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Circular RNA circ-MTO1 serves as a novel potential diagnostic and prognostic biomarker for gallbladder cancer

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Abstract. – OBJECTIVE: Primary gallbladder carcinoma (GBC) is one of the most common biliary malignancies in the gastrointestinal tract. In this work, we examined the roles of circular-mitochondrial translation optimization 1 (circ-MTO1) in GBC tissues and patient plasma.

PATIENTS AND METHODS: Circ-MTO1 expression in GBC tissues and patient plasma was evaluated by quantitative Real Time-PCR (qRT-PCR). The relationships between circ-MTO1 expression and the pathological characteristics of GBC were analyzed. Kaplan-Meier survival curve was applied to calculate overall survival (OS) and progression-free survival (PFS) in GBC patients with different circ-MTO1 expression. The univariate COX regression curve analysis method was employed to analyze the potential relationships between high circ-MTO1 expression and OS and PFS. At last, we assessed the diagnostic value of the circ-MTO1 level in GBC patient plasma.

RESULTS: Circ-MTO1 expression was significantly upregulated in tumor tissues and plasma in GBC patients. In addition, circ-MTO1 expression was associated with clinical-pathological characteristics in GBC. High circ-MTO1 expression served as an independent prognostic factor for poor OS and PFS in GBC patients. Moreover, upregulated plasma circ-MTO1 level was significantly associated with tumor development.

CONCLUSIONS: Circ-MTO1 is a potential early diagnostic and prognostic biomarker for patients with gallbladder cancer. Thus, our present work might provide a new understanding of the diagnosis and treatment of GBC.

Key Words:
GBC, Circ-MTO1, Diagnosis, Prognosis.

List of Abbreviations

GBC, Primary Gallbladder Carcinoma; OS, Overall Survival; PFS, Progression-Free Survival; circ-MTO1, Circular-Mitochondrial Translation Optimization 1; qRT-PCR, Quantitative Real-Time Polymerase Chain Reaction; MRE, MiRNA Response Element; TNM, Tumour, Node, Metastasis; AJCC, American Joint Committee on Cancer, ROC, Receiver-Operator Characteristic; AUC, Area Under the ROC Curve.

Introduction

Primary gallbladder carcinoma (GBC) is a kind of gastrointestinal tract tumor derived from the mucosal epithelium of the gallbladder[1]. Its major pathological type is adenocarcinoma, with only a small percentage of GBC patients are adenocarcinoma or squamous cell carcinoma[2]. Epidemiological surveillance results indicated that the incidence of gallbladder cancer was 2.5 cases per 1×10^5 people. Although the low incidence of this disease, gallbladder cancer-related mortality is significantly higher than other cancer types[3]. GBC often occurs with insidious onset without evident clinical symptoms in the early stage, similar to gallstones and gallbladder inflammation. Once the patients display symptoms, such as abdominal pain, yellow marks or an abdominal mass, GBC usually has progressed to the advanced stage. If surgery is not performed, the median survival time after symptom onset is only 6 months. The 1-year survival rate and the 5-year survival rate are less than 12% and 5%, respectively[4]. However, even if the patients receive radical surgery, the overall survival rate fails to increase significantly. This low survival rate is mainly caused by the early tumor metastasis through lymphatic vessels, nerves, bloodborne pathways, and direct invasion of the liver[5]. Therefore, the discovery of the potential gallbladder cancer targets and biomarkers has been becoming an urgent issue to be solved in gallbladder cancer treatment.

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Circular RNAs (circRNAs) are a type of special endogenous non-coding RNAs (ncRNAs) with a closed-loop structure without 5' and 3' ends[6]. CircRNAs are widely distributed in many eukaryotes and display an obvious tissue-specific expression pattern[7]. They are closely related to cardiovascular diseases such as atherosclerosis, nerve degeneration disease, cardiac hypertrophy, and diabetes mellitus[8]. In particular, circRNAs play important biological roles in the occurrence and development of tumors[9]. CircRNAs have a miRNA response element (MRE) that can bind to target miRNAs and function as a "miRNA sponge", thus inhibiting the activity of target miRNAs[10]. Bachmayr-Heyda et al[11] analyzed the proliferation of circRNA and linear RNA in normal colonic mucosa and tumor tissues by RT-qPCR. Their results indicated that the ratio of circRNA to linear RNA in tumor tissues was lower than that in normal tissues and colon cancer cells. In addition, this ratio was negatively associated with cell proliferation, suggesting that circRNA plays pivotal roles in tumorigenesis. Shang et al[12] reported that hsa_circ_0005075 expression was different between liver cancer tissues and normal tissues, and is closely related to tumor size and shows good diagnostic potential. Li et al[13] revealed that hsa_circ_002059 expression level in gastric cancer tissues was lower than that in adjacent non-tumor tissues. The low expression of hsa_circ_002059 was closely related to gender, age, gastric cancer metastasis, Tumour Node, Metastasis (TNM) stage suggestis the crucial role of circRNAs in regulating tumor development. However, to date, the functions of circRNAs in the pathological processes of GBC are far from being understood.

In this study, we investigated the potential role of circ-MTO1 in gallbladder cancer by evaluating the circ-MTO1 expression in GBC patients and its association with several GBC clinical characteristics.

Patients and Methods

GBC Specimens

All experimental specimens were approved by the Ethics Committee of Hechuan District People's Hospital before the acquisition (Ethics No. F0983312). All patients/healthy volunteers did not receive any surgery, chemotherapy, radiotherapy, neoadjuvant therapy, or targeted therapy and signed informed consent before obtaining the corresponding specimens. Tissue specimens were collected from June 2014 to February 2019. A total of 100 GBC patients and 100 healthy volunteers in the general surgery Department of Hechuan District People's Hospital were recruited to obtain cancer tissue and corresponding adjacent tissues. Paracancerous tissues were taken 4 cm from the edge of the tumor and evaluated by two experienced pathologists to confirm the absence of significant tumor cells. After obtaining the specimen under aseptic conditions, the specimen was washed with cold physiological saline. The specimens were cleaned and autoclaved with a scalpel, and immediately placed in a tissue-free tube without RNase. Subsequently, samples were stored in liquid nitrogen for further use. Meanwhile, 30 GBC patients who were hospitalized in the Department of general surgery of Hechuan District People's Hospital between July 2014 and March 2019 and 30 healthy volunteers were recruited. Each subject received 4 ml of peripheral blood using an EDTA-containing anticoagulant tube. The anticoagulation tube was centrifuged immediately within 30 minutes under the following conditions: 1000 g for 10 minutes. Plasma and cell fractions were separated. Plasma was transferred to a new RNase-free 1.5 mL centrifuge tube. Subsequently, the tubes were labeled and stored in liquid nitrogen for further analysis. Clinical and pathological data of patients and healthy volunteers included in the study were collected, including gender, age, tumor size, clinical stage and TNM stage, vascular invasion, lymph node invasion, and expression of CA19-9 and CEA in the blood. Clinical and TNM staging of cancer patients is based on the 8th edition of the cancer staging guideline published by the American Joint Committee on Cancer (AJCC)[14].

Total RNA Extraction and Reverse Transcription

Total RNA in tissues and plasma was extracted with RNApure Tissue&Cell Kit (CW0584, CWBio, China) and RNApure Blood Kit (CW0582, CWBio, China), respectively. The detailed operations were followed as required by the reagent instructions. Genomic DNA was removed using a gDNA Eraser which was supplied in the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). The extracted RNA purity (A260/A280) and integrity were determined using NanoDrop 3000 (Thermo Fisher Scientific, Waltham, MA, USA). Non-conforming specimens of quality inspections are not involved in subsequent experiments. The reverse transcription reaction was performed with PrimeScript^{TM} RTII

(#RR037A, TaKaRa, Otsu, Shiga, Japan). The obtained cDNA was stored at -80°C.

qRT-PCR Analysis

qRT-PCR experiments were performed according to the previous references, and β-actin stably expressed in tissues and plasma was finally selected as an internal reference[15]. The internal reference β-actin and circ-MTO1 primers were designed by Primer5 software. After BLAST alignment, the primers were synthesized by Sangon Biotech (Shanghai, China) Co., Ltd. Primer sequence: β-actin upstream 5'-TCCTCTCCCAAGTCCACACA-3', downstream 5'-GCACGAAGGCTCATCATTCA-3'; circ-MTO1 upstream 5'- GAGCTGTAGAAGATCTTATTC -3', downstream: 5'-CACAGGCCATCCAAGGCATC-3. SYBR® Premix Ex TaqII kit (RR820A, TaKaRa, Otsu, Shiga, Japan) was used in qRT-PCR assay according to the instruction provided by the manufacture. The SYBR® Green fluorescence signal after each amplification cycle was measured with ABI 7500 (Foster City, CA, USA). The thermal conditions were listed as following: pre-denaturation at 95°C, 30 s, 40 cycles of reaction: 95°C for 5 s, 60°C for 34 s. The dissolution curve was used to detect the purity of the amplified product. The obtained C_T values were statistically analyzed using a relative quantitative $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

The data were processed and analyzed using SPSS 22.0 statistical software (IBM Corp., Armonk, NY, USA). Continuous variable indicators, normal distribution, and homogeneity of variance were used to calculate the data distribution test. Data are presented as mean ± standard deviation. An independent sample *t*-test is used for comparison between two groups. On the other hand, data were expressed as the median (M) and interquartile range (QR). Comparisons between groups were performed using a nonparametric Mann-Whiney U test. GraphPad Prism 5.0 software (La Jolla, CA, USA) was employed for imaging. For the case group and the control group, the receiver operating curve (Receiver-operator characteristic, ROC) was plotted using SigmaPlot 12.5 (Sigma-Aldrich, St. Louis, MO, USA). The area under the ROC curve was compared using the Hanley-McNeil nonparametric test, with $p<0.05$ was considered as a significant difference.

Results

Circ-MTO1 Expression Is Upregulated in GBC Tissues and Plasma

Firstly, we analyzed circ-MTO1 expression in 100 pericarcinomatous tissues and the paired GBC tissues. The results suggested that circ-MTO1 expression in GBC tissues was significantly higher than that in the paired pericarcinomatous tissues ($p<0.001$) (Figure 1A). In addition, we examined the plasma circ-MTO1 level in 30 GBC patients and 30 healthy controls. The results suggested that circ-MTO1 level in GBC patient plasma was significantly up-regulated compared with that in

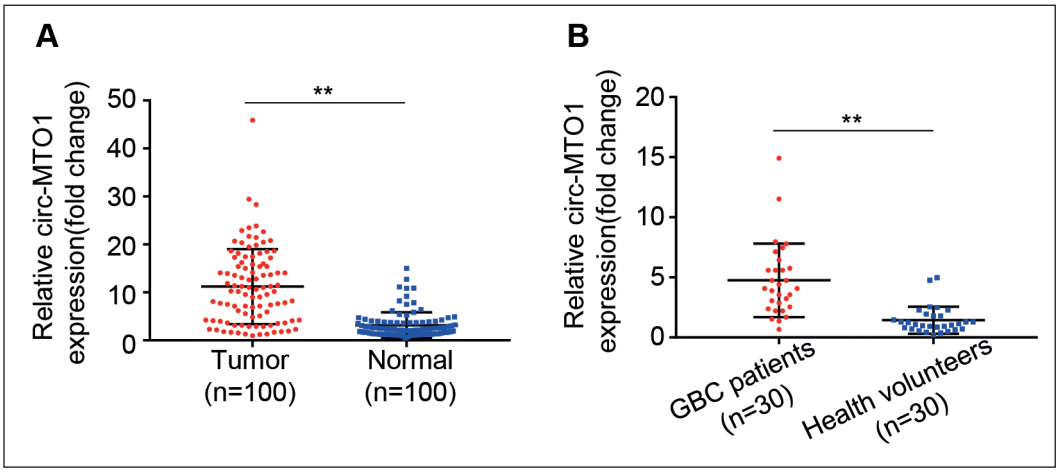

Figure 1. qRT-PCR analysis of the circ-MTO1 expression in tissues and plasma. **A**, qRT-PCR method for detecting the expression level of circ-MTO1 in 100 pairs of GBC adjacent and normal tissues. **B**, qRT-PCR method was used to detect the expression level of circ-MTO1 in plasma of 30 cases of GBC and 30 normal subjects. $^{**}p<0.01$.

healthy control plasma (p<0.001) (Figure 1B). Therefore, circ-MTO1 expression in tissue and plasma of GBC patients was upregulated compared with that in pericarcinomatous tissues and healthy controls.

Circ-MTO1 Expression Is Associated With Clinical-Pathological Characteristics in GBC

To study the potential relationships between circ-MTO1 expression and clinic pathological characteristics in GBC, we divided GBC patients into two groups, high circ-MTO1 expression group (N=50) and low circ-MTO1 expression group (N=50), based on the medium value of circ-MTO1 expression in 100 GBC tissues. The chi-square test was used to examine the relationship between circ-MTO1 expression and clinical-pathological data of GBC patients. The results indicated that circ-MTO1 expression was closely associated with tumor size, differentiation, TNM stage, lymph node metastasis, and distant metastasis (p<0.001) (Table I). However, no difference was observed between circ-MTO1 expression and patient age or gender.

High Circ-MTO1 Expression Predicts Poor Prognosis in GBC

In order to further validate the relationships between circ-MTO1 expression and clinical prognosis in GBC, we employed Kaplan-Meier survival curves to assess OS and PFS in GBC patients with circ-MTO1 low expression (n = 50) and circ-MTO1 high expression (n = 50). Figure 2A indicated that the OS of GBC patients with high circ-MTO1 expression was significantly shorter than that of patients with low circ-MTO1 expression (p<0.001). Meanwhile, Figure 2B showed that the progression-free survival time of GBC patients with high circ-MTO1 expression was significantly shorter than that of patients with low circ-MTO1 expression (p<0.001). These results indicate that high circ-MTO1 expression predicts poor prognosis in GBC patients.

High Circ-MTO1 Expression Is an Independent Prognostic Factor for Poor OS and PFS in GBC

In order to further investigate the role of circ-MTO1 in GBC, we used univariate COX regression curve analysis to evaluate the poten-

Table I. Clinical information and their relationships with circ-MTO1 expression.

Clinicopathological characteristics	Total	High expression	Low expression	χ^2	*p*-value
Gender					
Male	48	25	23	0.1603	0.6889
Female	52	25	27		
Age					
≤55	56	28	28	0	>0.999
>55	44	22	22		
Tumor size					
T1	36	13	23	6.694	0.019
T2	24	11	13		
T3	16	11	5		
T4	24	15	9		
Differentiation					
High	29	19	10	4.702	0.033
Moderate	37	18	19		
Poor	34	13	21		
Lymph node metastasis					
Positive	43	28	15	6.895	0.009
Negative	57	22	35		
TMN stages					
I	31	10	21	8.819	0.032
II	25	11	14		
III	19	12	7		
IV	25	17	8		
Distant metastasis					
Positive	46	31	15	10.306	0.01
Negative	54	19	35		

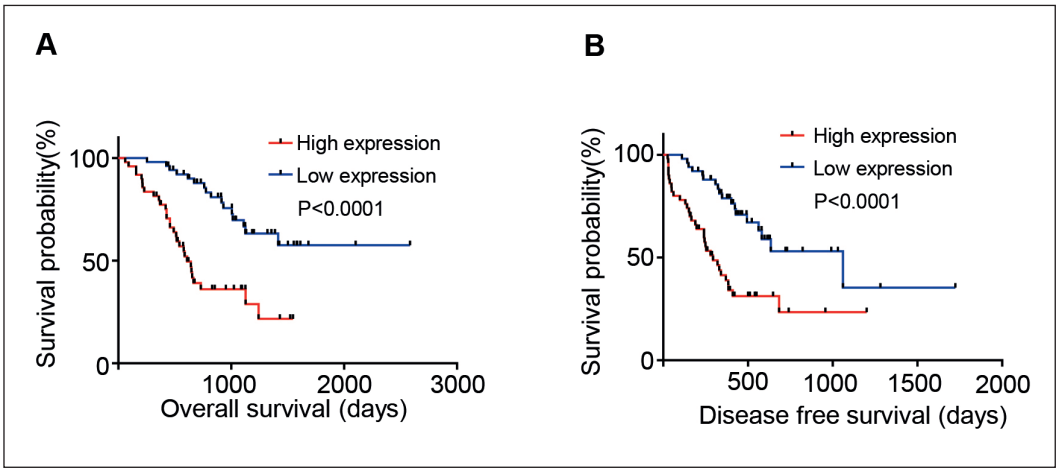

Figure 2. Overall survival and overall progress analysis of patients with low and high circ-MTO1 expression. **A**, Kaplan–Meier survival curves were used to assess overall survival in patients with circ-MTO1 low expression (n = 50) and circ-MTO1 high expression (n = 50) GBC. Kaplan–Meier survival curve assessment of progression-free survival in patients with circ-MTO1 low expression (n = 50) and circ-MTO1 high expression (n = 50) GBC.

tial overall survival prognostic indicators and progression-free survival prognostic indicators. Table II demonstrated statistically significant differences between high circ-MTO1 expression and tumor size, differentiation, TNM stage, lymph node metastasis, and distant metastasis (p<0.05). In contrast, no statistical difference was observed between high circ-MTO1 expression and patient age or gender. Moreover, in terms of free disease survival, statistically, significant differences were observed between high circ-MTO1 expression, TNM stage, lymph node metastasis, tumor differentiation and size, and distant metastasis (p<0.05). Again, no statistical difference could be detected between high circ-MTO1 expression and age or gender in GBC patients. In summary, high circ-MTO1 expression is an independent prognostic factor for poor OS and PFS in GBC.

Upregulated Plasma Circ-MTO1 Level Acts as a Potential Diagnostic Biomarker for GBC

Finally, we assessed the diagnostic value of circ-MTO1 in patient plasma. Pearson correlation coefficient analysis showed the positive correlation between circ-MTO1 expression in 30 tumor tissues and 30 paired plasma in GBC patients (p<0.001) (F). Meanwhile, qRT-PCR was employed to examine the expression of circ-MTO1 in 20 pairs of preoperative and postoperative GBC plasma. The plasma level of circ-MTO1 in GBC patients after surgery was significantly lower than that in GBC patients without surgery (p<0.001) (Figure 3C). In addition, the ROC curve was used to analyze the diagnostic value of circ-MTO1 level in GBC. The results revealed that the area under the ROC curve (AUC) of circ-MTO1 was

Table II. Correlation between the expression level of OIP5-AS1 and clinical characteristics of OC patients (n=52).

Overall survival	B	SE	Wald	d f	*p*-value	Exp (B)	95% Exp (B)	
							upper limit	lower limit
Circ-MTO1	-1.74	0.279	38.814	1	0	0.176	0.102	0.303
Gender	-0.062	0.233	0.071	1	0.789	0.94	0.595	1.484
Age	-0.332	0.235	2	1	0.157	0.717	0.453	1.137
Tumor size	0.549	0.115	22.803	1	<0.001	1.731	1.382	2.169
Tumor differentiation	-0.558	0.173	11.557	1	0.001	0.556	0.396	0.78
TMN stages	0.437	0.139	9.895	1	0.002	1.547	1.179	2.031
Lymphatic metastasis	0.256	0.284	0.812	1	0.368	1.292	0.74	2.254
Distant metastasis	0.768	0.248	9.611	1	0.002	2.156	1.327	3.505

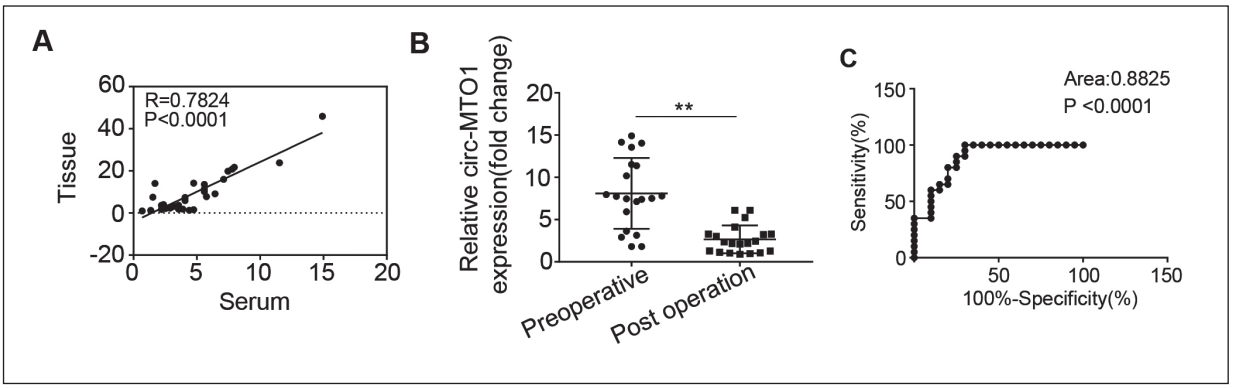

Figure 3. Diagnostic efficacy assessment of circ-MTO1 in GBC patients. **A**, Correlation analysis of circ-MTO1 expression in tissues and plasma of 30 patients with GBC by Pearson correlation coefficient. **B**, qRT-PCR method for detecting the expression level of circ-MTO1 in 20 pairs of GBC preoperative and postoperative plasma. **C**, ROC curve analysis of plasma circ-MTO1 level as diagnostic biomarker of GBC. $^{**}p<0.01$.

> 0.8 (Figure 3C). These results indicate that circ-MTO1 expression is significantly upregulated in both tumor tissues and plasma in GBC patients. Therefore, circ-MTO1 is an early diagnostic and prognostic marker for patients with gallbladder cancer.

Discussion

The pathogenesis of gallbladder cancer is still not fully demonstrated. Clinical studies have shown that gallstones are one of the risk factors for gallbladder cancer. Patients with stones over 3 cm in diameter have a greater incidence of gallbladder cancer than 10 times compared with the general population[16]. The possible cause of gallstone cancer in patients with stones is that the stones obstruct the bile duct, which causes the bile to accumulate. Therefore, inflammation of the gallbladder membrane induces gallbladder carcinogenesis[17]. Nishimura et al[18] revealed that gallbladder cancer incidence in patients with mirrizi syndrome was 27.8%, while the incidence of cancer in other patients with gallstones was only 2%. Therefore, the probability of gallbladder cancer in patients with mirrizi syndrome is significantly increased. In recent years, researchers have begun to study the pathophysiological mechanism of the origination and growth of gallbladder cancer at the molecular level[19]. Letelier et al[20] showed that the tumor suppressor genes expressed in the normal human gallbladder include: P73, P16, MGMT, APC, RARβ2, hMLH1. However, at least one gene promoter region is methylated in gallbladder carcinoma tissues, resulting in down-regulation or even loss of tumor suppressor gene expression. Salman et al[21] revealed that the positive rate of survivin expression in gallbladder carcinoma tissues was as high as 88.9%. However, the expression of survivin in normal gallbladder tissues and chronic cholecystitis tissues was low

Table III. Univariate and multivariate COX regression curves for potential progression-free survival prognosis in patients with GBC.

Free diseases survival	B	SE	Wald	d f	*p*-value	Exp (B)	95% Exp (B)	
							upper limit	lower limit
circ-MTO1	-0.888	0.273	10.593	1	0.001	0.412	0.241	0.702
Gender	-0.201	0.237	0.716	1	0.397	0.818	0.514	1.302
Age	0.016	0.241	0.004	1	0.947	1.016	0.634	1.63
Tumor size	0.649	0.142	20.772	1	<0.001	1.913	1.447	2.528
Tumor differentiation	-1.173	0.23	26.105	1	<0.001	0.309	0.197	0.485
TMN stages	0.909	0.204	19.787	1	<0.001	2.482	1.663	3.705
Lymphatic metastasis	0.956	0.317	9.108	1	0.003	2.602	1.398	4.843
Distant metastasis	0.796	0.332	5.741	1	0.017	2.217	1.156	4.251

or even not expressed. In addition, Kumari et al[22] showed that C-erB2 had a high expression rate in early gallbladder carcinoma. However, its expression level is low or even absent in advanced gallbladder carcinoma. It is concluded that the down-regulation or even loss of C-erB2 expression may be one of the early signs of gallbladder cancer. In summary, the occurrence and development of gallbladder cancer are complex processes involving multiple factors. Therefore, an in-depth study of the molecular mechanism of gallbladder cancer has important clinical and practical significance for the clinical treatment of gallbladder cancer.

The unique circular structure of circ RNA, unlike traditional linear RNA, does not exist at the 3' and 5' ends. There is no poly-A tail structure, which makes circ RNA evade nuclease digestion[23]. The expression of circ RNA in cells is more than ten times that of its isogenic linear RNA molecules[24]. Therefore, circRNA is an ideal biomarker for disease diagnosis with multiple advantages, including conserved sequences, stable, abundant, and tissue-specific. Many interesting circRNA molecules have been discovered in the study of cancer-related biomarker identification. CircRNA_100876 has been considered as a biomarker for small cell lung cancer. This molecule is closely related to the prognosis of small cell lung cancer[25]. Hsa_circ_0001895[26], hsa_circ_0000190[27], hsa_circ_002059[11], and circ PVT1[28] can be used as a marker for gastric cancer and are associated with clinical-pathological parameters. Hsa_circ_0004018[29] and hsa_circ_0001649[30] are closely related to the development and prognosis of liver cancer. Moreover, Hsa_circ_001988 is a low expression in colorectal cancer and can be used as a biomarker[31]. In addition, specific expression of circRNA was identified in other diseases, such as diabetes[32], coronary heart disease[33], intracranial aneurysms[34], preeclampsia[35], and idiopathic pulmonary fibrosis[11]. Therefore, this molecule could serve as a candidate biomarker for the diagnosis and treatment of multiple diseases. However, little evidence has been obtained about the circRNA role in gallbladder cancer. In this study, we have revealed that circ-MTO1 expression was significantly upregulated in gallbladder cancer tissue and plasma. High expression of circ-MTO1 as an independent factor was closely related to poor prognosis in GBC. In addition, circ-MTO1 is a potential early diagnosis and prognostic marker for patients with gallbladder cancer. Therefore, circ-MTO1 could provide useful information for the diagnosis and treatment of GBC disease.

Conclusions

In summary, circ-MTO1 is a potential diagnosis and prognostic biomarker for gallbladder cancer patients. This work presented here would provide detailed information diagnosis and treatment of GBC patients.

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Ethics Approval and Consent to Participate

The present study was approved by the Ethics Committee of Hechuan District People's Hospital.

Conflict of Interests

The authors declare that they have no competing interests.

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