Original Article

Long non-coding RNA *Linc00261* as a novel potential diagnostic and prognostic biomarker for gallbladder cancer

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Background: *Linc00261* is a lncRNA that plays key roles in tumor suppression. While gallbladder carcinoma (GBC) is one of the most common cancer of the bile duct. However, the study about Linc00261's correlation with the clinicopathological characteristics and postoperative outcomes of the GBC patients is few. Therefore, we want to explore *Linc00261* in GBC and assess its potential of clinical diagnosis.

Methods: Quantitative real-time PCR (qRT-PCR) was used to detect the expression of *Linc00261* in specimens of GBC and adjacent tissues as well as cell lines. Chi-square test has been used to research the correlation of the *Linc00261* expression in GBC with the clinicopathological features. The Cox model was used to assess the value of *Linc00261* in predicting the prognosis of GBC patients. ROC curve analysis was used to test the specificity and sensitivity of diagnostic method of serum *Linc00261* expression.

Results: The expression level of *Linc00261* in GBC was significantly lower than normal tissues' and it was also up-regulated after surgery. The *Linc00261* expression was significantly correlated with large tumor size (P<0.0001), late TNM stage (P=0.008), negative liver metastasis (P=0.027) and well differentiated phenotype (P=0.017). The patients with lower *Linc00261* expression had significantly worse outcomes in terms of overall survival (P=0.0188) and progression-free survival (P=0.0029), and the low expression of *Linc00261* was identified as an independent risk factor affecting postoperative survival rate of the patients (P<0.01). The expression of *Linc00261* in serum was down-regulated of GBC patients and increased in the patients after operation. *Linc00261* expressed in serum was also positively associated with its expression in GBC tissue of patients (P<0.0001). The GBC diagnosis efficacy of using the serum *Linc00261* level to identify the GBC has high specificity and sensitivity (AUC 0.805).

Conclusions: *Linc00261* could be identified a novel gene associated with GBC development and progression. It also may serve as a new diagnostic and prognostic biomarker for patients with GBC.

Keywords: Long non-coding RNA (lncRNAs); *Linc00261*; gallbladder cancer; prognosis

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Introduction

Gallbladder carcinoma (GBC) is the most common cancer of the bile duct, which accounts for 1.2% of all global cancer diagnoses and 1.7% of all cancer deaths (1,2). This carcinoma is infrequent in developed countries but more common in some developing countries. The incidence of GBC in female patients was significantly higher than that in male (3,4). Compared with other malignant bile duct tumors, GBC has a poorer prognosis, as the 5-year survival rate for patients with the disease was between 5% and 13% with a median survival of only about 6 months (5). Despite advances in the modalities used for GBC diagnosis and treatment, the clinical outcomes of GBC have not significantly improved because there are no specific symptoms in patients with early-stage carcinoma of the gallbladder and its diagnosis is often delayed (6,7). A surgical resection is currently the only effective treatment for GBC, but aggressive surgical approaches may result in high surgical morbidity and mortality rates (8,9). Therefore, studies aiming to elucidate the molecular mechanisms mediating GBC initiation and progression, improve the methods of early diagnosis, are urgently needed.

Long non-coding RNAs (lncRNAs) is a kind of extensive transcription with RNA transcripts longer than 200 nucleotides (nt) that lack of coding protein function(10). It has been proved that lncRNAs may involve in various physiological and pathological processes such as cell proliferation metastasis and carcinogenesis by regulating transcription, translation as well as post-transcriptional and post-translational processing (11,12).

Long intergenic non-protein coding RNA 261 (*Linc00261*) is a lncRNA, shown to play key roles in the tumor suppression (13-15). It has been revealed that level of *Linc00261*expression was significantly lower in choriocarcinoma tissues and cell lines (14). *Linc00261* overexpression in choriocarcinoma or hepatocellular carcinoma cell lines would decrease cell proliferation and inhibit cell invasion *in vitro* (14,16). However, the expressional level and functional role of *Linc00261* in GBC has remained largely unknown. We hypothesized that *Linc00261*, as an inhibitor of human tumorigenesis, may also play important role in GBC development and progression.

This research aimed to investigate the function of *Linc00261* associated with GBC development, which may enable clinicians and researchers to identify GBC-specific diagnostic biomarkers and to develop therapies capable of preventing cancer invasion and metastases.

Methods

Human sample

This study was approved by the ethical committee of the First People's Hospital in Yunnan Province. A total of 100 patients diagnosed with GBC in Hepatopancreatobiliary Surgery Department of the First People's Hospital in Yunnan Province were admitted to this study. Tumor tissues from 100 patients and their adjacent normal tissues (3 cm apart to the focus) were collected in sterile tube and stored in liquid nitrogen immediately. Serum *Linc00261* levels in 50 GBC patients and 30 healthy individuals were measured and compared. 20 GBC patients' blood samples were collected 1 week after surgery. Blood sample (2 mL) was placed in a vacutainer tube containing anticoagulant for isolation of serum. Supernatant was collected after centrifugation of blood. All simples were stored in liquid nitrogen. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the First People's Hospital of Yunnan Province (approval number: KY-E-2017-1-20). All patients showed their full intention to participate in this study, and written consent from each patient was collected.

Cell culture

Three GBC cell lines including EH-GB1 (RRID: CVCL_IU73), SGC-996 (RRID: CVCL_M737), GBC-SD (RRID: CVCL_6903), NOZ NOZ (RRID: CVCL_3079) and the human intrahepatic biliary epithelial cell line HIBEC were obtained from the Hepatopancreatobiliary Surgery Department of the First People's Hospital. All of these cells were cultured in the Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplied with 10% fetal bovine serum (FBS; Gibco, USA) at a 5% CO_2 atmosphere in a 37 ℃ incubator. After 24 h of culture, cells were digested and passage or used to extract RNA.

RNA extraction and qRT-PCR

Total RNA was extracted from human tissues, cultured cell lines and serum by TRIzol reagent (TaKaRa, China) with a concentration of 1 ml for each well in six-well plates. Cell lines and serum have been centrifuged and removed supernatant before extraction. cDNA was reversely

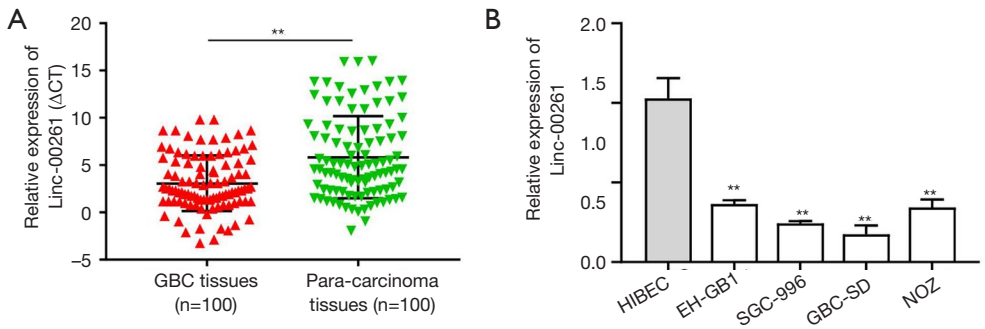

Figure 1 *Linc00261* expressed lower in Gallbladder carcinoma (GBC) tissues and cell lines compared with control. (A) qRT-PCR results showed the relative expression of Linc00261 in GBC was lower compared with para-carcinoma tissue (control). Expression levels at each sample were normalized to *Gapdh* (100 per samples were examined). (B) qRT-PCR results showed the relative expression of Linc00261 in human intrahepatic biliary epithelial cell line HIBEC or GBC cell lines including SGC-996, GBC-SD and NOZ. GBC cell lines expressed lower *Linc00261* compared with normal. Expression levels at each sample were normalized to *Gapdh*, which served as an internal control. **, P<0.01, Error bars indicate SD.

transcribed by Transcriptor First Strand cDNA Synthesis Kit (TaKaRa). RT-PCR was then performed using a Power SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies) and Bio-Rad CFX96 real-time PCR System. *Gapdh* was included as an internal control. The primers of *Linc00261* and *Gapdh* in previous report (17) were used.

The sequence of *Linc00261* primers are 5'-GTCAGAAGGAAAGGCCGTGA-3' (sense) and 3'-TGAGCCGAGATGAACAGGTG-5'(antisense). The sequence of *Gapdh* primers are 5'-GCTCTCTGCTCCTCCTGTTC-3' (sense) and 5'-ACGACCAAATCCGTTGACTC-3' (antisense).

Statistical analysis

All statistical data were analyzed by SPSS 19.0 version software. The relationship between the expressions of *Linc00261* in GBC with the clinicopathological features of the patients was estimated by the chi-square test. The differences in overall survival (OS) curves and the progress free survival (PFS) rate were compared using the Kaplan-Meier, and the independent prognostic factors of the patients (including gender, age, tumor size, TNM stage, differentiation, Lymph node metastasis) were analyzed by Cox model. The diagnosis efficacy of using the serum Linc00261 level to identify the GBC has been investigated by ROC curve analysis. A P value <0.05 (bilateral test) was considered statistically significant in all the examinations. Statistically significant values for <0.05, <0.01, and <0.001 are indicated by single (*), double (**) and triple (***) asterisk, respectively.

Results

Linc00261 is significantly down-regulated in GBC tissues and cell lines

First, we examined the *Linc00261* expression in 100 paired clinical GBC tissues and para-carcinoma tissues by qRT-PCR. As shown in *Figure 1A*, the expression of *Linc00261* was significantly down-regulated in GBC tissues compared with adjacent normal tissues (P<0.01). We also compared the level of *Linc00261* between normal human intrahepatic biliary epithelial cell line HIBEC and GBC cell lines (SGC-996, GBC-SD and NOZ) using qRT-PCR (*Figure 1B*). The results showed that GBC cell lines expressed lower *Linc00261* compared with normal.

The expression of Linc00261 is associated with clinicopathological characteristics of GBC patients

To elucidate the potential function of *Linc00261* in GBC, we analyzed the relationship between the *Linc00261* expression level and the clinicopathological factors of the GBC patients. As shown in *Table 1*, the findings revealed that the lower level of *Linc00261* expression was correlated with large tumor size (P<0.0001), late TNM stage (P=0.008), negative liver metastasis (P=0.027) and high differentiated

Table 1 Analysis of the relationship between the expression of Linc00261 and the features of GBC patients

Clinicopathological characteristics	Total	Low expression	High expression	χ^2	P value
Gender				0.360	0.548
Male	51	27	24		
Female	49	23	26		
Age, years				1.004	0.316
≤50	53	24	29		
>50	47	26	21		
Tumor size				16.000	<0.0001
T1	24	8	26		
T2	19	7	12		
T3	20	12	8		
T4	27	23	4		
Differentiation				8.174	0.017
High	34	20	10		
Moderate	33	18	15		
Poor	33	12	25		
Lymph node metastasis				4.889	0.027
Positive	61	33	22		
Negative	39	17	28		
TMN stages				11.934	0.008
I	21	5	16		
II	25	10	15		
III	25	15	10		
IV	29	20	9		

The median value of the *Linc00261* level in tissues of 100 GBC patients was considered as the cut-off value of all samples. 50 patients with *Linc00261* expression lower than the cut-off value was classified into low expression group. Other 50 patients with higher expression were in high expression group. The relationship between expression and features of patients has been analyzed by χ^2 test.

degree (P=0.017). But gender and age of patients has no significant association with *Linc00261* expression (P>0.05, respectively).

Low Linc00261 expression predicts poor prognosis in GBC

We have indicated that the expression of *Linc00261* has correlation with the clinicopathological features of GBC. To further determine the prognostic effect of *Linc00261* expression in GBC patients, we conducted a Kaplan-Meier survival analysis as shown in *Figure 2*. For the patients with low *Linc00261* expression, Kaplan-Meier analysis revealed that they had significantly worse outcomes in terms of overall survival (OS, P=0.0188) and progression-free survival (PFS, P=0.0029) (*Figure 2A*,*B*).

High Linc00261 expression is an independent prognostic factor for high OS and PFS rate in GBC

Moreover, to evaluate whether *Linc00261* expression was

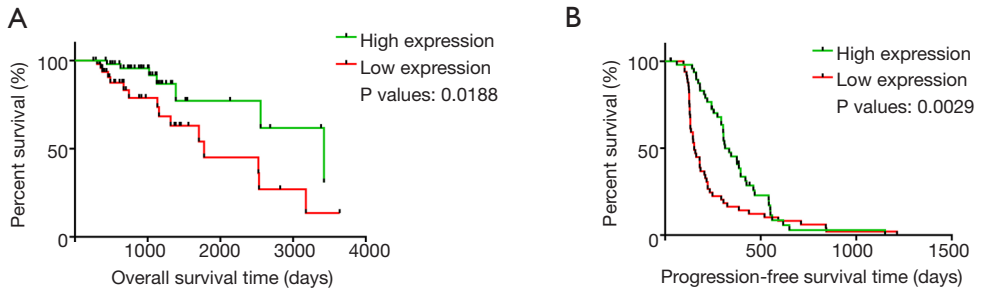

Figure 2 Kaplan-Meier curves of overall survival (OS) and progress free survival (PFS) for Gallbladder carcinoma (GBC) patients according to *Linc00261* expression. Kaplan-Meier curves were used to calculate the overall survival (OS) rate (A) and progress free survival (PFS) rate (B) in *Linc00261* high expression group and low expression (50 per samples were examined). Compared with the patients with low *Linc00261* expression, those with high Linc00261 expression had a higher OS rate (P<0.05). Compared with the patients with high *Linc00261* expression, those with low *Linc00261* expression had shorter PFS time (P<0.01).

Table 2 Univariate analysis of OS by Cox model in GBC

OS	B	SE	Wald	df	P value	Exp(B)	95% Exp(B)	
							Upper limit	Lower limit
Linc00261	−0.745	0.264	7.941	1	0.005	0.475	0.283	0.797
Gender	−0.046	0.228	0.041	1	0.840	0.955	0.611	1.492
Age	0.409	0.248	2.714	1	0.099	1.506	0.925	2.450
Tumor size	0.108	0.312	1.335	3	0.721	1.114	0.604	2.055
Differentiation	−0.025	0.277	0.973	2	0.615	0.975	0.567	1.678
Lymph node metastasis	0.670	0.311	4.625	1	0.032	1.954	1.061	3.597
TMN stage	−1.008	0.464	8.743	3	0.033	0.365	0.147	0.907

Univariate analyses of OS by Cox model were conducted to evaluate the potential prognostic factor in GBC. The candidate factors included the level of *Linc00261* expression, gender, age, tumor size and differentiated degree, lymph node metastasis and TNM stage. B means regression coefficient. SE means standard error. df means degree of freedom.

an independent prognostic factor for survival in GBC, univariate analyses by Cox model were conducted. The univariate analysis of OS shown in *Table 2* describes the significant prognostic factors for OS including the level of *Linc00261* expression (P=0.005), lymph node metastasis (P=0.032) and TNM stage (P=0.033). *Table 3* demonstrated the results of univariate analysis of PFS. The significant prognostic factors for PFS included the *Linc00261* expression (P<0.001), lymph node metastasis (P=0.005) and TNM stage (P=0.004) as well. However, other factors including gender, age, tumor size and differentiated degree had no significant effect both on OS and PFS (P>0.05). It is noteworthy that the low expression of *Linc00261* is the most significant unfavorable factor for OS and PFS rate compared with the other candidate factors.

Serum Linc00261 is significantly decreased in GBC and can be used as a potential diagnostic biomarker for GBC

We supposed that *Linc00261* expressed in serum may be affected by GBC. So, the level of *Linc00261* expression in GBC patients' or healthy volunteers' serum had been examined by qRT-PCR. The results showed that the expression of Linc00261 was down-regulated in the serum of GBC patients (*Figure 3A*). Additionally, the correlation analysis was used to study the relationship between *Linc00261* expression in GBC tissues and in serum of patient with GBC. As shown in *Figure 3B*, *Linc00261* expression in GBC tissue was positively associated with its expression in GBC patient's serum (P<0.0001). We also compared the serum *Linc00261* level in GBC patients and the patients

Table 3 Univariate analysis of PFS by Cox model in GBC

PFS	B	SE	Wald	df	P value	Exp(B)	95% Exp(B)	
							Upper limit	Lower limit
Linc00261	–1.183	0.283	17.520	1	0.000	0.306	0.176	0.533
Gender	–0.029	0.229	0.017	1	0.898	0.971	0.620	1.520
Age	0.446	0.241	3.418	1	0.065	1.563	0.973	2.508
Tumor size	0.412	0.330	1.649	3	0.648	1.510	0.790	2.885
Differentiation	–0.278	0.277	4.439	2	0.109	0.758	0.440	1.304
Lymph node metastasis	0.875	0.314	7.782	1	0.005	2.399	1.297	4.436
TMN stage	–1.460	0.453	13.111	3	0.004	0.232	0.096	0.564

Univariate analyses of PFS by Cox model were conducted to evaluate the potential prognostic factor in GBC. The candidate factors included the level of *Linc00261* expression, gender, age, tumor size and differentiated degree, lymph node metastasis and TNM stage. B means regression coefficient. SE means standard error. df means degree of freedom.

after surgery, which revealed that serum *Linc00261* level was increased in the patients after operation (*Figure 3C*). Then diagnosis efficacy of using the serum *Linc00261* level to identify the GBC was assessed. ROC curve analysis (*Figure 3D*) showed this diagnostic method has high specificity and sensitivity on GBC diagnosis (P=0.001, AUC 0.805).

Discussion

GBC is the most common and aggressive malignancy of the bile duct, and the worldwide incidence of the disease is increasing annually (18). Despite the recent advances in the management of GBC, the prognosis of patients with GBC remains relatively poor (19,20). The overall 5-year survival rate of GBC is less than 5%. But in early-stage disease, the 5-year survival rate reaching 75% can be achieved if stage-adjusted therapy is performed. Additionally, only a third of GBCs are preoperatively recognized. GBC is diagnosed by pathologists after routine cholecystectomy for a benign disease, which was termed "incidental or occult gallbladder carcinoma (IGBC)" (7). Therefore, it is significant that to find novel genes associated with GBC development and progression and to make them as new biomarkers for early diagnosis.

Previous studies have shown that *Linc00261* has lower expression in various kinds of cancer cell such as choriocarcinoma, gastric cancer, colon cancer and esophageal cancer cells (21,22) compared with normal cells. *Linc00261 has* been recognized as a tumor-suppressor gene. There was report that *Linc00261* can restrain β-catenin from cytoplasm into nuclei to down-regulate nuclear β-catenin, which could promote β-catenin degradation and inhibit activation of WNT pathway in human colon cancer cells (23). In gastric cancer cell lines, *Linc00261* could decrease the stability of Slug proteins and suppressing epithelial-mesenchymal transition (EMT) (21). Moreover, during lung cancer tumorigenesis and progression, *Linc00261* can suppress EMT correlated with its neighboring gene forkhead box A2 (FOXA2) (24). In our research, we found out that the expression of *Linc00261* was decreased in GBC tissues compared with para-carcinoma tissues just like reported researches mentioned above. And the expression level of *Linc00261* negatively correlated with advanced tumor status, clinical stage and poor prognostic outcome.

Many researches also identify *Linc00261* as a prognostic biomarker of many malignancies (17,21,24,25). In hepatocellular carcinoma (HCC), lower expression of *Linc00261* was considered as an independent risk factor affecting postoperative recurrence-free survival time of the patients (16,26). And *Linc00261* with other three lncRNAs (TRELM3P, GBP1P1, and CDKN2B-AS1) were identified as significantly correlated with overall survival (OS) of HCC patients (25). Cox model analysis result revealed that *Linc00261* expression was also an independent prognostic factor for survival of GBC patients in this report.

Moreover, we proposed using serum *Linc00261* expression level to reflect the status of GBC. The results showed that serum *Linc00261* is significantly decreased in patients with GBC. Serum *Linc00261* level was also increased in the patients after surgery. *Linc00261* expression in patient's serum was positively associated with its

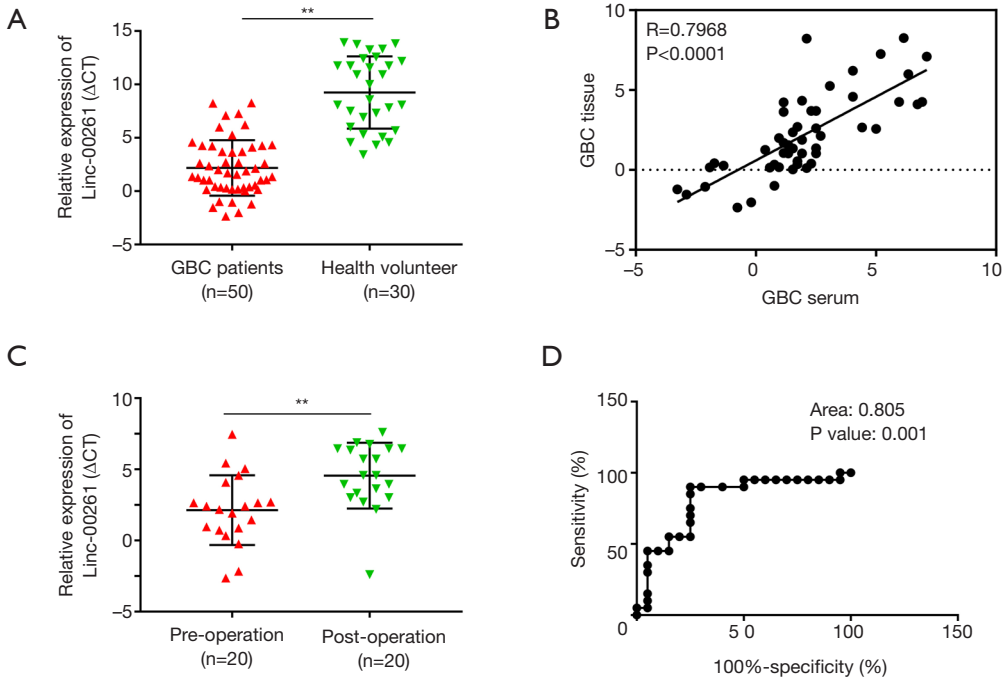

Figure 3 Serum *Linc00261* can be used as a potential diagnostic biomarker for Gallbladder carcinoma (GBC). (A) qRT-PCR results showed the relative expression of Linc00261 in the serum from GBC patients was lower than health volunteers. **, P<0.01. Error bars indicate SD. (B) The correlation of GBC tissue and *Linc00261* expression in serum are studied with Pearson correlation. (C) qRT-PCR results showed the relative expression of *Linc00261* in serum from GBC patients and the patients after surgery. The level of *Linc00261* in patients' serum was up-regulated after surgery. **, P<0.01. Error bars indicate SD. (D) ROC curve analysis was used to evaluate the diagnostic efficiency of *Linc00261* expression level in serum. Area under curve (AUC) >0.8.

expression in GBC tissues.

Conclusions

In conclusion, this research evidenced that *Linc00261* was downregulated in GBC and its expression level was associated with OS and PFS rate in GBC, which proved that *Linc00261* can be an independent potential biomarker for the prognosis of GBC. *Linc00261* in tissues or serum might be used as a potential marker for the prognosis of GBC, which may contribute to early diagnosis and treatment of GBC.

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Footnote

Data Sharing Statement: Available at http://dx.doi.org/10.21037/tcr-20-1091

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr-20-1091). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the First People's Hospital of Yunnan Province (approval number: KY-E-2017-1-20) and informed consent was taken from all the patients.

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