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Original Article

High expression of fibulin-5 is significantly associated with the poor prognosis of patients with esophageal cancer

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Abstract: Fibulin-5 is an extracellular matrix protein that plays a multifaceted role in cancer. To date, the role of Fibulin-5 in esophageal cancer is poorly understood. The focus of the present study was to investigate the relationship between Fibulin-5 expression and the clinical index of esophageal cancer, especially the prognosis of patients. The results of this study indicated that Fibulin-5 was localized in both the cytoplasm and nucleus in all of the esophageal cancer samples with higher expression in cancer tissues when compared to para-carcinoma tissues ($P = 0.000$, $P = 0.004$). Pearson analysis revealed that Fibulin-5 cytoplasmic expression was both significantly and positively correlated with Fibulin-5 nuclear expression ki67 (marker of cellular proliferation) expression in cancer tissues ($r = 0.283$, $P = 0.004$; $r = 0.211$, $P = 0.035$). The clinical index correlation analysis showed that Fibulin-5 expression in the cytoplasm was positively associated with histological grading, lymph node stage and clinical stage ($r = 0.314$, $P = 0.001$; $r = 0.280$, $P = 0.005$; $r = 0.280$, $P = 0.006$), while nuclear Fibulin-5 expression was only correlated with lymph node stage ($r = 0.253$, $P = 0.012$). Survival analysis revealed that patients with high Fibulin-5 cytoplasmic expression had a poorer prognosis than those with low Fibulin-5 expression (0.0% versus 23.2%, $P = 0.024$). However, nuclear Fibulin-5 expression did not have an influence on patient prognosis of esophageal cancer. However, Fibulin-5 cytoplasmic expression was not determined to be an independent predictor of esophageal cancer. In conclusion, our data firstly demonstrated the Fibulin-5 oncogenic function in esophageal cancer. Further investigation is necessary to elucidate the molecular mechanism of Fibulin-5 in esophageal cancer.

Keywords: Fibulin-5, tissue microarray, immunohistochemistry, esophageal cancer, prognosis

Introduction

As one of the four most common cancers, approximately 500,000 new esophageal cancer cases were presented during 2015 in China [1]. Although the incidence rate of esophageal cancer has decreased in recent decades, the morbidity levels in China for this cancer remained relatively high [2]. Therefore, it is necessary to investigate the molecular events associated with esophageal cancer and discover new diagnostic and therapeutic strategies for this disease.

The Fibulin family of extracellular matrix proteins interacts with endothelial cells and participates in multiple normal functions, including tissue and organ development, remodeling as well as restoration. As a member of the Fibulin family, Fibulin-5 also plays multiple roles in different biological pathways, including hepatic fibrosis, angiogenesis and tumorigenesis [3-5]. Recently, the role of Fibulin-5 in different types of cancers has gained more attention as due to its conflicting functions. Mostly, reports indicate that Fibulin-5 acts as a tumor suppressor in cancers such as ovarian cancer, hepatocellular carcinoma, glioma cancer, lung cancer and urothelial carcinoma [6-11]. In contrast, Fibulin-5 also promotes cancer cell proliferation, motility and invasion, and plays a critical role in tumorigenesis in some cancers, including breast cancer, nasopharyngeal carcinoma and pancreatic cancer [12-14]. To our knowledge, the role of Fibulin-5 in esophageal cancer remains poorly understood.

In the present study, we investigated the potential role of Fibulin-5 in esophageal cancer using a tissue microarray containing 100 esophageal

Table 1. Difference of Fubulin-5 expression in esophageal cancer tissues and para-carcinoma tissues

Histology	No.	Fubulin-5 expression in cytoplasm	*P*-value	No.	Fubulin-5 expression in nuclear	*P*-value
Esophageal cancer tissue	72	50.01% ± 37.42%	0.000	73	22.26% ± 26.61%	0.004
Adjacent tissue	72	4.54% ± 9.55%		73	13.99% ± 15.77%	

Mean ± Std. Deviation.

Table 2. Correlation between Fibulin-5 expression and ki67 expression in esophageal cancer

		Fibulin-5 cytoplasm expression	Fibulin-5 nuclear expression	ki67 expression
Fibulin-5 cytoplasm expression	Pearson Correlation	1	0.283**	0.211*
	P value		0.004	0.035
	Number	100	100	100
Fibulin-5 nuclear expression	Pearson Correlation	0.283**	1	-0.002
	P value	0.004		0.981
	Number	100	100	100

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).

cancer tissues and 80 adjacent tissues. Statistical analysis was performed to reveal the relationship between Fibulin-5 expression and the prognosis for esophageal cancer.

Materials and methods

Clinical materials

The esophageal cancer tissue microarray (HEso-Squ180Sur-04) was obtained from Shanghai Outdo Biotech Co., Ltd., which includes 100 cancer tissue samples and 80 para-carcinoma tissues. The standard protocol to generate microarray was employed as previously described.

The clinical factors of esophageal cancer patients showed that 74 members were male and 26 were female; the age ranged from 48 to 82 with a median age of 65; and the tumor size ranged from 1.2 cm to 10 cm. The clinical grading distribution showed that four cases were Stage 1, 42 cases were Stage 2, 50 cases were Stage 3, zero cases were Stage 4. Four of the cases lacked clinical staging information. Details of the clinical information for all of the patients can be found in **Table 3**.

The follow-up information for the esophageal cancer patients was reported as follows: the operation time was from January 2006 to October 2008 and the eventual follow-up time started from September 2014, which followed 0-97 months. During the follow-up period, 81 patients died from esophageal cancer with a median overall survival time of 14 months (0-60 months), 18 patients were alive with a median overall survival time of 80 months (71-97 months), one patient was lost to follow-up in September 2013 and was included in the survival group used in the statistical analysis. All patients were diagnosed as esophageal squamous cell carcinoma and received no additional treatment prior to surgery.

Immunohistochemistry

The tissue microarray was treated with citrate buffer and freshly prepared 0.3% hydrogen peroxide in methanol in order to retrieve the antigen and remove the endogenous peroxidase activity. Tissue sections were blocked with goat serum and subsequently incubated with primary antibody (anti-Fibulin-5, 1:200, 20079, ProMab, Richmond, CA, USA) at 4°C overnight, then incubated with secondary antibody (HRP-labeled anti-mouse antibody, Dako), washed with PBS, and visualized using the diaminobenzidine (DAB) system and hematoxylin re-dying. Over 100 cells of the three visual fields from random areas of each specimen were chosen, and Fibulin-5 expression was scored according to the percentage of positive cells in that field: ≤85% positive cells were classified as the low expression group while >85% positive cells was classified as the high expression group.

Table 3. Correlation between clinical factors and Fubulin-5 expression in esophageal cancer tissues

Clinical factors	No. patients	Cytoplasm expression		*P* value	No. patients	Nuclear expression		*P* value
		Low	High			Low	High	
Gender				0.695				0.230
Male	74	57	17		74	70	4	
Female	26	21	5		26	26	0	
Age				0.315				0.436
≤60	32	23	9		32	30	2	
>60	68	55	13		68	66	2	
Tumor size				0.972				0.733
≤5 cm	56	47	9		56	53	3	
>5 cm	29	23	6		29	28	1	
Lost	15	8	7		5	5	0	
Grade				0.001				0.488
I	6	6	0		6	5	1	
II	66	56	10		66	64	2	
III	28	16	12		28	27	1	
T staging				0.293				0.595
T1	4	4	0		4	4	0	
T2	11	9	2		11	10	1	
T3	79	60	19		79	77	2	
T4	3	2	1		3	2	1	
Lost	3	3	0		3	3	0	
N staging				0.005				0.012
N0	45	40	5		45	45	0	
N1	31	23	8		31	30	1	
N2	17	10	7		17	15	2	
N3	5	3	2		5	4	1	
Lost	2	2	0		2	2	0	
cTNM staging				0.006				0.055
1	4	4	0		4	4	0	
2	42	37	5		42	42	0	
3	50	33	17		50	46	4	
Lost	4	4	0		4	4	0	

Statistical analysis

The difference between Fibulin-5 expression in esophageal cancer tissues and the adjacent tissues was analyzed by paired T-test. The Pearson test was used to analyze the association among Fibulin-5 cytoplasm expression, Fibulin-5 nuclear expression and ki67 expression. The correlations between Fibulin-5 expression and clinical pathological parameters of esophageal cancer were calculated using Spearman's correlation analysis. Univariate analysis between Fibulin-5, clinical factors and survival time was estimated using the Kaplan-Meier method and the log-rank test. Then, statistically significant variables identified following the univariate analysis were included in the Cox multivariate regression survival analysis. All statistical analyses were conducted using SPSS 22.0 software. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of Fubulin-5 in esophageal cancer tissues

The immunohistochemistry results revealed that Fibulin-5 was localized in the cytoplasm and nucleus of all of the esophageal cancer specimens (see **Figure 1**). Paired t-test revealed that both Fibulin-5 cytoplasmic expression (50.01% ± 37.42% vs. 4.54% ± 9.55%, P = 0.000) and nuclear expression (22.26% ± 26.61% vs. 13.99% ± 15.77%, P = 0.004) were significantly higher in esophageal cancer tissues than in para-carcinoma tissues (see **Table 1**).

Relationship between the expression of fibulin-5 and ki67 in esophageal cancer

In order to further understand the molecular mechanism of Fibulin-5 in esophageal cancer, we considered the clinical immunohistochemical data for ki67, which is a proliferation marker of tumor cell., The relationship between Fibulin-5 and ki67 in esophageal cancer was analyzed by Pearson analysis The results revealed that Fibulin-5 cytoplasmic expression was both significantly and positively associated with Fibulin-5 nuclear expression (r = 0.283, P = 0.004) and ki67 expression (r = 0.211, P = 0.035) in esophageal cancer tissues (**Table 2**). However, the relationship between Fibulin-5 nuclear expression and ki67 expression was not significant (r = -0.002, P = 0.981).

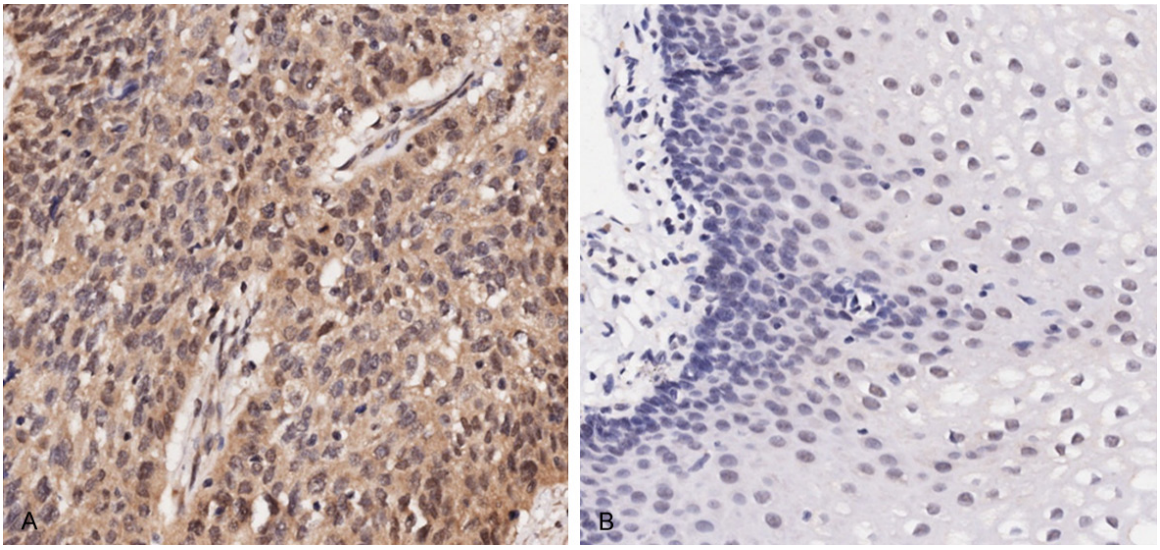

Figure 1. Representative immunohistochemistry of Fibulin-5: Fibulin-5 was expressed in both the cytoplasm and nucleus of esophageal cancer tissues and para-carcinoma tissues. The Fibulin-5 cytoplasmic and nuclear expression in esophageal cancer tissues (A) were both significantly higher than that observed in para-carcinoma tissues (B). (Magnification: ×200).

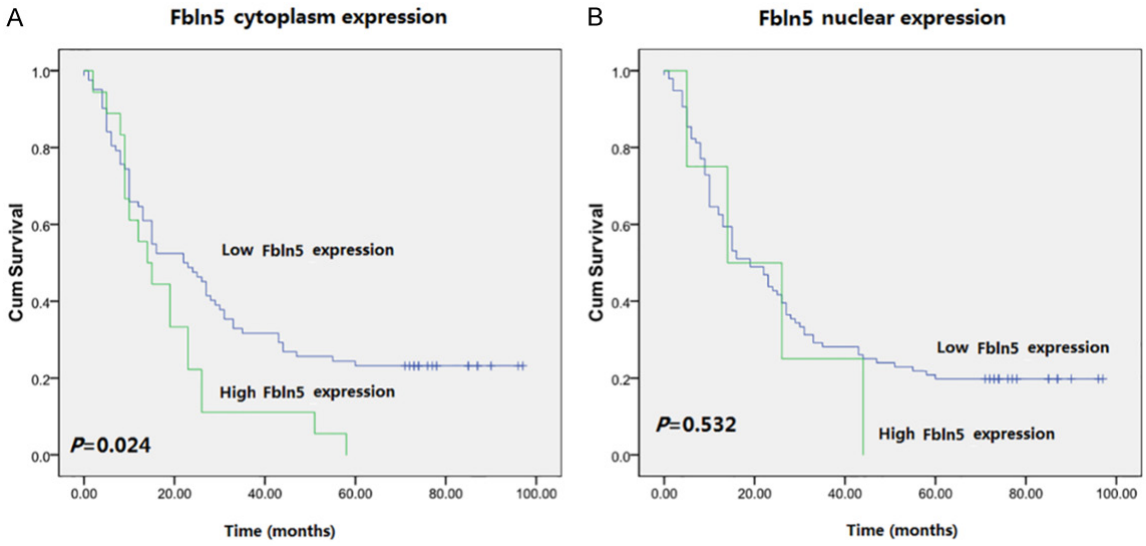

Figure 2. Kaplan-Meier survival curves dependent on Fibulin-5 expression in (A) cytoplasm (B) nuclear *P* values were calculated using the log-rank test.

Correlation between fibulin-5 expression and the clinical factors in esophageal cancer

The correlation between cytoplasmic and nuclear Fibulin-5 expression and clinical factors of esophageal cancer were evaluated by Spearman analysis (see **Table 3**). Patients were divided into low or high Fibulin-5 expression groups in each database to test the relationship. As shown in **Table 3**, Fibulin-5 cytoplasmic expression was positively associated with histological grading, lymph node stage and clinical stage (r = 0.314, P = 0.001; r = 0.280, P = 0.005; r = 0.280, P = 0.006), while Fibulin-5 nuclear expression was only related to lymph node stage (r = 0.253, P = 0.012).

Correlation of high fibulin-5 cytoplasmic expression and the prognosis of esophageal cancer

Kaplan-Meier analysis and log-rank test were applied to determine the association between Fibulin-5 cytoplasmic expression, Fibulin-5

Table 4. Cox regression analysis under inclusion of clinical factors and Fibulin-5 expression

	Overall survival	
	HR (95% CI)	*P*
Fibulin-5 cytoplasm expression	1.082 (0.569-2.055)	0.811
Gender	0.618 (0.293-1.300)	0.205
Tumor size	1.510 (0.878-2.597)	0.137
T	1.599 (0.802-3.189)	0.183
N	1.149 (0.740-1.785)	0.537
cTNM	1.722 (0.740-4.008)	0.207

nuclear expression, ki67 expression, clinical indexes and the prognosis of esophageal cancer. The results revealed that Fibulin-5 cytoplasmic expression had a significant inverse correlation with the overall survival time of the patients in esophageal cancer (see **Figure 2A**). However, Fibulin-5 nuclear expression showed no relationship with the prognosis of esophageal cancer (see **Figure 2B**). Furthermore, gender, tumor size, T staging, N staging and clinical staging were all significantly correlated with the prognosis of the patients; while age and pathological grading were not related (data was not shown). Subsequently, all of the factors related to the prognosis of esophageal cancer were included in the Cox multivariate analysis. The results revealed that none of these factors could serve as an independent prognostic marker (**Table 4**).

Discussion

Fibulin-5, also known as FBLN-5, DNACE or EVEC, is the newest member of the Fibulin family of extracellular matrix proteins. Previous reports have shown that Fibulin-5 exhibits various biological functions in different types of cancers, showing both oncogenicity and tumor suppressor properties. Prior to this report, the relationship between Fibulin-5 and esophageal cancer had not been reported. Here, we investigated the correlation between Fibulin-5 expression and occurrence, development as well as the prognosis of esophageal cancer.

The results indicated that Fibulin-5 was localized in both the cytoplasm and the nucleus in all esophageal cancer samples with significantly higher expression in cancer tissues than in para-carcinoma tissues (P = 0.000, P = 0.004). Pearson analysis showed that Fibulin-5 cytoplasm expression was both significantly and positively correlated with Fibulin-5 nuclear expression and ki67 expression in cancer tissues (r = 0.283, P = 0.004; r = 0.211, P = 0.035), while the relationship between Fibulin-5 nuclear expression and ki67 expression was not significant (r = -0.002, P = 0.981). The clinical index correlation analysis showed that Fibulin-5 expression in the cytoplasm was positively associated with histological grading, lymph node stage and clinical stage (r = 0.314, P = 0.001; r = 0.280, P = 0.005; r = 0.280, P = 0.006), while the Fibulin-5 expression in nuclear was only correlated with lymph node stage (r = 0.253, P = 0.012). Survival analysis revealed that patients with high Fibulin-5 cytoplasmic expression had a poorer prognosis compared to those patients with low Fibulin-5 cytoplasmic expression (0.0% versus 23.2%, P = 0.024); however, Fibulin-5 nuclear expression had no influence on the prognosis of esophageal cancer. In addition, gender, tumor size, T, N and clinical stage were all significantly related to the prognosis of esophageal cancer. However, Cox multivariate analysis showed that none of these esophageal cancer indicators could serve as an independent predictor of esophageal cancer, possibly due to their significant correlation with each other.

Taken together, we speculate that Fibulin-5 plays the role of an ocogenenic protein in esophageal cancer. The high expression of Fibulin-5 in cancer tissues may enhance ki67 expression and thus promote cancer cell proliferation. Another hypothesis under consideration in regards to Fibulin-5 oncogenic function is that Fibulin-5 may promote cancer cells to metastasize into the lymph node, and thus reduce the survival time of patients. Moreover, Fibulin-5 cytoplasmic expression seemed to play the leading function, comparing to Fibulin-5 nuclear expression.

Recently, there were many reports about the relationship between Fibulin-5 expression and cancer which mostly indicated that Fibulin-5 exhibits tumor suppressive functions [6-11]. In contrast, tumor promoting roles of Fibulin-5 were also reported. For example, Hwang et al. revealed that patients with low Fibulin-5 expression had longer survival time than those with high Fibulin-5 expression, and that Fibulin-5 could improve the cancer cell proliferation,

invasion and metastasis. The AKT protein kinase pathway may participate in this process, and Fibulin-5-siRNA could reverse the oncogenic function of Fibulin-5 [14]. In addition, research regarding the relationship between Fibulin-5 and pancreatic cancer has revealed that 1) the proliferation of pancreatic cancer cells and angiogenesis were both decreased in Fibulin-5 null mice, and the concentration of reactive oxygen species was augmented in Fibulin-5 null pancreatic cancer cells [15]; 2) anti-oxygen agents could recruit the proliferation and angiogenesis of pancreatic cancer cells in vivo [12]; and 3) most importantly, the synthesis of reactive oxygen species was negatively regulated by Fibulin-5 *in vitro*. Based on these findings, the investigators anticipated that Fibulin-5 may promote the proliferation of pancreatic cancer by mitigating the reactive oxygen species [16].

These findings were similar to the results of this paper, thus we hypothesized that Fibulin-5 might promote tumorigenic process via the similar pathway in esophageal cancer. Further study was needed to investigate the mechanism of Fibulin-5 in esophageal cancer. In conclusion, our date firstly demonstrated the Fibulin-5 oncogenic function in esophageal cancer. To investigate the regulatory network of Fibulin-5 in esophageal, further work about Fibulin-5 expression function in esophageal cancer cells is needed to be done by constructing Fibulin-5+/Fibulin-5-esophageal cancer cell lines.

Disclosure of conflict of interest

None.

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