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Clinicopathological Characteristics of Patients with Gastric Cancer according to the Expression of LIN28A

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Background/Aims: Although LIN28A is known to potentially play a role in the oncogenesis of various cancers, whether LIN28A expression is a predictor of poor prognosis in patients with gastric cancer has not been fully explored. We sought to evaluate clinicopathological characteristics according to the expression of LIN28A in numerous gastric cancer tissue samples. **Methods:** LIN28A expression was evaluated by immunohistochemical (IHC) analysis of a tissue microarray comprising 288 gastric cancer tissues and 288 adjacent normal tissues. Clinicopathological characteristics, including overall survival, were compared according to LIN28A expression. **Results:** The IHC staining score was lower for the cancer tissues than the normal tissues (p<0.001). However, no significant differences were observed in the clinicopathological characteristics between the low and high LIN28A expression groups. In addition, the 5-year overall survival rate did not differ between the two groups: 75.3% (95% confidence interval [CI], 69.3% to 81.7%) versus 71.6% (95% CI, 63.3% to 80.9%) for low versus high expression, respectively. **Conclusions:** The expression of LIN28A did not appear to play a distinct role in predicting the clinicopathological characteristics of patients with gastric cancer. In addition, LIN28A expression was not an independently associated factor for overall survival in patients with gastric cancer. **(Gut Liver 2016;10:714-718)**

Key Words: LIN28A; Stomach neoplasms; Survival

INTRODUCTION

While the survival rates of patients with early gastric cancer have improved with the increase of earlier diagnoses in Korea and Japan due to the use of endoscopy screening,[1-4] the long-term oncologic outcomes of treatment in patients with advanced gastric cancer are still relatively poor.[5,6] Although ongoing collaborative sequencing efforts have highlighted recurrent somatic genomic aberrations in gastric cancer, the outcomes of patients have not sufficiently improved.[7] Better understanding of the molecular pathogenesis of gastric carcinogenesis may be needed for the advancement of gastric cancer treatments.

LIN28A is a highly conserved RNA-binding protein that was originally recognized as a key regulator of developmental timing in *Caenorhabditis elegans*.[8] Recently, a study on LIN28A showed that it is overexpressed in various human cancers and functions as an oncogenes.[9] p53 activates the expression of *miR-34a* and *miR-145*, which inhibit the expression of several stem-cell factors including OCT4, KLF4, LIN28A, and SOX2.[10] Additionally, it has been shown that tristetraprolin induced by p53 in cancer cells increases the level of *let-7*, a known tumor suppressor, via downregulation of LIN28A.[11] Blockade of the processing of various microRNAs, including *let-7*, has been suggested as one of the pathogenetic mechanisms of LIN28A in human carcinogenesis.[12-15] In addition, LIN28A has been reported to suppress many other factors including OCT4, SOX2, and NANOG, which are involved in reprogramming human somatic cells, by post-transcriptional regulation.[16,17]

Expression of LIN28A has been identified in various human cancers, including breast cancer,[18] ovarian cancer,[19] hepatocellular carcinoma,[9] and colorectal cancer.[20] As for gastric cancer, one study from China showed that positive expression of LIN28A was correlated with poor overall survival.[21] However, expression of LIN28A was found to be more common in corresponding normal tissue than in gastric cancer tissue in that study. In addition, the study did not fully adjust for TNM stages

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in the survival analysis. Therefore, we cannot be certain whether LIN28A expression is a poor prognostic factor in patients with gastric cancer. In the current study, we aimed to evaluate clinicopathological characteristics according to the expression of LIN28A by using a large number of gastric cancer tissue samples.

MATERIALS AND METHODS

1. Study design and patient population

In order to evaluate the relationship between the clinicopathological characteristics of gastric cancers and the expression of LIN28A, we performed immunohistochemical (IHC) staining for LIN28A using tissue microarrays (TMAs). The TMAs were constructed from 288 pairs of gastric cancer tissue samples and corresponding normal tissues that were obtained from patients who had undergone curative surgery for gastric cancer at the same center between April 2001 and December 2003. We collected the following data from the patients' medical records: demographic information, tumor location, histology, TNM staging, and overall survival duration. This study was approved by the Institutional Review Board of Severance Hospital.

2. Tissue microarray construction

The TMAs were constructed as previously described.[22] On hematoxylin and eosin-stained slides of tumors, a representative area was selected, and the corresponding spot was marked on the surface of the paraffin block. Using a biopsy needle, the selected area was punched out, and a 2-mm tissue core was placed into an 8×6 recipient block. Gastric cancer tissues and corresponding normal tissues were then extracted. Each tissue core was assigned a unique TMA location number that was linked to a database containing other clinicopathological data.

3. Immunohistochemistry

IHC analyses were performed on formalin-fixed, paraffin-embedded tissues, using the TMA. The slides were deparaffinized in xylene and rehydrated through an ethanol gradient. Endogenous peroxidase was blocked with 3% H_2O_2 for 10 minutes. The slides were immersed in 10 mM citrate buffer (pH 6.0) and heated for 10 minutes for antigen retrieval. Nonspecific binding was blocked by preincubation with a protein blocking agent (Immunotech Laboratories Inc., Monrovia, CA, USA), and the slides were incubated at room temperature for 1 hour with antibody against LIN28A (rabbit polyclonal, 1:500; Abcam, Cambridge, MA, USA). The samples were washed three times with phosphate-buffered saline (PBS) and then incubated with secondary antibodies for 30 minutes. After an additional three washes in PBS, the slides were incubated with the streptavidin-horseradish peroxidase complex (Dako, Glostrup, Denmark) for 30 minutes. The slides were visualized with diaminobenzidine (Dako) and then counterstained with hematoxylin. The expression of the antibodies was assessed semiquantitatively by estimating the percentage of tumor cells with positive cytoplasm staining among the entire population of tumor cells. All slides were examined and scored independently by two experienced investigators to avoid subjective biases. Each slide was examined in its entirety under a light microscope, and a proportion score was initially assigned, which represented the estimated proportion of positive tumor cells (0, none; 1, 0% to 10%; 2, 10% to 50%; and 3, 50% to 100%). Next, an intensity score was assigned, which represented the average intensity of the positive tumor cells (0, none; 1, weak; 2, intermediate; and 3, strong). The proportion and intensity scores were then multiplied to obtain a total score, which ranged from 0 to 9, and high expression of LIN28A was defined as a total score ≥ 2.[21]

4. Statistical analysis

Statistical tests used to compare the measured results included the t-test, Mann-Whitney U test, chi-square test, and Fisher exact test. The Kaplan-Meier method and log-rank test were used for survival analysis. In addition, the Cox proportional hazards model was used to adjust for possible confounding variables including TNM stage. A value of p less than 0.05 was regarded as a statistically significant difference for comparisons between groups. All statistical procedures were conducted using the statistical software SPSS for Windows version 18.0 (SPSS Inc., Chicago, IL, USA) with the exception of the survival analysis, which was performed using R version 2.15.3 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

1. Comparison of LIN28A expression between cancer tissues and corresponding normal tissues

IHC staining was performed on 288 pairs of normal and cancer tissue samples to analyze the difference in LIN28A expression. The mean IHC staining score was higher in the normal tissues than in the cancer tissues (normal vs cancer [mean±standard deviation], 2.0±1.6 vs 1.1±1.5; p<0.001). The proportion of tissues with LIN28A expression was higher in the normal tissues than in the cancer tissues (normal vs cancer, 63.5% vs 35.4%; p<0.001).

2. LIN28A expression and clinicopathological characteristics of gastric cancer

The clinicopathological characteristics of the enrolled patients according to the LIN28A expression of cancer tissues on IHC staining are shown in Table 1. High expression of LIN28A was shown in 102 of 288 patients (35.4%). The mean patient age and the proportion of men did not differ between the groups (p=0.469 and p=0.217, respectively). Neither tumor location nor histology differed between the groups (p=0.148 and p=0.827, respectively). In addition, T, N, and TNM stages did not differ

Table 1. Clinicopathological Characteristics according to LIN28A Expression via Immunohistochemical Staining

Variable	Low expression on IHC staining (IHC staining score <2) (n=186)	High expression on IHC staining (IHC staining score ≥2) (n=102)	p-value
Age, yr	56.2±12.1	55.1±12.8	0.469
Sex			0.217
Male	116 (62.4)	56 (54.9)	
Female	70 (37.6)	46 (45.1)	
Location			0.148
Upper third	34 (18.3)	29 (28.4)	
Middle third	34 (18.3)	14 (13.7)	
Lower third	115 (61.8)	59 (57.8)	
Anastomosis site	3 (1.6)	0	
Histology			0.827
AWD or AMD	45 (24.2)	28 (27.5)	
APD or SRC	129 (69.4)	68 (66.7)	
Others*	12 (6.5)	6 (5.9)	
T stage			0.284
pT1	3 (1.6)	0	
pT2	31 (16.7)	25 (24.5)	
pT3	65 (34.9)	34 (33.3)	
pT4	87 (46.8)	43 (42.2)	
N stage			0.375
pN0	74 (39.8)	36 (35.3)	
pN1	54 (29.0)	25 (24.5)	
pN2	32 (17.2)	26 (25.5)	
pN3	26 (14.0)	15 (14.7)	
TNM stage			0.725
IIa	59 (31.7)	30 (29.4)	
IIb	65 (34.9)	34 (33.3)	
IIIa	28 (15.1)	19 (18.6)	
IIIb	14 (7.5)	11 (10.8)	
IIIc	20 (10.8)	8 (7.8)	

Data are presented as mean±SD or number (%).
IHC, immunohistochemical; AWD, adenocarcinoma well differentiated; AMD, adenocarcinoma moderated differentiated; APD, adenocarcinoma poorly differentiated; SRC, signet ring cell carcinoma.
*This category included mucinous adenocarcinoma, lymphoepithelioma-like carcinoma, and glandular endocrine carcinoma.

between the groups (p=0.284, p=0.375, and p=0.725, respectively).

3. Survival analysis according to LIN28A expression

Kaplan-Meier plots were used to illustrate overall survival according to the expression of LIN28A (Fig. 1). The median follow-up durations were 69.4 months (interquartile range [IQR], 60.6 to 77.4 months) and 66.9 months (IQR, 40.8 to 76.7 months) in the low and high expression groups, respectively (p=0.158). The 5-year overall survival was 75.3% (95% confidence interval [CI], 69.3% to 81.7%) in the low expression group and 71.6% (95% CI, 63.3% to 80.9%) in the high expression group. The duration of overall survival did not differ between the groups (p=0.405).

A Cox proportional hazards model showed that high LIN28A expression was not an independently associated factor for death after adjusting for TNM stage (hazard ratio, 1.191; 95% CI, 0.758 to 1.872) (Table 2).

DISCUSSION

LIN28A has been considered an oncogene in the development of various human cancers.[9,18-20] One of the possible mechanisms of LIN28A as an oncogene is downregulation of the *let-7* family, well-known microRNAs that function as tumor suppressors.[12-14] Under this hypothesis, Xu *et al*.[21] performed a study on the expression of LIN28A in gastric cancer patients via real-

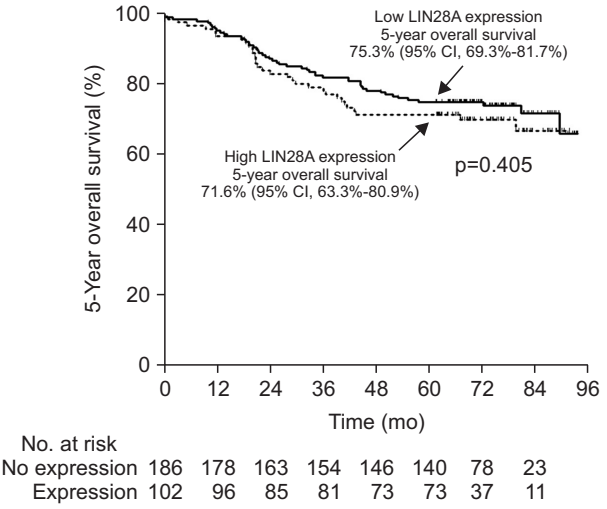

Fig. 1. Kaplan-Meier plots for the overall survival rate according to LIN28A expression. The median follow-up durations of the low- and high-expression groups were 69.4 months (interquartile range [IQR], 60.6 to 77.4 months) and 66.9 months (IQR, 40.8 to 76.7 months), respectively.

time polymerase chain reaction analysis and IHC staining. In their study, however, the expression level of LIN28A in normal tissues was higher than that in cancer tissues (normal vs cancer, 62.8% vs 46.0%). Although it was shown that LIN28A expression was independently associated with poor overall survival, the authors could not clearly explain how LIN28A expression affected the overall survival of patients with gastric cancer. We questioned whether the expression of LIN28A really has a negative influence on overall survival in patients with gastric cancer.

In our study, similar proportions of cancer tissues and corresponding normal tissues showed expression of LIN28A on IHC staining (normal vs cancer, 63.5% vs 35.4%) compared to the results of Xu *et al.*[21] However, no associated clinicopathological factor for the high LIN28A expression in gastric cancers was identified. Unlike Xu *et al.*,[21] we classified gastric cancers into four or five groups according to T stage (pT1, pT2, pT3, and pT4), N stage (pN0, pN1, pN2, and pN3), and TNM stage (IIa, IIb, IIIa, IIIb, and IIIc). These detailed classifications were selected to stratify the gastric cancers more accurately for survival analysis. As a result, we showed that LIN28A expression was not an independently associated factor for overall survival. The expression of LIN28A seemed to have no distinct role in predicting clinicopathological characteristics including survival outcomes in patients with gastric cancer.

Our results, which are in conflict with the usual expectation that LIN28A may have a role as an oncogene, are likely due to the various roles of LIN28A aside from repressing *let-7*. First, overexpression of LIN28A induces the expression of several transcription factors that are involved in early embryonic-cell fate decisions. Moreover, LIN28A functions independently of *let-7*.[23] Second, LIN28A directly regulates various metabolisms

Table 2. Cox Proportional Hazards Model for Predicting Death

Variable	Hazard ratio (95% CI)	p-value
TNM stage		
IIa	1	
IIb	1.361 (0.672–2.757)	0.392
IIIa	3.302 (1.608–6.779)	0.001
IIIb	5.172 (2.340–11.434)	<0.001
IIIc	7.897 (3.826–16.296)	<0.001
High LIN28A expression	1.191 (0.758–1.872)	0.448

CI, confidence interval.

including oxidative phosphorylation or glucose metabolism.[24,25] Third, LIN28A may facilitate the global translational suppression of endoplasmic reticulum-associated mRNAs in undifferentiated stem cells.[26] We believe that LIN28A may have a role not only in gastric cancer cells but also in normal gastric cells. In addition, the heterogeneity of gastric cancers could contribute to the discrepancy between the results of Xu *et al.*'s study and ours.[27,28] A better understanding of the molecular biology of gastric cancers and appropriate selection of the patient population might be necessary to clarify the role of LIN28A in gastric carcinogenesis.

Although this was the largest study to date that evaluated clinicopathological characteristics according to the expression of LIN28A in patients with gastric cancer, it has several limitations. First, we did not assess the lesion characteristics according to well-known associated factors for gastric cancer, including *Helicobacter pylori* infection status and family history of gastric cancer. Unfortunately, *H. pylori* infection status and family history were unavailable in our gastric cancer cohort for TMAs. Second, various associated factors for LIN28A including p53, Ki-67, *miR-34a*, *miR-145*, and *let-7*, were not analyzed in the study. Evaluating these factors might support the negative results of our study in patients with gastric cancer. The lack of an analysis of LIN28B was another limitation of the study. Although the study was performed to validate the results of the previous study,[21] expression of LIN28B should be investigated to better understand LIN28 in patients with gastric cancer.[29,30] Despite these limitations, our data may form the basis of a system that can be used to understand the clinicopathological characteristics based on LIN28A expression.

The expression of LIN28A seemed to have no distinct role in predicting clinicopathological characteristics in gastric cancer. Additionally, LIN28A expression was not an independently associated factor for overall survival in patients with gastric cancer.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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