphatidylinositol signaling system and endocytosis (**Table 1**).

The accuracy of important miRNAs as biological marker of BDC

Among down-regulated miRNAs, AUC of hsa-miR-483-5p, hsa-miR-675 and hsa-miR-139-

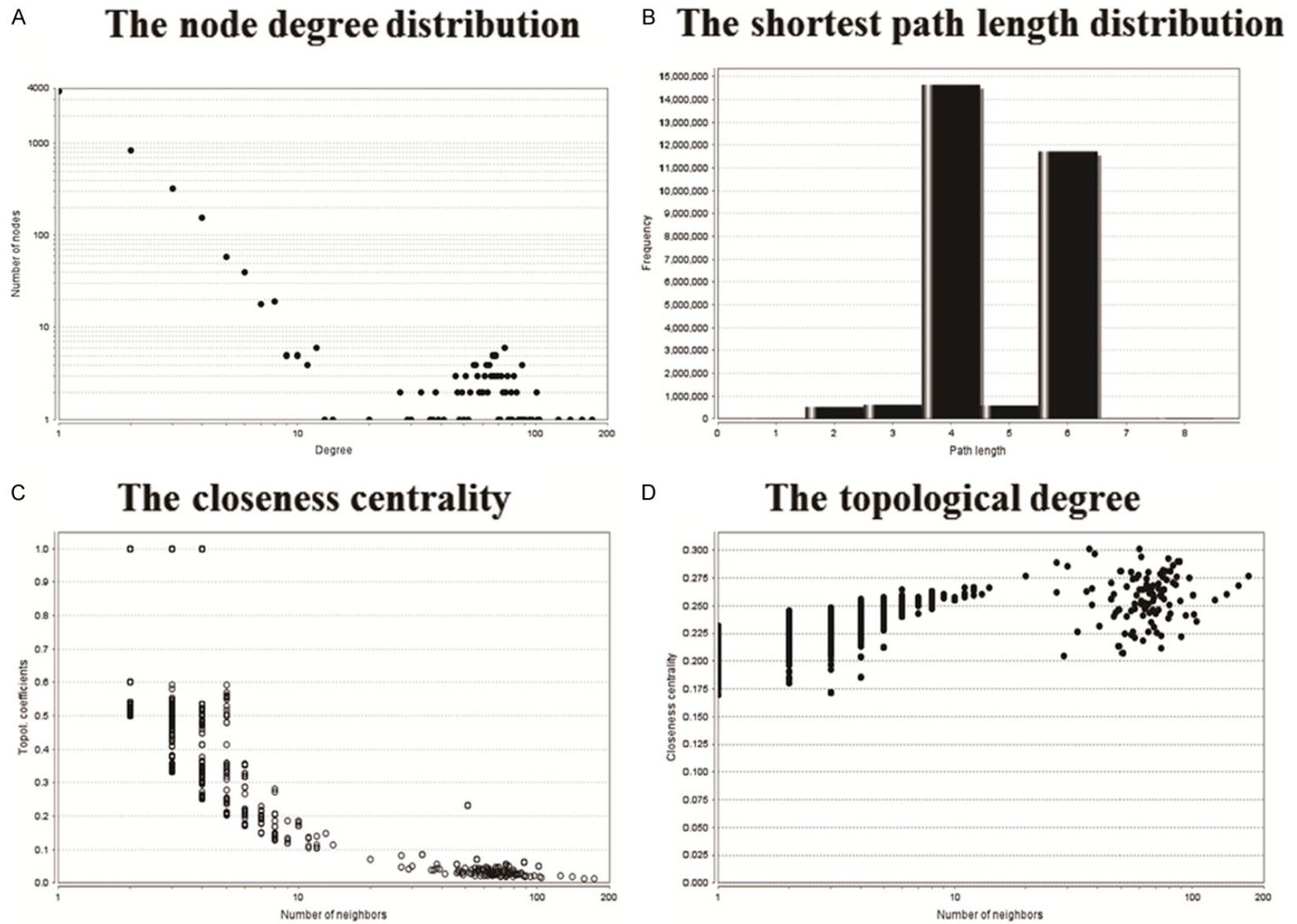


Figure 6. Topological properties of Interaction network of differentially expressed miRNAs and target genes. A. The node degree distribution; B. The shortest path length distribution; C. The closeness centrality; D. The topological degree.

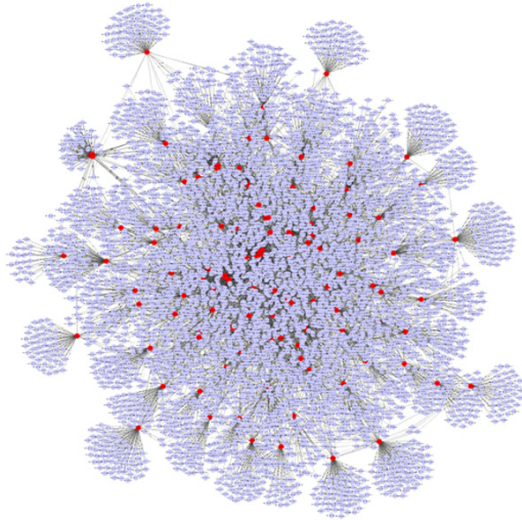


Figure 7. Interaction network of differentially expressed miRNAs and target genes. Gray spots show target genes and red spots show miRNA.

3p were 0.997, 0.978 and 1.000, respectively. While among up-regulated miRNA, AUC of hsa-miR-598, hsa-miR-625 and hsa-miR-187 were 0.818, 0.846 and 0.676, respectively (**Figure 8**). These results of AUC indicated that those six miRNAs shared high diagnosis accuracy as biological markers of BDC, as AUC was a index of the diagnosis accuracy.

Discussions

In this study, we identified total 120 differentially expressed miRNAs after principal component analysis, such as miRNA-21, miR-302, let-7a, let-7b and miR-204. More importantly, six important miRNAs, hsa-miR-483-5p, hsa-miR-675, hsa-miR-139-3p, hsa-miR-598, hsa-miR-625 and hsa-miR-187, were predicted as biomarkers in prognosis and prediction of BDC. Then target genes of those differentially expressed miRNAs were acquired and executed for functional annotation by DAVID. Meanwhile, both significantly altered GO terms and KEGG pathways, such as cation binding, Jak-STAT signaling pathway, ion binding and graft-versus-host disease, were related with ion binding. Moreover, top 3 important pathways sharing the highest influence were nuclear receptors in lipid metabolism and toxicity, phosphatidylinositol signaling system and endocytosis.

In this study, miRNA-21 was significantly up-regulated, which was in accordance with previ-

ous results that miRNA-21 were highly over-expressed in malignant cholangiocytes [10]. MiRNA-21 directly targeted some anti-oncogenes, such as TPM1, PDCD4 (programmed cell death 4), maspin and PTEN (phosphatase and tensin homolog) [19]. Besides, maspin as the target gene of miRNA-21 might participate in the process of biology function during cholangiocarcinoma. Moreover, literatures pointed that miRNA-21 might promote cell proliferation by down-modulating the expression of tumor suppressor phosphatase and tension homolog [10, 20] and might increase migration properties of tumor [21]. In addition, miRNA-21 was suggested to contribute to chemoresistance in cholangiocarcinoma by regulating the chemotherapy-induced apoptosis [22]. Meanwhile the down-regulating of miRNA-21 could enhance the sensitivity of BDC cells to anticancer drugs gemcitabine [10]. Also, miRNA-21 might play an important role in cholangiocarcinoma by inhibiting the proliferation and accelerating the apoptosis of cholangiocarcinoma RBE cells [10, 23]. All in all, miRNA-21 might affect the propagation, invasion and translation of tumor by down-regulating anti-oncogenes.

As members of let-7 family, both let-7a and let-7b were down-regulated in BDC, while target gene of let-7a was anti-oncogene NF-2 (neurofibromin 2). Considering that NF-2 could negatively regulate the expression of stat-3 (signal transducer and activator of transcription 3) [24, 25], who was the conditional signal sensing and activating transcription factor, we suggest that let-7a might modulate the procession and enhanced drug resistance [24] of BDC through regulating Stat-3. In addition, oncogene HMGA2 (high mobility group A2) was the target gene of let-7. let-7 negatively regulate HMGA2 and then depress propagation of lung cancer [26], which implies let-7 might play tumor suppressor functions.

MiRNA-302 which was down-regulated in our study targeted the MCL-1 (myeloid cell leukemia 1), an anti-apoptotic Bcl-2 (b-cell lymphoma 2) family member, and MCL-1 was shown to be regulated by miRNAs [27]; while Bcl-2, another anti-apoptotic Bcl-2 family member, was targeted by miR-204, who was also down-regulated in our study and other papers [8]. By analogy, we reasoned that those down-regulated miRNAs might contribute to the pathogenesis of BDC through modulating proapoptotic

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Table 1. Enriched KEGG pathway classification of top 3 modules

Term	Genes
module 1 h_nuclearRsPathway: Nuclear Receptors in Lipid Metabolism and Toxicity	NM_177435, NM_001017535, NM_000376, NM_004391, NM_000778
module 2 hsa04070: Phosphatidylinositol signaling system	NM_001105540, NM_005185, NM_001135637, NM_001135638, NM_002220, NM_003557, NM_003646, NM_001135636, NM_019892, NM_201533, NM_201532
module 3 hsa04144: Endocytosis	NM_015470, NM_152284, NM_001135637, NM_001135638, NM_003557, NM_001135636, NM_018209, NM_024591, NM_019619, NM_175609, NM_015075, NM_001111125, NM_014043

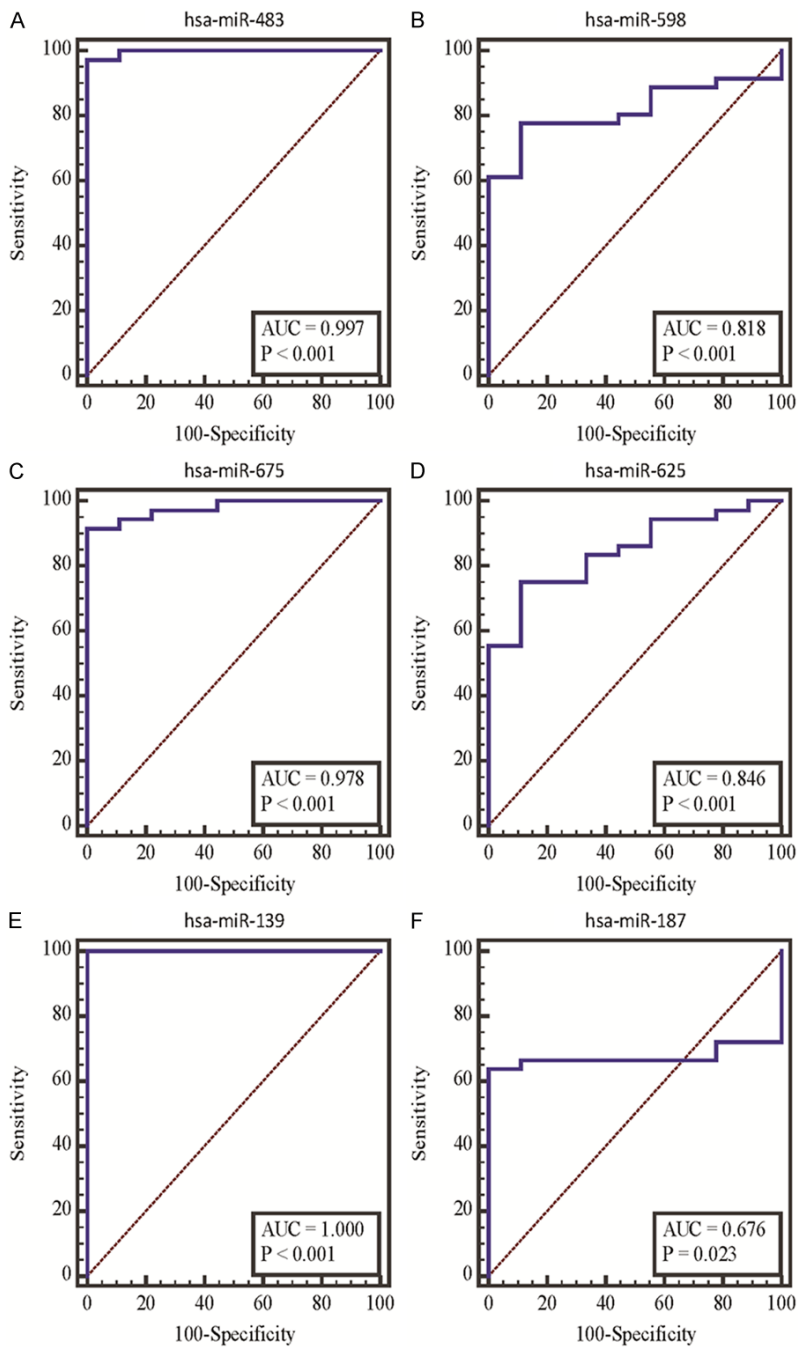


Figure 8. ROC curves analysis of significantly altered miRNAs. The abscissa axis represents specificity and the longitudinal axis represents sensitivity. AUC is area under curve.

mechanisms. It was in urgent need to explore the exactly pathogenesis.

The GO terms and KEGG pathways of target genes of differentially expressed miRNAs showed that the generation of BDC affected the ion combining capacity of target protein which was important for maintaining normal physiological function. For instance, protein phosphorylation site was impacted and induced incorrect phosphorylation which might lead to the change of micro-environment or milieu, so dysfunction of protein activation and inactivation were induced. As a result, the interaction between protein and DNA was changed as well. Ion combining correlated protein, such as calcium-binding proteins A4 [28], and ion combining tyrosine phosphorylation mediation genes, such as phosphoinositide-3-kinase [29] and protein kinase A RII-like [30], were proved to be closely related with invasion and translation of malignant tumor. As mentioned above, it might hit us clues that expression difference of those target genes might participate in progress of BDC.

Notably, early detection, diagnosis and treatment were the key to decrease mortality and improve the prognosis of cancer. Consi-

dering that the expression of miRNAs was closely related with metastasis [31] and drug tolerance of tumor [32], we predicted six important significantly expressed miRNAs as a biomarker for predicting BDC prognosis, which was confirmed by ROC curves analysis. Although, many works had done to investigate genetic variants and abnormal miRNAs, such as KRAS (kirsten rat sarcoma viral oncogene homolog), BRAF (B-Raf proto-oncogene, serine/threonine kinase), EGFR (epidermal growth factor receptor) and TP53 (tumor protein p53), clinical outcome of the disease has not been improved [33]. Our study identified new miRNAs and target genes, hoping to provide a new thought on BDC therapy to improve treatment effectiveness.

More importantly, the accuracy analysis results showed six miRNAs, hsa-miR-483-5p, hsa-miR-675, hsa-miR-139-3p, hsa-miR-598, hsa-miR-625 and hsa-miR-187 could be served as biomarkers for BDC. MiR-483-5p was proved to upregulate in adrenocortical cancer [34] and suppress the proliferation of glioma cells [35]. Besides, miR-139 could suppress metastasis and progression of hepatocellular carcinoma [36]. However, those six miRNAs were determined as biomarkers, and were persuasive and feasible for important implications in clinical practice about targeted therapy of BDC first time.

Conclusions

In summary, we identified miRNA-21, miR-302, let-7a, let-7b and miR-204 which might relate with pathogenesis of BDC. More importantly, we predicted six miRNAs, hsa-miR-483-5p, hsa-miR-675, hsa-miR-139-3p, hsa-miR-598, hsa-miR-625 and hsa-miR-187, as biomarkers prognosis and predicting in BDC, implying the application for diagnostic and prognostic tools and treatments.

Disclosure of conflict of interest

None.

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