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Original Article

Increased expression of LncRNA GAPLINC is associated with tumor progression and predicts a poor prognosis in hepatocellular carcinoma patients

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Abstract: Long non-coding RNAs (lncRNAs) have been investigated to be correlated with the behaviors and prognosis of hepatocellular carcinoma (HCC). However, the functional role of lncRNA GAPLINC in HCC has not been elucidated yet. The present study found that the expression of GAPLINC was up-regulated in HCC tissues and cell lines in comparison with tumor adjacent tissues and normal hepatocytes, respectively. Besides, high GAPLINC level was investigated to be correlated with tumor size ($P < 0.001$), number of tumors ($P < 0.001$) and TNM stage ($P < 0.001$) of HCC. Specially, patients with high GAPLINC expression displayed significantly lower overall survival rate and progression-free survival rate. Moreover, both univariate and multivariate COX regression analyses identified high GAPLINC expression as a risk factor of HCC poor prognosis. In addition, GAPLINC was verified to promote the proliferation and metastasis of HCC cells *in vitro* assays. In conclusion, GAPLINC could promote HCC proliferation and metastasis and it may serve as a potentially prognostic marker and therapeutic target of HCC.

Keywords: Long non-coding RNA GAPLINC, hepatocellular carcinoma, prognosis, metastasis, proliferation

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequently happened cancers worldwide and the second leading cause of cancer-related death in men. Specially, China alone accounted for about 50% of the total number of HCC cases and deaths worldwide [1]. Standard treatment strategies, including surgical resection, liver transplantation and sorafenib could only provide limited survival benefits due to high postsurgical recurrence rates [2]. Besides, most HCC patients are usually diagnosed at an advanced stage for lacking of typical symptoms at early stage. The median survival after the first treatment is only 23 months in China [3]. Therefore, further investigations of the molecular pathogenesis of HCC are urgently needed for identifying new diagnostic and prognostic markers, which may shed light on HCC early detection and promote the development of novel therapeutic strategies, and thus improve the overall prognosis of HCC patients.

Recently, long non-coding RNAs (lncRNAs), with a length of 200 bp-100 kbp that lack protein-coding potential, have been focused on by many investigators for its potentially extensive functions in almost every aspect of cell biology from nuclear organization and epigenetic regulation to post-transcriptional regulation and splicing [4]. Although most of underlying mechanisms of lncRNAs functions remain obscure at present, accumulating evidence has indicated lncRNAs play essential roles in cancer development and progression through various regulatory pathways, hierarchies and networks [4, 5]. LncRNA GAPLINC is a recently discovered non-coding RNA, and has been reported to regulate cell invasiveness of colorectal cancer, and associates with the metastasis and poor prognosis of gastric cancer [6, 7]. However, to the best of our knowledge, the functional role of GAPLINC in HCC has not been illustrated to date.

The present study confirmed that lncRNA GAPLINC was overexpressed in HCC tissues and cell lines in comparison with paired adjacent non-tumorous tissues and normal hepatocytes, respectively. Besides, high GAPLINC expression

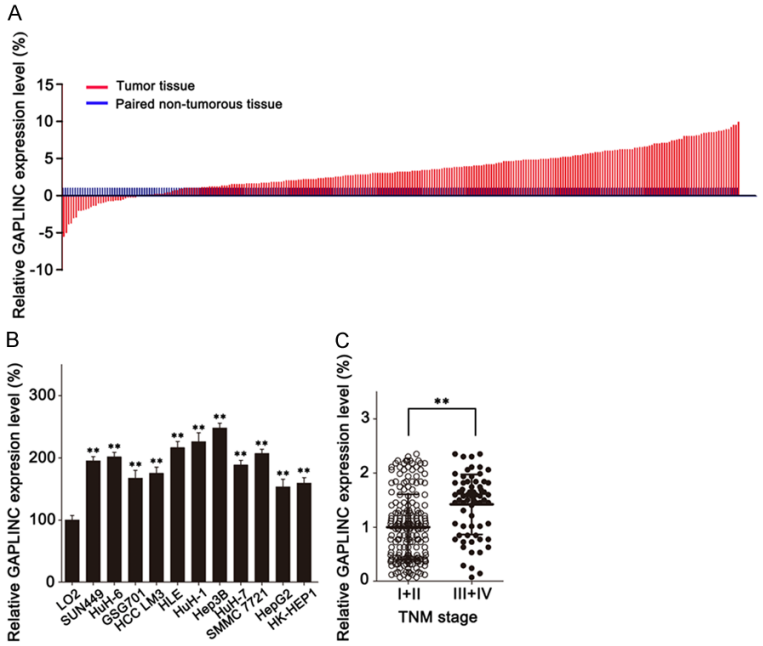

Figure 1. LncRNA GAPLINC is overexpressed in HCC. A. GAPLINC expression was analyzed in 274 HCC tissues and paired adjacent non-tumorous tissues by qRT-PCR assay. B. qRT-PCR assay was performed to detect the expression of GAPLINC in HCC cell lines and normal hepatocytes (LO2). C. The expression levels of GAPLINC were compared on qRT-PCR assay for patients with TNM stage I+II and stage III+IV. $^{**}P < 0.01$ based on the Student's *t*-test. Data are represented as M ± SD.

was implicated to be associated with HCC progression and poor prognosis. Furthermore, multivariate Cox regression analysis revealed that high GAPLINC expression was an independent risk factor of HCC prognosis. *In vitro* assays, GAPLINC was showed to promote the proliferation and metastasis of HCC cells. These results indicated that lncRNA GAPLINC may be considered as a potential prognostic marker and therapeutic target of HCC patients.

Materials and methods

Cell lines and patient samples

The SUN449, HuH-6, GSG701, HCC LM3, HLE, HuH-1, Hep3B, HuH-7, SMMC 7721, HepG2 and HK-HEP1 human HCC cell lines and the LO2human immortalized normal hepatocytes were obtained from the Cell Bank of Type Culture Collection (Chinese Academy of Sciences, Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) or RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) and incubated at 37°C in an atmosphere of 5% CO_2.

The clinical data were obtained from 274 cases of patients who underwent liver cancer radical resection surgery during May 2009 to November 2011 at People's Hospital of Wuhan University and Inner Mongolia People's Hospital. The specimens were frozen in liquid nitrogen immediately after surgery and stored at -80°C. Neither radiotherapy nor chemotherapy was carried out for patients before surgery, and no other co-occurring cancers were diagnosed. The paired adjacent non-tumorous tissues were isolated from at least 2 cm away from the tumor border and confirmed to lack tumor cells by microscopy. The diagnosis was confirmed by pathological results. Cancer staging was determined based on pathological findings according to the American Joint Committee on Cancer (AJCC).

The latest follow-up was terminated on June 30, 2016. Overall survival (OS) was defined as the interval between the date of surgery and death or when censored at the latest date. Progression-free survival (PFS) was defined as the time from the date of surgery to the date of disease relapse/progression or the date of death or when censored at the latest date. Patients died from causes other than HCC were censored. This study was approved by the Ethical Committee of the People's Hospital of Wuhan University and Inner Mongolia People's Hospital, and written informed consent was obtained from each patient.

RNA extraction and real-time quantitative PCR analysis (qRT-PCR)

Total RNA was extracted from frozen tissues or cultured cell lines using TRIzol Reagent (Takara, Dalian, China). Then, cDNA was reversely transcribed using the PrimeScript RT reagent kit (Takara, Dalian, China) with 1 microgram of total RNA. qRT-PCR was conducted to evaluate the expression level of lncRNA GAPLINC using SYBR Green PCR Master Mix (Takara, Dalian,

Table 1. Correlation between GAPLINC expression and clinicopathological characteristics of hepatocellular carcinoma

Parameters	NO. of patients		*P* value
	Low GAPLINC expression (n=145)	High GAPLINC expression (n=129)	
Age			0.869
< 55 years	84	76	
≥ 55 years	61	53	
Gender			0.374
Male	95	91	
Female	50	38	
Etiology			0.592
No virus	20	15	
Virus	125	114	
Liver cirrhosis			0.169
With	78	80	
Without	67	49	
Differentiation grade			0.081
Well + Moderate	98	74	
Poor	47	55	
Tumor size			< 0.001
< 5 cm	109	69	
≥ 5 cm	36	60	
Number of tumor			< 0.001
Solitary	121	80	
Multiple	24	49	
Macro-vascular invasion			0.018
Present	40	53	
Absent	105	76	
Micro-metastases			0.704
Absent	82	70	
Present	63	59	
Encapsulation			0.593
No	71	59	
Complete	74	70	
TNM stage			< 0.001
I+II	124	80	
III+IV	21	49	

China), which was performed on the ABI 7500 Fast Real Time PCR system (Applied Biosystems, CA, USA). Comparative quantification was determined with the $2^{-\Delta\Delta Ct}$ method. GAPDH used as an internal control. The primer sequences for GAPLINC were 5'-ACACACAGCAGCCTGGTTTC-3' (sense) and 5'-ATGGCACAATCAGGGCTCTT-3' (antisense); the primers for GAPDH were 5'-GCACCGTCAAGGCTGAGAAC-3' (sense) and 5'-GGATCTCGCTCCTGGAAGATG-3' (antisense).

Colony formation assay

The HCC cell lines were cultured in 6-well plates at a density of 100 cells per well. Cells treated with different strategies were incubated for 14 days. After being washed with PBS and fixed with methanol, cell colonies were stained with crystal violet. Number of colonies containing more than 50 cells was counted under a microscope.

Cell migration and invasion assay

The migration and invasion abilities of HCC cells were assessed by Transwell assay and Matrigel assay with boyden chambers (8 μm pore size) (BD Biosciences, USA). For Transwell assay, a volume of 200 μl HCC cells (2.5×10^5/ml), suspended in serum-free medium, was plated in the upper chamber. The lower chamber was filled with 600 μl medium supplemented with 10% FBS. Then, the cells were incubated in a humidified incubator supplemented with 5% CO_2 at 37°C for 24 hours. Subsequently, cells attached to the upper side of the chamber were removed, and cells migrated to the lower surface of the membrane were fixed and stained. For quantification, counts were obtained from five random fields at 100 × magnification. The Matrigel assay was conducted similarly to the Transwell assay except that the boyden chambers were precoated with 50 μl of 1 mg/ml Matrigel matrix (BD Biosciences, USA) to form a matrix barrier. Besides, the incubation time was 48 hours for invasion assay.

Statistical analyses

All data was presented as M ± SD. For statistical comparisons, the χ^2 test, the Fisher's exact test, the one-way analysis of variance and the two-tailed Student's *t*-test were performed where appropriate. Kaplan-Meier method and the log-rank test were conducted to evaluate the difference of OS and PFS rates. Univariate and multivariate Cox proportional hazards models were conducted to evaluate the survival data. $P < 0.05$ was considered to be statistically significant. All statistical analyses were

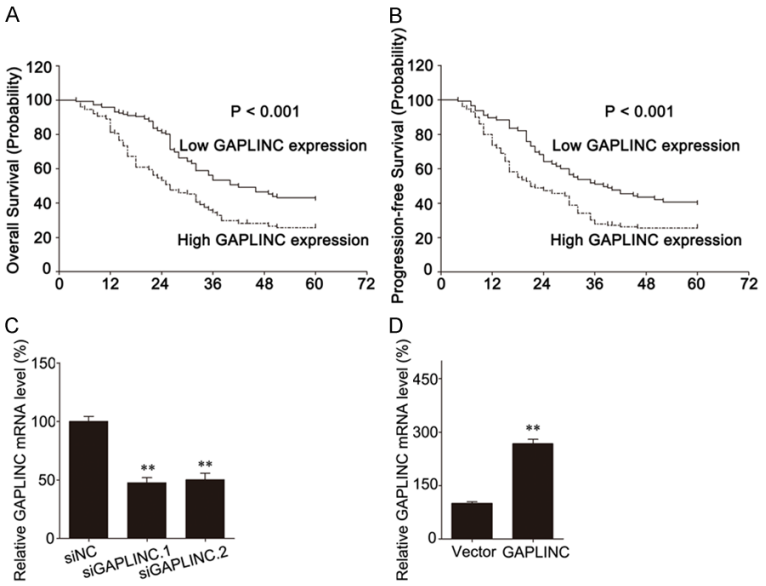

Figure 2. LncRNA GAPLINC overexpression is correlated with HCC poor OS rate and PFS rate. (A and B) The median GAPLINC expression level was used as the cutoff and all the patients were divided into 2 groups based on the cutoff, including high GAPLINC expression group and low GAPLINC expression group. Kaplan-Meier curves were used to estimate the overall survival rate (A) and progression free survival rate (B). (C and D) The expression of GAPLINC in HepG2 cells transfected with GAPLINC siRNAs (C) or cDNA (D) was investigated by qRT-PCR assay. $^{**}P < 0.01$ based on the Student's *t*-test or log-rank test. Data are represented as M ± SD.

carried out with the SPSS 19.0 software package (SPSS, Chicago, USA).

Results

LncRNA GAPLINC is overexpressed in HCC tissues and cell lines

To investigate the functional role of lncRNA GAPLINC in HCC, the expression level of GAPLINC in HCC tissues was evaluated by qRT-PCR assay. It was revealed that GAPLINC was significantly overexpressed in HCC tissues relative to paired adjacent non-tumorous tissues ((1±0.813)% *vs.* (2.035±1.143)%, $P < 0.001$). (**Figure 1A**) Further detection of GAPLINC expression in HCC cell lines showed that the expression of GAPLINC in HCC cell lines was much higher than in normal hepatocytes. (**Figure 1B**) These results suggested that lncRNA GAPLINC may play anoncogenetic role in HCC.

LncRNA GAPLINC associates with HCC progression and poor prognosis

To further verify the function of GAPLINC in HCC, the patients enrolled in this study were divided into two groups, including low GAPLINC expression group and high GAPLINC expression group, with the median GAPLINC expression level working as the cutoff. Correlation analysis between GAPLINC expression and HCC clinicopathological characteristics discovered that high GAPLINC expression was correlated with tumor size ($P < 0.001$), number of tumors ($P < 0.001$) and TNM stage ($P < 0.001$) of HCC (**Table 1**). Moreover, patients with advanced TNM stage displayed a much higher GAPLINC expression level ((1±0.61)% *vs.* (1.42±0.55)%, $P < 0.001$) (**Figure 1C**).

Specially, survival analysis showed that patients with low GAPLINC expression had a higher overall survival (OS) rate and a longer progression-free survival (PFS) period than the patients with high GAPLINC expression (**Figure 2A** and **2B**). Additionally, univariate COX regression analysis identified number of tumor, macro-vascular invasion, TNM stage and high GAPLINC expression as risk factors of HCC OS and PFS (**Table 2**). By further analyzing these factors with multivariate analysis, number of tumor, macro-vascular invasion and high GAPLINC expression were discovered to be independent risk factors of HCC OS and PFS (**Table 2**). Collectively, these results suggest that GAPLINC may serve as a potential recurrence indicator and prognostic marker in HCC.

LncRNA GAPLINC could promote the proliferation and metastasis of HCC cells

To extend our investigation, the functional role of GAPLINC in HCC was analyzed *in vitro* assay. The expression of GAPLINC was knocked-down with siRNA in Hep3B cells and overexpressed in HepG2 cells (**Figure 2C** and **2D**). As a result, GAPLINC interference dramatically suppressed the proliferation of Hep3B cells on MTT assay and colony formation assay (**Figure 3A** and **3B**). In contrast, GAPLINC overexpression obviously led to improved proliferation abilities of HepG3

Table 2. Univariate analysis and multivariate analysis of clinicopathologic features for OS and PFS of hepatocellular carcinoma patients

Parameters	OS			PFS		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Univariate analysis						
Age	1.287	0.959-1.725	0.092	1.250	0.934-1.672	0.133
≥ 55 years vs. < 55 years						
Gender	1.066	0.782-1.453	0.688	1.083	.0798-1.471	0.608
Male vs. Female						
HBs antigen	1.264	0.802-1.992	0.314	1.238	0.786-1.950	0.357
Negative vs. Positive						
Liver cirrhosis	1.068	0.796-1.435	0.660	1.091	0.816-1.460	0.556
Without vs. With						
Differentiation	1.037	0.767-1.401	0.814	0.991	0.734-1.337	0.952
Poor vs. Well + Moderate						
Tumor size	1.178	0.868-1.599	0.293	1.141	0.842-1.546	0.394
≥ 5 cm vs. < 5 cm						
Number of tumor	1.888	1.376-2.591	< 0.001	1.806	1.318-2.473	< 0.001
Multiple vs. Solitary						
Macro-vascular invasion	1.599	1.188-2.151	0.002	1.645	1.225-2.208	0.001
Present vs. Absent						
Micro-metastases	1.291	0.963-1.730	0.088	1.298	0.971-1.735	0.078
Absent vs. Present						
Encapsulation	1.213	0.905-1.627	0.197	1.152	0.862-1.540	0.338
No vs. Complete						
TNM stage	1.541	1.111-2.137	0.010	1.459	1.053-2.020	0.023
(III+IV) vs. (I+II)						
GAPLINC	1.837	1.370-2.463	< 0.001	1.698	1.271-2.270	< 0.001
High vs. Low						
Multivariate analysis						
Number of tumor	1.925	1.208-3.067	0.006	1.899	1.195-3.017	0.007
Multiple vs. Solitary						
Macro-vascular invasion	1.518	1.125-2.049	0.006	1.560	1.159-2.100	0.003
Present vs. Absent						
TNM stage	0.845	0.517-1.382	0.502	0.814	0.499-1.326	0.408
(III+IV) vs. (I+II)						
GAPLINC	1.660	1.222-2.255	0.001	1.527	1.126-2.069	0.006
High vs. Low						

Abbreviation: OS, overall survival; PFS, progression free survival; HR, hazard ratio; CI, confidence interval.

cells on MTT assay and colony formation assay (**Figure 3C** and **3D**). Besides, decreased GAPLINC expression suppressed the migration ability of Hep3B cells on Transwell assay (**Figure 3E**, up). Consistently, the invasion ability of Hep3B cells was also reduced accompanied with GAPLINC depletion. (**Figure 3E**, bottom) Consistently, GAPLINC ectopic expression significantly increased the migration and invasion of HepG2 cells (**Figure 3F**). In conclusion, GAPLINC could promote the migration and invasion of HCC cells.

Discussion

HCC patients are usually asymptomatic at early stage and have no access to surgical resection, the only curative treatment strategy at present, when diagnosed. Besides, adjuvant therapeutic strategies are relatively rare for patients

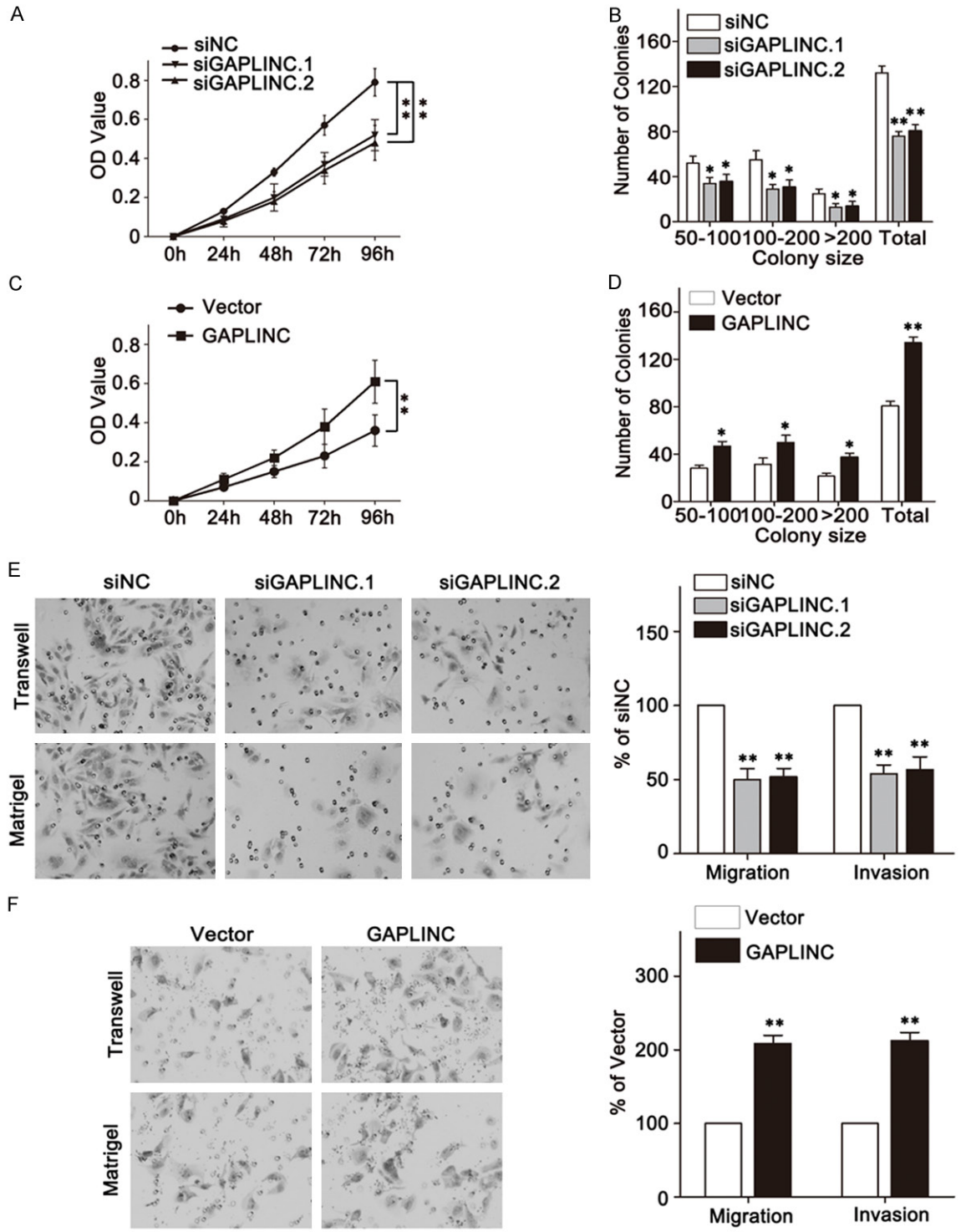

Figure 3. GAPLINC promotes proliferation and metastasis of HCC cells *in vitro* assay. Hep3B cells with GAPLINC silencing and overexpression were subjected to MTT (A and C), colony formation (B and D), Transwell (E and F, top) and Matrigel (E and F, bottom). (A) Cell proliferation of the siNC group, siGAPLINC.1 group and siGAPLINC.2 group was examined by MTT assay. (B) Colony formation assay was carried out to measure cell proliferation of the siNC group, siGAPLINC.1 group and siGAPLINC.2 group. Colonies containing more than 50 cells were counted and plotted. (C) Cell proliferation of the Vector group and GAPLINC group was examined by MTT assay. (D) Colony formation assay was carried out to measure cell proliferation of the Vector group and GAPLINC group. Colonies containing more than 50 cells were counted and plotted. (E) Representative images of the siNC group, siGAPLINC.1 group and siGAPLINC.2 group on Transwell assay and Matrigel assay. Corresponding quantification of migrated cells through

the membrane and invaded cells through matrigel was shown relative to control. (F) Representative images of the Vector group and GAPLINC group on Transwell assay and Matrigel assay. Corresponding quantification of migrated cells through the membrane and invaded cells through matrigel was shown relative to control. $^{**}P < 0.01$ and $^{*}P < 0.05$ based on the Student's *t*-test. Data are represented as M ± SD.

with advanced stage. The average 5-year survival rate is less than 12% [8], and only 3% in advanced disease [9]. Therefore, early diagnosis is crucial for curative treatment. The essential roles of protein-coding genes in HCC development and progression have been well illuminated, but these genes only account for 1%-2% of transcribed RNAs [10-12]. Non-coding RNAs, including lncRNAs, are abundant in human tissues and are investigated to be crucial regulators of cellular transcription and translation [10, 13].

Although only a small number of functional lncRNAs have been well characterized till now, they have been shown to control every level of the multi-level regulated gene expression pathways, including chromatin remodeling, RNA maturation (splicing, editing), transport and protein synthesis [14]. Recent studies implicated that lncRNAs constitute an important component of tumorbiology [15, 16]. In addition, human plasma provides a convenient way for the early detection of cancer. However, the existing markers for HCC diagnosis are lack of enough specificity and sensitivity. LncRNAs could be measured in human plasma, thus it may be a relatively non-invasive and potentially effective tool for early diagnosis and prognosis evaluation. Furthermore, aberrant expression of lncRNAs in cancer marks the spectrum of disease progression and may serve as an independent predictor for patient outcomes [17, 18].

Numerous lncRNAs have been identified to be involved in hepatocarcinogenesis, and mediate HCC metastasis and proliferation [19-21]. Some lncRNAs have been reported to be correlated with HCC recurrence and prognosis [22-31]. Therefore, lncRNAs are supposed to be promising therapeutic targets of cancer [32, 33]. LncRNA GAPLINC is a 924-bp long lncRNA. Hu et al. [6] reported that GAPLINC could regulate gastric cancer invasiveness via mediating CD44 as a molecular decoy for miR211-3p, and associated with gastric cancer prognosis. Peng et al. [7] showed that GAPLINC could promote colorectal cancer invasion by targeting snail family zincfinger 2 (SNAI2) through binding with PTB-associated splicing factor (PSF) and non-POU-domain-containing octamer binding (NONO) protein, and concluded that GAPLINC may serve as a promising target for colorectal cancer diagnosis and therapy. However, the role of lncRNA GAPLINC in HCC has not been elucidated.

The present study found that GAPLINC expression was up-regulated in HCC tissues and cell lines in comparison with tumor adjacent tissues and normal hepatocytes, which suggested that GAPLINC may also play an oncogenic role in HCC. Indeed, correlation analysis between GAPLINC expression and clinical features showed that high GAPLINC level was correlated with tumor size ($P < 0.001$), number of tumors ($P < 0.001$) and TNM stage ($P < 0.001$) of HCC. Kaplan-Meier method and the log-rank test further detected that patients with high GAPLINC expression displayed significantly shorter OS rate and PFS rate. Moreover, high GAPLINC expression was recognized as risk factors of HCC poor prognosis on multivariate COX regression analysis. *In vitro* assays, GAPLINC was confirmed to promote the proliferation and metastasis of HCC cells. Together, these results indicated that lncRNA GAPLINC could be considered as a predictor of HCC recurrence and prognosis, and a potential therapeutic target of HCC patients. However, there are also some limitations of this study. The detailed mechanisms of GAPLINC accelerating HCC progression were not further explored, and its therapeutic value needs more clinical evidence.

In conclusion, our results confirmed that lncRNA GAPLINC could promote HCC proliferation and metastasis and it may serve as a potentially marker for HCC recurrence and prognosis. In addition, it may be a promising therapeutic target for individual treatment of HCC patients.

Disclosure of conflict of interest

None.

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