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Original Article

Lin28B is a novel prognostic marker in gastric adenocarcinoma

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Abstract: Lin28B, a homologue of Lin28, represses biogenesis of let-7 microRNAs with a role in tumorigenesis and is considered a potential therapeutic target for various human cancers. The aim of the study was to identify the clinical significance of Lin28B in gastric adenocarcinoma (GAC). We examined the expression of Lin28B in 97 human gastric cancer samples with 32 samples of non-dysplastic tissues by immunohistochemistry. In the 97 GAC cases, 42 were with high Lin28B expression. The expression levels of Lin28B proteins in GAC were higher than in corresponding adjacent normal tissues (P=0.001). Significant correlations were noted between Lin28B expression and lymph node status (P=0.005), TNM stage ($P < 0.001$), tumor invasion (P=0.036), and differentiation (P=0.001) of GAC patients. The Kaplan-Meier estimates showed a negative correlation of overall 5-year survival rate with Lin28B expression where higher expression resulted in poorer prognosis in GAC. In univariate analysis, lymph node metastasis, TNM stage, serosal invasion, Lin28B expression as well as differentiation grade could predict the prognosis of GAC patients (P=0.002, $P < 0.001$, P=0.003, $P < 0.001$, P=0.001, respectively). Multivariate analysis revealed that the expression level of Lin28B ($P < 0.001$), TNM stage ($P < 0.001$) as well as differentiation grade ($P < 0.001$) were the three potential independent prognostic factors in our study [Hazard ratio (HR)=2.108 and P=0.017, HR=1.994 and P=0.018, HR=1.939 and P=0.046, respectively]. Our findings point to the prognostic role of Lin28B in GAC, and indicate Lin28B as a potential therapeutic target of GAC patients.

Keywords: Lin28B expression, gastric adenocarcinoma, overall 5-year survival rate, multivariate Cox proportional hazards model analysis, prognosis

Introduction

Currently, gastric cancer remains the main common cancers worldwide and one of the leading causes for cancer-related death in China [1, 2], with an estimated 934,000 new cases per year in 2002 [3]. In China, the overall 5-year survival rate of patients with gastric cancer is lower than 40%. Identifying biological markers to predict prognostic risk in gastric carcinoma is still lacking. To improve the clinical outcome of gastric carcinoma patients, it is necessary to target novel biomarker genes, which appear to be involved in carcinoma development, as described previously [4, 5]. Because adenocarcinoma accounts for approximately 90% of gastric cancer [6], patients with gastric adenocarcinoma (GAC) were selected in this study.

Lin28B protein is a homologue of Lin28 [7], a RNA-binding protein originally identified as a key regulator of developmental timing in Caenorhabditis elegans [8]. Similar to Lin28, Lin28B contains a cold shock domain and retroviral-type CCHC zinc fingers that confer RNA-

binding ability [8, 9] and inhibit biogenesis of tumor-suppressive microRNAs of the let-7 family [10-12]. Evidences demostrate that Lin28B is implicated in multiple developmental processes, largely as a consequence of its ability to repress let-7 biogenesis [8, 9, 13-17]. Induction of expression with exogenous Lin28B promotes cancer cell proliferation [7]. Lin28B is also induced by Myc and plays an important role in Myc-dependent cellular proliferation [18], which has been demonstrated to enhance cell migration, invasion, and metastasis [13-15].

Though Lin28 and Lin28B share similar structures, they show different functions [19, 20]. For example, Lin28, in use with factors of Oct4, Nanog, Sox2, Klf4 and c-Myc, has been found to be able to reprogram sarcoma cells into mature connective cells with concomitant abrogation of tumori-genicity [21]. In contrast, Lin28B showed important functions during cell transformation from inflammation to malignancy [22]. Recent studies show that Lin28 and Lin28B are upregulated in human tumors and function as oncogenes promoting transformation and tumor progression, where Lin28B overexpression in human cancers seems to occur more frequently [14], pointing to Lin28B as perhaps the more relevant homologue in tumorigenesis. Until now, Lin28 and Lin28B expression has been reported distinctively or exclusively in several tumours including colorectal, gonad, esophagus cancer, oral squamous cell carcinoma (OSCC), hepatocellular and breast tumours [19, 23-26].

Lin28B overexpression was observed in breast cancer [27], lung cancer [28], ovarian cancer [29], hepatocellular cancer [30], esophageal cancer [25], colorectal cancer [31], melanoma [30] and OSCC [26]. High expression of Lin28B is associated with poor clinical outcome and patient survival in HCC, colon, esophageal cancer, ovarian cancer and OSCC [25, 26, 30, 32, 33]. These findings indicate Lin28B a potential antibody-based theraputic target. Therefore, understanding the existence and expression status of Lin28B will have profound implications in the prognosis and treatment of cancer.

Though a recent study shows that positive expression of Lin28 is correlated with poor survival in gastric carcinoma [34], considering the different function of Lin28 and Lin28B, it is necessary to investigate the role of Lin28B in GAC, which as so far has not been investigated. We hypothesize that Lin28B may also play a role in the oncogenesis of GAC as Lin28, and the expression of Lin28B served as a prognostic factor. To elucidate the prognostic value of Lin28B in GAC, we analyzed the expression of Lin28B with immunohistochemistry and assessed their associations with various clinicopathological parameters and overall 5-year survival rate (OS) outcomes of patients in the study. This study implicates a role for Lin28B expression in GAC and finds its overexpression correlating with reduced OS.

Materials and methods

Patients and tissue specimens

For this retrospective study, archival formalin-fixed paraffin-embedded (FFPE) specimens from 97 GAC patients admitted to West China Hospital, Sichuan University from 2001 to 2003 were attained. As shown in **Table 1**, Cases (74 male, 23 female) with available follow-up and clinical data were included for immunohistochemical studies. Patients receiving chemotherapy or radiation therapy before surgery were excluded. Of the 97 GAC patients, 32 cases were combined with normal counterpart non-dysplastic tissue. Among the 97 GAC cases, 13 cases located in corpus gastricum, 42 cases located in Fundus gastricus and 42 cases located in Sinus ventriculi. Information on sex, age, stage of disease, and histopathological parameters were retrieved from the medical records. The tumors were confirmed as malignant after surgery by pathologists from West China Hospital. The study was approved by the Ethics Committees of West China Hospital, Sichuan University. Informed consent was obtained from all participating patients.

Immunohistochemistry

For immunohistochemistry, 4 μm-thick sections cut from the FFPE tissue blocks were deparaffinized and rehydrated using xylene and a graded series of ethanol (absolute, 95%, 80%, 50%), followed by two 5 min washes in phosphate buffered saline with Tween-20 (PBST). Antigen retrieval was performed in 10 mmol sodium citrate buffer (pH 6.0), which was microwaved at 90-100°C for 20 min and

Table 1. Clinical characteristic of GAC patients

Characteristics	*N cases (%)*
n	97
Age	
≤50	24 (24.7)
>50	73 (75.3)
Sex	
Male	74 (76.3)
Female	23 (23.7)
Tumor location	
Corpus gastricum	13 (13.4)
Fundus gastricus	42 (43.3)
Sinus ventriculi	42 (43.3)
Lymphnode metastasis	
negative	36 (37.1)
positive	61 (62.9)
TNM Stage	
I + II	57 (58.8)
III + IV	40 (41.2)
Therapy	
Surgery	47 (48.4)
Surgery + others	50 (51.6)
Serosal invasion	
negative	60 (61.9)
positive	37 (38.1)
Differentiation	
Poor	56 (64.4)
Well/Moderate	41 (35.6)

washed in PBST for 2×5 min. The sections were then incubated for 30 min in 3% (v/v) hydrogen peroxide in methanol to block endogenous peroxidase activity, washed in PBST for 3×5 min, blocked at room temperature for 30 min by using 2% normal goat serum, 2% bovine serum albumin (BSA), and 0.1% triton-X in phosphate buffered saline (PBS), and incubated in a humidified chamber overnight at 4°C with the primary antibodies anti-Lin28B (1:50 dilution; Proteintech, US). The sections were then washed in PBST (3×5 min) and incubated at room temperature for 1 h with the secondary antibodies (goat-anti-rabbit, SP-9002, Zhongshan Golden Bridge Inc, China). After a wash with PBST (3×5 min), the sections were incubated with ready-to-use streptavidin peroxidase at room temperature for 30 min and well rinsed with distilled water. Colors were developed with a DAB kit. The sections were then counterstained with hematoxylin, dehydrated, and mounted. Negative controls were prepared by substituting PBS for the primary antibodies.

Immunoreactivity scoring

For evaluation of Lin28B protein expression, a reproducible semiquantitative method that takes both staining intensity and area scores into account was adopted.

The staining intensity was scored as follows: 1 (weak staining = light yellow), 2 (moderate staining = yellow brown) and 3 (strong staining = brown) [35]. The staining area was the percentage of positive tumor cells, which was scored as follows: 0 (no tumor cell stained), 1 (1%-30% positive tumor cells), 2 (31%-60% positive tumor cells), 3 (61%-90% positive tumor cells), 4 (91%-100% positive tumor cells) [36]. The final immunoreactivity score (IS) for each specimen was obtained by adding the staining intensity and area scores. Using the median value 4.5 of IS as a cut-off value [37], Lin28B expression was divided into Lin28B-high (IS ≥ 5) and Lin28B-low (IS < 5) groups. Each section was assessed by two histopathologists independently, who were blinded to patient information. Positive samples were defined as those showing brown signals in the cytoplasm and/or nuclei of cancer cells.

Statistical analysis

As the study endpoint, overall survival time is the time from surgery until the date of death or last follow-up (March, 2012). To assess correlations of demographic and clinical variables with Lin28B expression, *chi*-square test and Fisher's exact test were used for categorical variables and two-sample *t*-test for continuous variables. *Chi*-square test was also performed to compare the expression of Lin28B between tumor tissues and adjacent benign tissues. Hazard ratios (HR) and their 95% confidence intervals (CI) were estimated using multivariate Cox's proportional hazards model adjusted for age, sex, clinical stage, histologic grade, and therapeutic modality. Overall patient survival was estimated with Kaplan-Meier analysis with a log-rank score for determining statistical significance. All *P* values were two-sided. A $P \leq 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS16.0 for Windows (SPSS Inc., Chicago, IL).

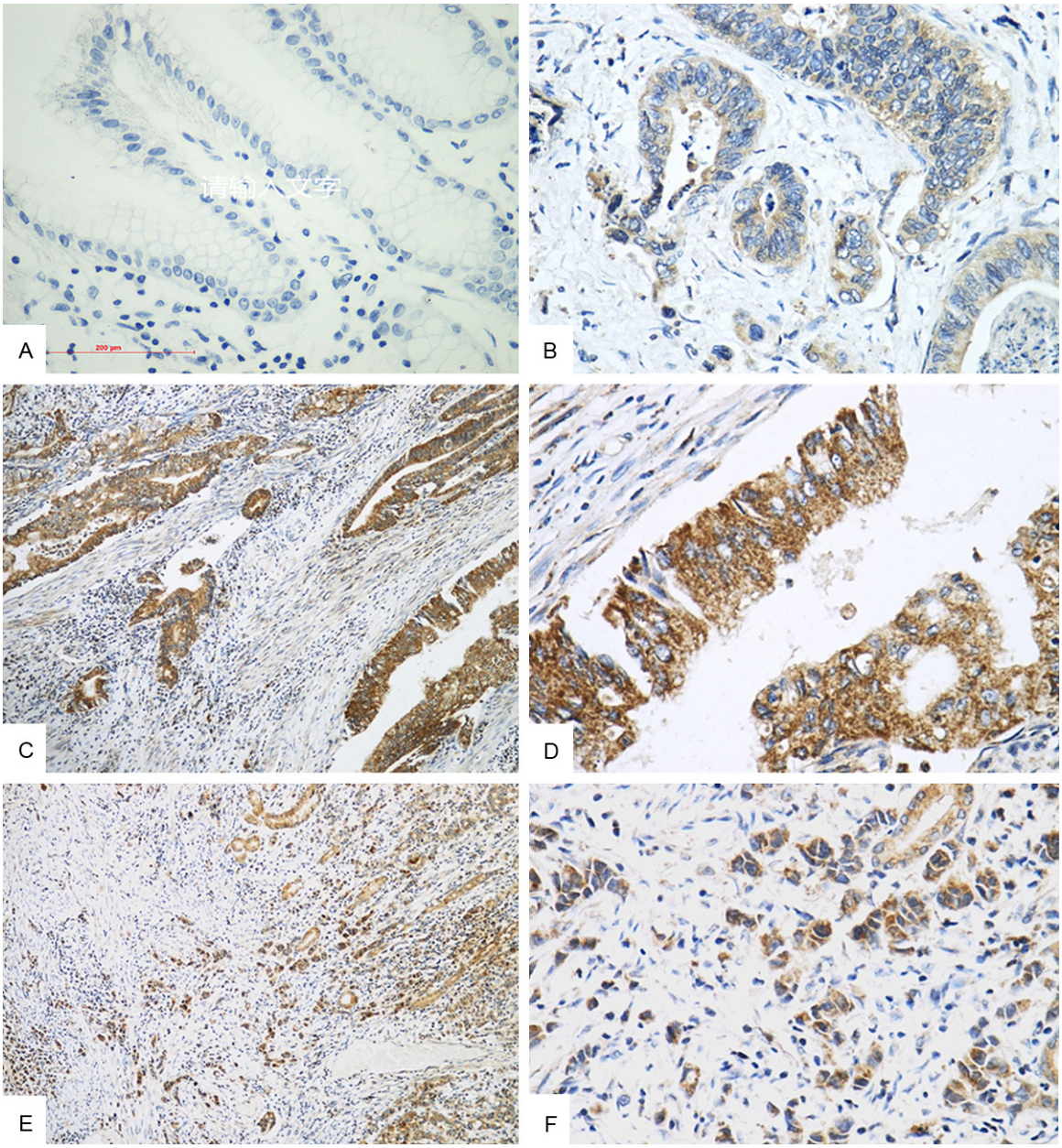

Figure 1. Expression of Lin28B in GAC patients. A. Low expression of Lin28B in normal counterpart non-dysplastic tissue (x 400). B. Low expression of Lin28B in GAC tissues (x400). C and D. High expression of Lin28B in GAC tissue (x100, x400). Lin28B immunoreactivity was predominantly localized in the cytoplasm of the tumor cell of GAC. E and F. Nuclei staining was also evident in some cancer cells (x100, x400).

Results

Increased Lin28B expression in GAC

Expression levels of Lin28B in GAC were determined by immunohistochemistry (**Figure 1**). We compared 97 samples of GAC tissues with 32 samples of non-dysplastic tissues, which had enough normal counterparts adjacent to cancer tissues from the 97 cases, GAC tissues. Lin28B immunoreactivity was predominantly localized in the cytoplasm of the tumor cell of GAC, though nuclei staining was also evident in some cancer cells as illustrated in **Figure 1**. Most adjacent non-neoplastic cells were not stained, although weak staining could be found in some normal counterpart non-dysplastic tissue (**Figure 1A**). High and moderate staining

Table 2. Comparison of Lin28B expression between gastric carcinoma (GAC) and adjacent normal tissue (Control)

Groups	Lin28B expression	*P* Value
	Mean staining score	
GAC	4.90	0.001
Control	1.45	

could be found in the tumor cells. High expression of Lin28B was found in 43.3% (42/97) of GAC tissues. In contrast, low expression of Lin28B was found in most of nondysplastic tissues (28/32), and strong expression of Lin28B was only detected in 4 samples (12.5%) of them. The expression levels of Lin28B proteins in GAC were higher than in corresponding adjacent normal tissues (*P*=0.001, **Table 2**).

Association of expression levels of Lin28B with the clinicopathological parameters of GAC

In order to know the clinical role of Lin28B in GAC, we further assessed the correlations between Lin28B expression level and clinicopathological parameters, including sex, age, location, invasion, tumor differentiation, lymph nodes status, and TNM stage and therapeutic modality of GAC patients (**Table 2**).

Among 97 GAC cases examined, the presence of high LIN28B was detected in carcinoma cells of 42 cases. Statistical analysis showed that Lin28B expression was not significantly correlated with such clinical parameters as age, sex, tumor location, and therapeutic modality of GAC patients *(P*>0.05). Interestingly, significant correlations were noted between Lin28B expression and lymph node status (*P*=0.005), TNM stage (*P* < 0.001), tumor invasion (*P*=0.036), and differentiation (*P*=0.001) of GAC patients.

Expression level of Lin28B for prognosis in patients with GAC

Expression levels of LIN28B, as described between Lin28B-low and Lin28B-high expression groups, were evaluated for correlations with overall 5-year survival (OS) by using Kaplan-Meier analysis. The results showed that patient OS was negatively correlated with the Lin28B expression level, where higher expression of Lin28B resulted in poorer OS (**Figure 2A**). Of the 97 GAC patients, the MST was 24 months and the 5-YSR was 31% for the 42 patients with high Lin28B expression, significantly lower compared with the remaining 55 patients with low Lin28B expression (MST>136 months, 5-YSR=65.5%; *P* < 0.001, **Figure 2A**).

Further, in a univariate Cox regression analysis, lymph node metastasis, TNM stage, serosal invasion, Lin28B expression as well as differentiation grade could predict the prognosis of GAC patients (*P*=0.002, *P* < 0.001, *P*=0.003, *P* < 0.001, *P*=0.001, respectively; **Table 3**). Use of the Cox regression model in multivariate analysis revealed that the expression level of Lin28B (*P* < 0.001) (**Figure 2A**), TNM stage (*P* < 0.001) (**Figure 2B**) as well as differentiation grade (*P* < 0.001) (**Figure 2C**) were the three potential independent prognostic factors in our study [Hazard ratio (HR)=2.108 and *P*=0.017, HR=1.994 and *P*=0.018, HR=1.939 and *P*=0.046, respectively; **Table 4**].

Discussion

This effect of Lin28 and Lin28B, which seems similar to an oncogene, is largely due to its ability to inhibit the let-7 microRNA family [10-12]. Despite their high degree of homology, Lin28 and Lin28B function through distinct mechanisms to block let-7 processing [19]. Lin28 recruits a TUTase (Zcchc11/TUT4) to let-7 precursors to block processing by Dicer in the cell cytoplasm. Unlike Lin28, Lin28B represses let-7 processing through a Zcchc11-independent mechanism. Lin28B functions in the nucleus by sequestering primary let-7 transcripts and inhibiting their processing by The Microprocessor. It is concluded that this distinction derives from the differential subcellular localization of these two proteins: Lin28 localizes primarily to the cytoplasm, whereas Lin28B contains functional nuclear localization signals and specifically localizes to nucleoli. In the current study, the result was inconsistent, whereas Lin28B immunoreactivity was predominantly localized in the cytoplasm of the tumor cell of GAC, though nuclei staining was also evident in some cancer cells. This may due to the reason that though Lin28B-mediated repression of let-7 expression is Zcchc11 (TUT4) independent in multiple different cell types, it remains possible that in certain contexts or cell types including GAC, Lin28B may localize to the cytoplasm and utilize Zcchc11/TUT4 to repress let-7 biogenesis. For example, uridylated pre-let-7was previously detected in Huh7 cells, and Lin28B is reportedly localized to the cytoplasm in Huh7 cells [7, 11]. Another previous study

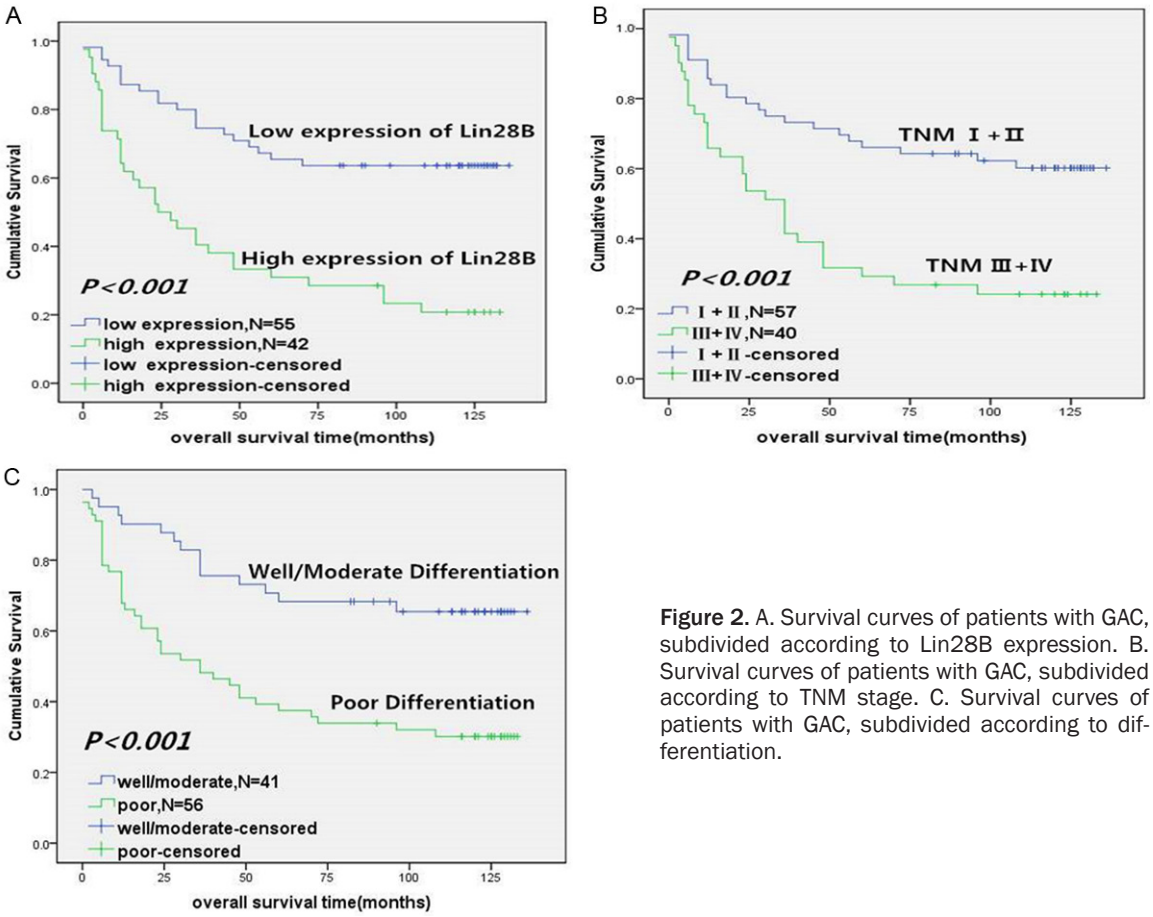

Figure 2. A. Survival curves of patients with GAC, subdivided according to Lin28B expression. B. Survival curves of patients with GAC, subdivided according to TNM stage. C. Survival curves of patients with GAC, subdivided according to differentiation.

that distribution of LIN28B is cell cycle-regulated, LIN28B was predominantly present in the cytoplasm of G1phase cells, nuclear accumulation of LIN28B was observed in S phase and G2 phase cells [7], may also explain this inconformity. The mechanism for this different distribution of Lin28B in cancer cells should be studied more in details in future.

A previous study has demonstrated that Lin28B, but not Lin28, is associated with human puberty and menopause [38]. It seems that Lin28B was more often expressed in the digestive system neoplasm [23, 32], while Lin28 was expressed in germ cell development and gonadal tumours [36, 39-41]. A recent study showed that Lin28 was lower expressed in gastric carcinoma tissues than corresponding normal tissues [34]. In our study, the expression levels of Lin28B proteins in GAC were higher than in corresponding adjacent normal tissues (P=0.001). Most adjacent non-neoplastic cells were not stained, although weak staining could be found in some normal counterpart non-dysplastic tissue. In contrast, high and moderate staining of Lin28B could be found in the tumor cells of GAC. In consideration of the above differences between Lin28 and Lin28B, which may indicate their different clinical role in GAC patients, we conducted current work aiming to determine the clinical significance of Lin28B expression in GAC. To our knowledge, there are no reports concerning the clinical roles of expression level of Lin28B in GAC.

Early studies only used positive tumor cell rate as an index to evaluate the sensitivity of Lin28B staining in cancer cells [25, 32]. In consideration of the varied staining intensity of Lin28B among specimens or even in a same carcinoma section, and the diverse effect of different expression levels on patient outcome, we used semi-quantitative scoring including both staining area and intensity to assess the immunostaining as previously described [26]. This method is more efficient to assess protein expression levels and for association analysis [42, 43].

Recent study has shown in ovarian cancer, the level of Lin28B expression is correlated with

Table 3. Correlation between Lin28B expression and various clinicopathological features of patients with GAC

	Lin28B expression		*P* Value
	low	high	
n	55	42	
Age			
≤50	12 (12.4)	12 (12.4)	
>50	43 (44.3)	30 (30.9)	0.445
Sex			
Male	42 (43.3)	32 (33.0)	
Female	13 (13.4)	10 (10.3)	0.984
Tumor location			
Corpus gastricum	6 (6.2)	7 (7.2)	
Fundus gastricus	23 (23.7)	19 (19.6)	
Sinus ventriculi	26 (26.8)	16 (16.5)	0.572
Lymph node metastasis			
negative	27 (27.8)	9 (9.3)	
positive	28 (28.9)	33 (34.0)	**0.005**
TNM Stage			
I + II	42 (43.3)	15 (15.5)	
III + IV	13 (13.4)	27 (27.8)	**< 0.001**
Therapy			
Surgery	27 (27.8)	20 (20.6)	
Surgery + others	28 (28.9)	22 (22.7)	0.886
Serosal invasion			
negative	39 (40.2)	21 (21.6)	
positive	16 (16.5)	21 (21.6)	**0.036**
Differentiation			
Poor	24 (24.7)	32 (33.0)	
Well/Moderate	31 (32.0)	10 (10.3)	**0.001**

P value < 0.05 was indicated in bold.

tumor stage and lymph node metastasis [33]. The results about the association between Lin28B and pathological features in our study showed that Lin28B expression predicted TNM stage, serosal invasion, lymph nodes metastasis, and tumor differentiation of GAC patients. A recent study demonstrated that constitutive expression of Lin28B expression in colon cancer cells confers metastatic ability by showing that mice xenografted with Lin28B expressing colon cancer cells developed much more metastasis in the liver, lung, and mesenterium compared with mice of the empty vector control group [25, 32]. Thus, Lin28B expression may have an important role in cancer metastasis. Meanwhile, the TNM stage system and lymph node status are two prognostic indexes widely used in clinic for GAC [44, 45], and poorly differentiated cancer cells of gastric cancer often show stronger aggressive and metastatic ability [46]. The relevance between Lin28B expression and the above clinicopathological characteristics indicates that Lin28B could be used as a potential factor to predict tumor progression and poor prognosis in GAC.

Lin28B functioning as an oncogene has been demonstrated in a few previous studies, For example, Lin28B promotes epithelial-mesenchymal transition and the Lin28B knockdown inhibit tumorigenecity and growth [7, 18, 19, 21, 23]. Indeed, several recent reports demonstrated that Lin28 expression correlates with survival of patients with malignant diseases [7]. In ovarian cancer, patients with high Lin28B expression had shorter progression-free and overall survival times than those with low Lin28B expression [33]. In another recent report, high Lin28B staining intensity in stage I/II colon cancers correlated with reduced survival and increased probability of tumour recurrence [32]. And high expression of Lin28B was with poor prognosis of patients with esophageal cancers [25] and Oral squamous cell carcinoma [26].

The results of the current work indicate a strong correlation between the expression of Lin28B and the OS in GAC patients. Kaplan-Meier analysis showed that patient survival time is negatively correlated with the Lin28B expression level, where higher Lin28B expression points to shorter survival time in GAC patient. Cox regression results suggested Lin28B, TNM stage, and differentiation were independent prognosis predictors for GAC patient. Our result of the correlation between high expression of Lin28B and poor prognosis of patients with GAC is compatible with the above studies. Thus, Lin28B expression may be clinically relevant prognostic marker in various malignancies including GAC.

In conclusion, our findings points to Lin28B as a potential predictive factor for the prognosis of GAC and indicate Lin28B as a potential therapeutic target. Further studies will be performed to determine initiated Lin28B activation in GAC, and the related different functional pathways and molecular mechanisms from Lin28.

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Table 4. Results of univariate and multivariate Cox's models for overall survival of GAC

		Univariate analysis		multivariate analysis	
	N	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age					
≤50	24	0.749 (0.416-1.346)	0.333		
>50	73				
Sex					
Male	74	0.777 (0.400-1.511)	0.457		
Female	23				
Tumor location					
Corpus gastricum	13	0.949 (0.648-1.388)	0.786		
Fundus gastricus	42				
Sinus ventriculi	42				
Lymph node metastasis					
Negative	36	2.762 (1.448-5.267)	0.002	1.811 (0.846-3.875)	0.126
Positive	61				
Stage					
I + II	57	2.762 (1.598-4.774)	<0.001	1.994 (1.124-3.535)	0.018
III + IV	40				
Therapy					
Surgery	47	1.594 (0.918-2.769)	0.098		
Surgery + others	50				
Serosal invasion					
negative	60	2.266 (1.318-3.896)	0.003	1.877 (0.940-3.745)	0.074
positive	37				
Lin28B					
Low	55	4.816 (2.465-9.410)	<0.001	2.108 (1.142-3.889)	0.017
High	42				
Differentiation					
Poor	56	2.853 (1.545-5.268)	0.001	1.939 (1.011-3.719)	0.046
Well/Moderate	41				

Abbreviations: HR = hazard ratio; 95% CI = 95% confidence interval.

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Disclosure of conflict of interest

None.

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