Decreased expression of hsa-miR-372 predicts poor prognosis in patients with gallbladder cancer by affecting chloride intracellular channel 1

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Abstract. It has been reported that hsa-microRNA (miRNA/miR)-372 functions as a tumor suppressor or oncogene in various digestive system tumors, however, its roles in gallbladder cancer (GBC) are yet to be established. The present study aimed to determine the expression and clinical relevance of hsa-miR-372 in GBC. The expression of hsa-miR-372 in 80 pairs of human GBC tissues and adjacent normal gallbladder tissues was measured by reverse transcription-quantitative polymerase chain reaction. Subsequently, the associations between hsa-miR-372 expression levels and the clinicopathological characteristics of patients with GBC were determined using χ^2 test. Furthermore, Kaplan-Meier method and Cox regression analysis were performed to evaluate the association between hsa-miR-372 expression and the prognosis of patients with GBC. Furthermore, a dual-luciferase reporter assay and western blot analysis were performed to predict and verify the target gene of hsa-miR-372. The results demonstrated that markedly lower hsa-miR-372 expression was observed in GBC tissues, which was associated with poor prognosis in patients with GBC. Downregulated expression of hsa-miR-372 was negatively associated with tumor histological grade, tumor-node-metastasis stage, lymph node metastasis and distant metastasis, however, no association was observed between reduced hsa-miR-372 expression and patient gender, age, tumor size and gallbladder stones. Multivariate Cox regression analysis revealed that hsa-miR-372 expression, histological grade and lymph node metastasis were independent prognostic factors for overall survival in patients with GBC. Chloride intracellular channel 1 (CLIC1) was previously reported to be an effective biomarker for predicting the prognosis of GBC. Notably, the results of the present study indicated that CLIC1 may be a direct target gene of hsa-miR-372. In conclusion, the current study provides the first statistically convincing evidence that downregulation of hsa-miR-372 may occur in GBC tissues, which may be associated with aggressive and progressive tumor behavior by affecting CLIC1 expression.

Introduction

Gallbladder cancer (GBC) is the most prevalent biliary malignancy, and is the sixth most common type of gastrointestinal cancer in the United States of America, with >10,000 novel cases of GBC diagnosed each year (1). GBC is a highly progressive and aggressive malignancy, with survival in the majority of patients being ≤1 year after diagnosis. Early diagnosis of GBC is difficult due to a lack of specific clinical symptoms, and a large number of patients with GBC are diagnosed at an advanced stage with substantial metastasis and invasion to other organs (2,3). Currently, cholecystectomy is the only effective treatment strategy for ~10 % of early-stage patients with GBC (4,5). Therefore, identification of clinical biomarkers that may be used to accurately evaluate tumor metastasis and prognosis may allow the most appropriate treatment regimen to be selected for individual patients.

MicroRNAs (miRNAs/miRs) are a class of non-coding RNAs that consist of 18-25 nucleotides with no protein-coding function (6,7). Previous reports have demonstrated that the aberrant expression miRNAs have important roles in the regulation of carcinogenesis and progression in various tumor types, including brain, lung, breast, liver, bladder and colorectal cancers (8,9). For example, miRNAs modulate epigenetic regulation, the immune system and the tumor microenvironment in lung cancer (10). In addition, miRNA-21 was reported to have diagnostic, prognostic and predictive value in patients with breast cancer, their daughters and healthy individuals (11), and miRNA-143 was demonstrated to be a predictive factor for the response to fluoropyrimidine-based chemotherapy in patients with colorectal cancer metastasis (12).

Although the functions of aberrant miRNA expression in malignancies have not been fully illustrated, miRNAs may

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become novel diagnostic biomarkers or therapeutic targets for GBC (13). hsa-miR-372 is transcribed from the human genomic region on chromosome 19q13.42. Together with hsa-miR-371a, hsa-miR-371b and hsa-miR-373, hsa-miR-372 forms part of the miR-371-373 cluster, which is reported to exhibit a key role in tumorigenesis and progression (14). However, to the best of our knowledge, the clinical effects of aberrant hsa-miR-372 expression have not been previously reported in GBC.

The present study investigated hsa-miR-372 expression levels in 80 pairs of GBC tissues and adjacent normal gallbladder tissues, and assessed their association with the prognosis of patients with GBC. Furthermore, a dual-luciferase reporter assay and western blot analysis demonstrated that chloride intracellular channel 1 (CLIC1) was a direct target gene of hsa-miR-372.

Materials and methods

Patients. A total of 80 samples from patients with pathologically confirmed GBC were collected from Hunan Provincial People's Hospital, The First Affiliated Hospital of Hunan Normal University (Changsha, China) between 2008 and 2012 following cholecystectomy. None of the patients that participated in the present study were treated with radiotherapy, chemotherapy or other treatments prior to cholecystectomy. The patients included 30 males and 50 females, with an average age of 48.2±2.6 years. The pathological stage of GBC was classified according to 6th edition of the tumor-node-metastasis (TNM) classification of the American Joint Committee on Cancer (15). Poorly differentiated tumor tissues exhibit poor maturity and high degree of malignancy, while well-differentiated tumor tissues are similar in appearance to normal cells and exhibit a low degree of malignancy. The histological grade was evaluated by hematoxylin and eosin staining. Tumor invasion and lymph metastasis were assessed according to the standard criteria proposed by the Chinese Research Society for GBC (16). The clinicopathological features of all patients are presented in Table I. The overall survival time of each patient was measured from the date of initial surgical operation to mortality. The follow-up time ranged from 1 to 60 months.

This medicinal study was approved by the Research Ethics Committee of Hunan Provincial People's Hospital, The First Affiliated Hospital of Hunan Normal University, and written informed consent was also obtained from all patients or their families. All patient specimens were made anonymous and handled according to the legal and ethical standards for protecting human rights.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from 80 samples of fresh GBC tissues and adjacent normal gallbladder tissues or GBC cell lines were extracted using a miRNeasy mini kit (Qiagen, Inc., Valencia, CA, USA). Subsequently, for hsa-miR-372, the cDNA was synthesized by using One Step Prime script miRNA cDNA Synthesis kit (Qiagen, Inc.) according to the manufacturer's protocol. qPCR was performed on the Roche Light Cycler 480 II real-time PCR System using a SYBR Premix Ex Taq II kit (Takara Bio, Inc., Otsu, Japan). The qPCR conditions were: 95°C for 5 min then 40 cycles of denaturation at 95°C for 10 sec and for the

annealing/elongation step, 60°C for 30 sec. Hsa-miR-372 was amplified by using forward primers with the DNA sequence of the mature miRNA (5'-ACTATTCTGATGTCCAAG TGGA-3') and common reverse primers provided in the First Strand cDNA Synthesis kit. U6 small nuclear RNA was used as an internal control with the following primers: forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-ACG CTTCACGAATTTGCGT-3'. For CLIC1, the first-strand cDNA was synthesized by using a RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. qPCR was also performed on the Roche Light Cycler 480 II real-time PCR System using a SYBR Premix Ex Taq II kit. The qPCR conditions were 98°C for 10 min and 40 cycles of denaturation at 98°C for 10 sec and for the annealing/elongation step, 65°C for 30 sec. GAPDH was used as an internal control. The primer sequences of CLIC1 and GAPDH were as follows: CLIC1 forward, 5'-TCTGAACCC TGAGTCCAAC-3' and reverse, 5'-GAGTCCCTTCTCCAG ATTGT-3'; and GAPDH forward, 5'-GAAGGTGAAGGT CGGAG-3' and reverse, 5'-GAAGATGGTGATGGGAT-3'. All reactions were run in triplicate, the relative expression levels of hsa-miR-372 and CLIC1 were normalized to the expression of U6 and GAPDH, respectively using the $2^{-\Delta\Delta Cq}$ method (17).

Target gene prediction and plasmid construction. Target genes of hsa-miR-372 were predicted by using TargetScanHuman (version 7.1; www.targetscan.org) and miRanda (2010 version; www.microrna.org) algorithms.

To generate a reporter plasmid for the dual-luciferase reporter assay, the full length 3'-untranslated region (UTR) of the CLIC1 gene was synthesized by PCR. The following primers were used: 5'-GCCCCTCCTGGGACTCCCTCA ACCC-3' (forward) and 5'-TTGCGTAAAAACACTTGATTT TTAT-3' (reverse). Subsequently, PCR products were cloned into a psiCHECK-2 vector (Promega Corporation, Madison, WI, USA) to produce psiCHECK-2-CLIC1 wild type luciferase reporter plasmid. The correct clones were confirmed by Sanger DNA sequencing. The mutant CLIC1 3'-UTR sequence plasmid was synthesized by GeneCopoeia (GeneCopoeia, Inc., Rockville, MD, USA) and also inserted into the psiCHECK-2 vector to construct psiCHECK-2-CLIC1 mutant (MUT) luciferase reporter plasmid.

Cell culture and dual-luciferase reporter assay. G-415, OCUG-1 and SGC-996 human GBC cell lines were purchased from the Japan Health Science Research Resources Bank (Osaka, Japan). All cells were cultured in Dulbecco's modified Eagle's medium/F12 (Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO_2 at 37°C.

GBC cells were seeded onto 24-well plates ($4x10^5$/well) and cotransfected with 100 nM hsa-miR-372 mimics (5'-AAA GUGCUGCGACAUUUGAGCGU-3') or corresponding mimics negative control (5'-CAGCUCAAGUGUCGCCAU GUTCU-3') and WT or MUT luciferase reporter plasmids (100 ng) by using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. After transfection for 24 h, the luciferase activities

Table I. Association between hsa-miR-372 expression in patients with gallbladder cancer and clinicopathological characteristics.

Variable	Number of cases	hsa-miR-372 expression		P-value
		Low	High	
Age, years				0.105
<50	47	29	18	
≥50	33	26	7	
Gender				0.071
Male	30	17	13	
Female	50	38	12	
Tumor size, cm				0.191
≤3	27	16	11	
>3	53	39	14	
Histological grade				<0.001
G1-G2	16	5	11	
G3-G4	64	50	14	
TNM stage				<0.001
I-II	23	8	15	
III-IV	57	47	10	
Lymph node metastasis				0.014
Negative	35	19	16	
Positive	45	36	9	
Distant metastasis				<0.001
Negative	42	18	24	
Positive	38	37	1	
Gallbladder stones				0.228
Absent	40	25	15	
Present	40	30	10	

miR, microRNA; TNM, tumor-node-metastasis.

were analyzed using a dual luciferase reporter gene assay kit (Promega Corporation) in a TD-20/20 luminometer (Turner Designs, San Jose, CA, USA). *Renilla* luciferase activity was normalized to firefly luciferase activity.

Western blot analysis. Total protein from G-415, OCUG-1 and SGC-996 cells was extracted by radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) at 65°C and quantified using a bicinchoninic assay kit (Thermo Fisher Scientific, Inc.). Subsequently, the quantified proteins (50 μg) were denatured and subjected to 15% SDS-PAGE, and blotted onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were then blocked with 5% non-fat milk for 2 h at 37°C. Following washing with TBS-Tween-20 (0.1%) three times, the membranes were incubated with mouse anti-human CLIC1 (1:1,000; cat. no. ab77214; Abcam, Cambridge, MA, USA) and anti-GAPDH (1:2,000; cat. no. ab8245; Abcam) antibodies overnight at 4°C.
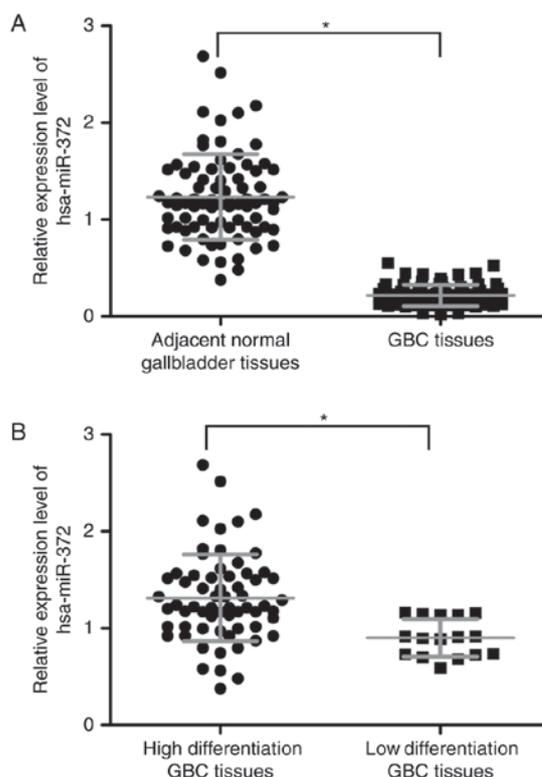

Figure 1. Expression of hsa-miR-372 in GBC tissues and adjacent normal gallbladder tissues from patients with GBC. (A) Relative hsa-miR-372 expression was significantly decreased in GBC tissues compared with adjacent normal gallbladder tissues. (B) Relative hsa-miR-372 expression was lower in the GBC tissues with low differentiation compared with highly differentiated tissues. *P<0.05, as indicated. miR, microRNA; GBC, gallbladder cancer.

Then, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG (1:2,000; cat. no. 7076; Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 h at 37°C and visualized using an enhanced chemiluminescence western blot detection kit (GE Healthcare, Pittsburgh, PA, USA) according to the manufacturer's protocols.

Statistical analysis. Statistical analysis was performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Each experiment was repeated at least three times. Data are presented as the mean ± standard deviation. A paired Student's t-test was performed to compare hsa-miR-372 expression in GBC tissues with adjacent normal gallbladder tissues. The χ^2 test was used to detect the association between hsa-miR-372 expression and clinicopathological features. The expression of hsa-miR-372 between the three GBC cell lines was compared using one-way analysis of variance and Dunnett's post-hoc test. Overall survival curves were evaluated by the Kaplan-Meier method and logrank test. Univariate Cox regression analysis was performed for prognostic parameters, and the importance of multiple parameters for survival was assessed by the Cox's proportional hazards model for multivariate analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

hsa-miR-372 expression is reduced in GBC tissues. The expression of hsa-miR-372 was determined in 80 GBC tissue

Table II. Univariate and multivariate Cox regression analyses of prognostic parameters patients with gallbladder cancer.

	Univariate Cox regression		Multivariate Cox regression	
Variable	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, ≥50 vs. <50	0.991 (0.525-1.467)	0.724	-	-
Gender, male vs. female	1.275 (0.838-1.529)	0.522	-	-
Tumor size, ≤3 vs. >3 cm	1.643 (0.587-2.965)	0.458	-	-
Histological grade, G1-G2 vs. G3-G4	2.047 (1.198-4.325)	0.026[a]	2.013 (1.112-4.214)	0.024[a]
TNM stage, I-II vs. III-IV	1.028 (0.468-2.093)	0.791	-	-
Lymph node metastasis, negative vs. positive	2.431 (1.752-7.043)	0.001[a]	2.419 (1.682-6.931)	0.001[a]
Distant metastasis, negative vs. positive	1.125 (0.369-2.467)	0.652	-	-
Gallbladder stones, absent vs. present	1.019 (0.728-1.646)	0.435	-	-
hsa-miR-372 expression, low vs. high	2.559 (1.578-4.927)	0.006[a]	2.410 (1.467-4.532)	0.004[a]

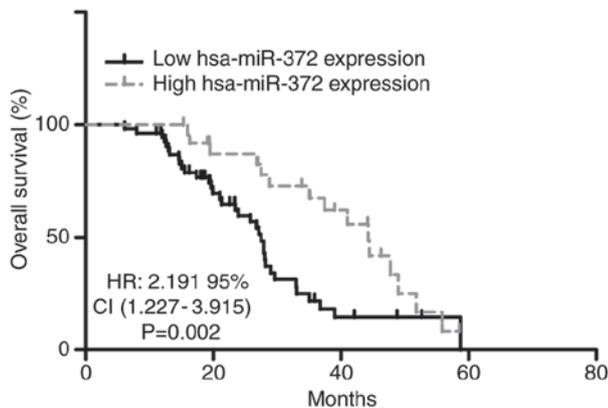HR, hazard ratio; CI, confidence interval; TNM, tumor-node-metastasis; miR, microRNA. [a]P<0.05.

Figure 2. Reduced expression of hsa-miR-372 is associated with poor prognosis in patients with GBC. Kaplan-Meier survival curves were established for high and low hsa-miR-372 expression groups, which were set at the median value of the hsa-miR-372 expression levels in 80 patients with GBC. The high hsa-miR-372 group exhibited improved prognosis compared with the low hsa-miR-372 group. miR, microRNA; GBC, gallbladder cancer; HR, hazard ratio; CI, confidence interval.
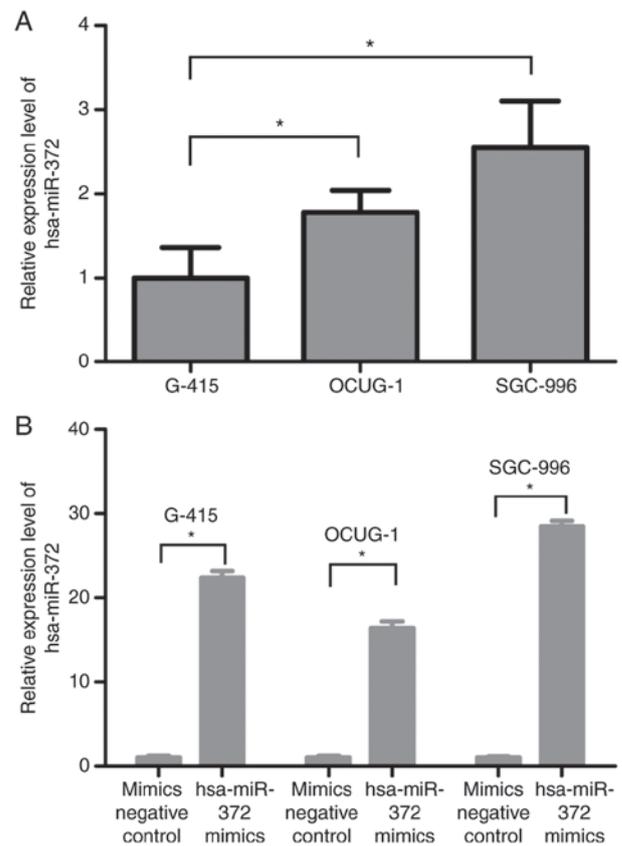

Figure 3. Endogenous expression levels of hsa-miR-372 in three GBC cell lines. (A) RT-qPCR was performed to determine the expression of hsa-miR-372 in G-415, OCUG-1 and SGC-996 cells. (B) The relative expression levels of hsa-miR-372, determined by RT-qPCR, in three GBC cell lines following treatment with hsa-miR-372 mimics were significantly increased compared with cells transfected with mimics negative control. *P<0.05, as indicated. miR, microRNA; GBC, gallbladder cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

samples and adjacent normal gallbladder tissues by RT-qPCR. Following normalization to U6, the hsa-miR-372 expression in GBC tissues was significantly lower compared with adjacent normal gallbladder tissues (P=0.002; Fig. 1A). Additionally, hsa-miR-372 expression was also downregulated in GBC tissues with low differentiation compared with highly differentiated tissues (P=0.042; Fig. 1B).

Association between hsa-miR-372 expression and clinicopathological parameters of patients with GBC. RT-qPCR results indicated that hsa-miR-372 expression was significantly downregulated in patients with GBC. In order to further determine the potential associations between hsa-miR-372 expression and the clinicopathological features, 80 samples of GBC tissues were divided into low and high expression groups based on the average expression of hsa-miR-372 in GBC tissues. Patients with GBC that exhibited expression of hsa-miR-372 at levels lower than the average expression

level (2.41) were assigned to the low expression group (n=55), and those samples with expression equal to or above the average value were assigned to the high expression group (n=25).
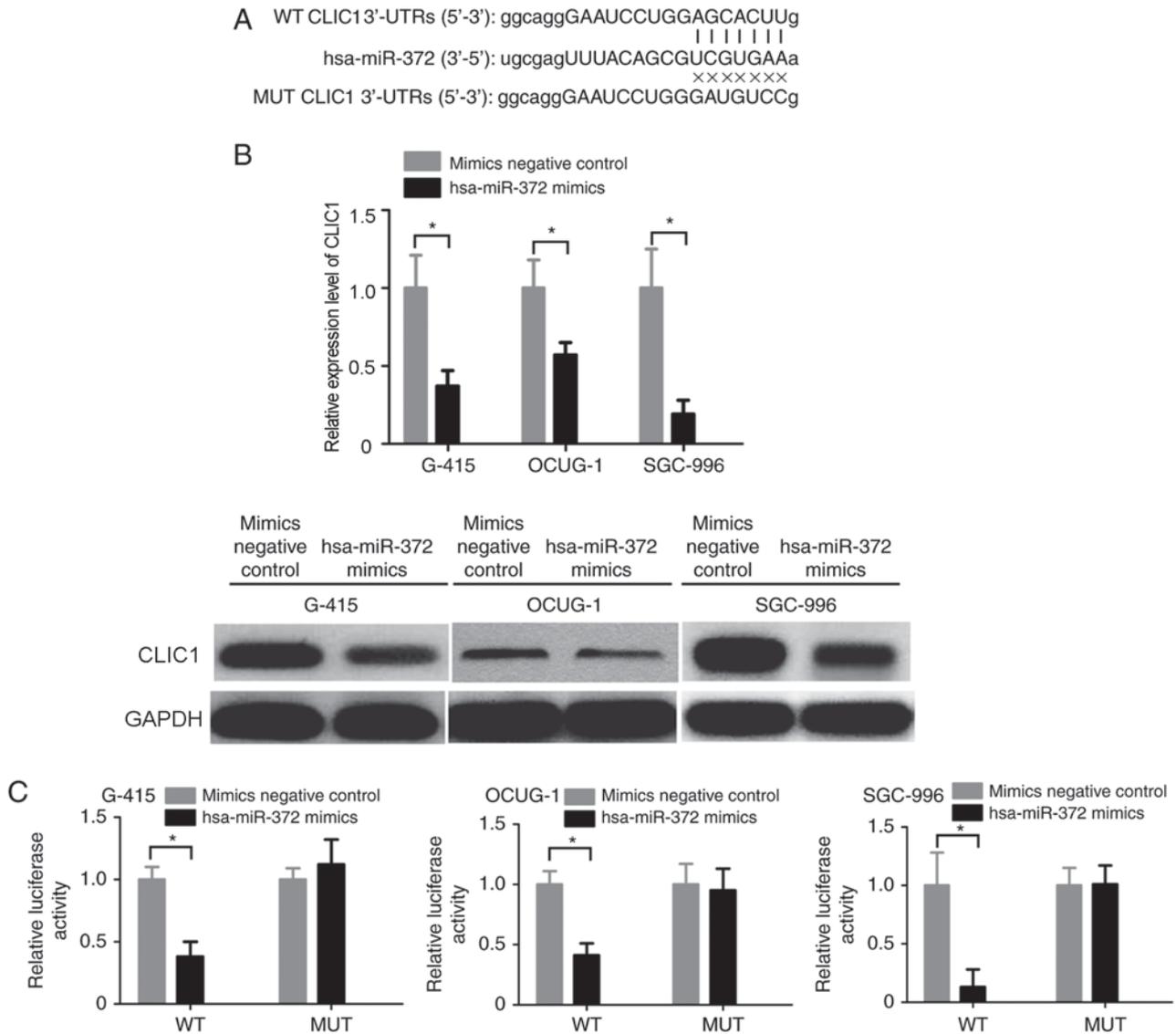
Figure 4. CLIC1 is a direct target of hsa-miR-372 in GBC cells. (A) Sequence alignment of hsa-miR-372 and the CLIC1 3'-UTR using miRanda algorithm. (B) Reverse transcription-quantitative polymerase chain reaction and western blot analysis was performed to determine CLIC1 expression in G-415, OCUG-1 and SGC-996 cells following transfection with hsa-miR-372 mimics or mimics negative control. GAPDH was used as an internal control. (C) G-415, OCUG-1 and SGC-996 cells were cotransfected with hsa-miR-372 mimics or mimics negative control, and the luciferase reporter plasmid containing a fragment of the CLIC1 3'-UTR harboring either the WT hsa-miR-372 binding sites or MUT binding sites. *$P<0.05$, as indicated. CLIC1, chloride intracellular channel 1; miR, microRNA; GBC, gallbladder cancer; UTR, untranslated region; WT, wild-type; MUT, mutant.

As summarized in Table I, low hsa-miR-372 expression was significantly associated with the presence of GBC histological grade ($P<0.001$), TNM stage ($P<0.001$), lymph node metastasis ($P=0.014$) and distant metastasis ($P<0.001$). However, there were no significant differences in the gender of patients with GBC ($P=0.071$), patient age ($P=0.105$), tumor size ($P=0.191$) or the presence of gallbladder stones ($P=0.228$) between the high and low hsa-miR-372 expression groups. Collectively, these results indicate that decreased expression of hsa-miR-372 may be relevant to tumor differentiation and metastasis, which are involved in tumorigenesis and the progression of GBC.

Reduced expression of hsa-miR-372 is associated with poor prognosis in patients with GBC. The association between hsa-miR-372 expression and the overall survival of GBC patients was evaluated by Kaplan-Meier analysis.

As demonstrated in Fig. 2, low hsa-miR-372 expression was associated with a shorter overall survival time compared with patients with high hsa-miR-372 expression ($P=0.002$). Furthermore, multivariate Cox regression analyses indicated that hsa-miR-372 expression ($P=0.004$), histological grade ($P=0.024$) and lymph node metastasis ($P=0.001$) maintained an independent prognostic influence on the overall survival of patients with GBC (Table II).

Endogenous expression levels of hsa-miR-372 in three GBC cell lines. The endogenous expression level of hsa-miR-372 in OCUG-1 and SGC-996 cells were relatively higher compared with G-415 cell, as determined by RT-qPCR (Fig. 3A). The expression levels of hsa-miR-372 in G-415, OCUG-1 and SGC-996 cells transfected with hsa-miR-372 mimics were significantly upregulated by 22.33-, 16.38- and 28.45-fold, respectively, when compared with levels in the mimics

negative control transfection group (Fig. 3B), indicating that the transfection efficiency is suitable for further cell experiments.

CLIC1 is a direct target of hsa-miR-372 in GBC cells. As miRNAs primarily function by inhibiting their target genes, the targets of hsa-miR-372 in GBC were predicted by using bioinformatics analysis. Previous research demonstrated that CLIC1 expression was closely associated with GBC progression and may be regarded as an effective biomarker for predicting the prognosis of GBC (18,19). Complementary sequences were identified between hsa-miR-372 and the 3'UTR of CLIC1 (Fig. 4A), which indicated CLIC1 may be a target gene of hsa-miR-372. To further investigate whether hsa-miR-372 inhibits CLIC1 expression in GBC cells, G-415, OCUG-1 and SGC-996 cells were treated with hsa-miR-372 mimics or mimics negative control. RT-qPCR and western blot analysis demonstrated that transfection with hsa-miR-372 mimics markedly decreased the expression of CLIC1 mRNA and protein in all three GBC cell lines (Fig. 4B). In addition, a dual-luciferase reporter assay was performed to confirm the direct interaction between hsa-miR-372 and the 3'UTR of CLIC1. Notably, the results demonstrated that the relative luciferase activity of the wild-type CLIC1 3'UTR plasmid decreased in response to treatment with hsa-miR-372 mimics compared with negative control mimics (Fig. 4C). By contrast, hsa-miR-372 mimics exhibited no inhibitory effect on luciferase activity when transfected with the mutant CLIC1 3'UTR plasmid (Fig. 4C).

Discussion

At present, GBC is associated with a high mortality rate and poor prognosis (2,3). Although certain clinicopathological parameters have been the standard for predicting the clinical outcome of patients with GBC, these classification schemes are not precise indexes of prognosis for patients with GBC. Thus, it is essential to identify novel and effective biomarkers that are associated with advanced GBC progression to allow early diagnosis and treatment.

hsa-miR-372 has been reported to function as either an oncogenic gene or a tumor suppressor gene in several types of human cancer. For example, Cho *et al* (20) demonstrated that hsa-miR-372 has an oncogenic function by directly targeting the tumor suppressor gene large tumor suppressor kinase 2 in gastric cancer. In addition, Yamashita *et al* (21) indicated that the upregulation of hsa-miR-372 was an independent prognostic factor in colon cancer, and was strongly associated with synchronous liver metastasis. Furthermore, hsa-miR-372 was reported to regulate glioma cell proliferation and invasion by targeting PH domain and leucine rich repeat protein phosphatase 2 (22), and was downregulated and potentially contributes to tumorigenesis in human cervical cancer by targeting cyclin-dependent kinase 2 and cyclin A1 (23). hsa-miR-372 was also demonstrated to downregulate the expression of the oncogene ATPase family AAA domain containing 2 (ATAD2) to affect hepatocellular carcinoma cell proliferation and metastasis (24). In addition, hsa-miR-372 was reported to be downregulated in breast cancer (25), and may regulate protein folding by suppressing two chaperones [DnaJ

heat shock protein family (Hsp40) members A2 and C9] and Sec61 translocon α 2 subunit, which is considered to be an essential complex for correct protein folding (25).

The present study demonstrated that hsa-miR-372 expression was decreased in human GBC tissues compared with adjacent normal gallbladder tissues. hsa-miR-372 was already established as a potential marker and regulated a member of the ATPase family, ATAD2, in lung cancer (24). In addition, the results of the current study indicated that the downregulated expression of hsa-miR-372 in patients with GBC was associated with advanced tumor progression. Kaplan-Meier analysis demonstrated that patients with low hsa-miR-372 expression exhibited poorer overall survival compared with those with higher expression. Furthermore, multivariate Cox regression analysis demonstrated that low hsa-miR-372 expression was a statistically significant risk index influencing overall survival rates in patients with GBC, which indicated that downregulation of hsa-miR-372 in GBC is a predictor of overall survival as well as a grade-dependent factor. These results were consistent with previous studies (24,25), confirming the downregulation pattern of hsa-miR-372 in GBC and indicating a potential important role for hsa-miR-372 in the regulation of GBC progression.

Increasing evidence has demonstrated that miRNAs regulate genes expression at transcriptional and/or post-transcriptional levels via mRNA degradation and/or translational repression (26,27). At present, it is estimated that ~60% of human genes may be controlled by >1,900 identified miRNAs (28). miRNAs mediate translational repression and/or mRNA degradation by binding to the 3'UTRs of their target genes (29,30). CLIC1 is a novel member of the p64 chloride channel protein family, which has been reported to have important roles in various tumor types (31-34). A recent study indicated that CLIC1 expression is associated with total postoperative survival of patients with GBC, and CLIC1 was a potential poor prognostic factor for GBC (35). The present study identified CLIC1 as a direct target of hsa-miR-372 in GBC, as hsa-miR-372 binds to the 3'-UTR of CLIC1 mRNA *in vitro*, which indicates that the association between the downregulation of hsa-miR-372 and poor prognosis of patients with GBC may occur via CLIC1.

In conclusion, the present study provides evidence that hsa-miR-372 expression was downregulated in GBC tissues, which was associated with advanced tumor progression. Notably, the present study, to the best of our knowledge, is the first to demonstrated that hsa-miR-372 may regulate GBC progression and development by directly targeting CLIC1. As a result, the manipulation of hsa-miR-372 may be an effective and promising therapeutic method for patients with GBC in the future.

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