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RTN4/Nogo is an independent prognostic marker for gastric cancer: preliminary results

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Abstract. – OBJECTIVE: Gastric cancer is the fourth most common malignant cancer and is the second leading cause of cancer death worldwide. We evaluated the association of the immunohistochemical RTN4 expression with clinicopathological variables and patient outcome, and to evaluate its prognostic value.

PATIENTS AND METHODS: Histological samples from 95 primary gastric carcinoma patients were retrospectively studied with monoclonal antibody to RTN4.

RESULTS: Tumors with high RTN4 expression were found in 57.9% of patients. High RTN4 were associated with advanced stages (p = 0.0377) and different histology (p = 0.0030). In the overall population (median follow-up 42 months), patients with high RTN4 had shorter survival time than those with low RTN4 expression (p = 0.0119). In Cox multivariate analysis, high RTN4 (p = 0.0160) is an independent prognostic factor for overall survival of gastric cancer patients.

CONCLUSIONS: Our data suggest that RTN4 may contribute to the malignant progression of gastric cancer and serve as a novel prognostic indicator for gastric cancer patients.

Key Words:
RTN4, Nogo, Gastric cancer, Prognosis, Survival analysis.

Introduction

Gastric cancer is the fourth most prevalent malignant tumor worldwide and second leading cause of cancer-related deaths each year[1,2]. Although the incidence and mortality of gastric cancer has gradually decreased in East Asia, or develop recurrence in few years after surgery, and the 5-year survival rate of these patients is very poor[3]. Therefore, further study of potential biomarkers for gastric cancer is pivotal for its early diagnosis and the development of targets for new therapies.

Nogo proteins, encoded by gene reticulon-4 (RTN4), are myelin-associated endoplasmic reticulum proteins, including three major isoforms, namely, Nogo-A, B, and C[4,5]. These three isoforms share a conserved reticulum homology domain (RHD), which contains a 66-aa loop domain termed Nogo-66[6,7]. Recent studies have shown that Nogo-B plays a role in cell adhesion and migration[8]; the amino terminus of Nogo-B promotes the adhesion and migration of endothelial cells and negatively regulates platelet-derived growth factor-induced migration in smooth muscle cells. RTN4 is ubiquitously expressed in various normal human tissues, such as the central nervous system[4,9], cultured endothelial and smooth muscle cells[10], as well as intact blood vessels[11]. There is increasing evidence indicating that RTN4 interacts with apoptosis-inducing proteins[12] and mediates transmembrane signaling pathways, thereby regulating cell adhesion, migration, metastasis and apoptosis[9,12,13]. Over expression or constitutive activation of RTN4 is widely implicated in various cancers[14-17]. However, there have been no studies reporting the relationship between RTN4 and clinical characters of gastric cancers

In this study, we analyzed RTN4 expression in gastric cancers using immunohistochemistry and further evaluated the clinicopathologic features and prognostic importance of RTN4 expression in gastric cancer.

Patients and Methods

Patients

Gastric cancer tissues were obtained from 95 patients who underwent gastrectomy and D2 lymphadenectomy at the Affiliated Hospital of Qingdao University Medical College from May 2008 to July 2009. 72 para-cancer tissues which were more than 5 cm away from the edge of tumor were

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randomly selected. None had received chemotherapy or radiotherapy before surgery. The clinical and pathological data were recorded according to the World Health Organization (WHO) and the Japanese Classification of Gastric Carcinoma (JCGC). All tumors were staged based on the American Joint Committee on Cancer TNM staging system (7th edition) (Washington, 2010). Follow-up information was collected by phone calls, letter, or the outpatientclinical database. The followed up study was made from the date of surgery until death or 31 December 2013. The median follow-up period was 35.6 months (range, 3-55 months) (range, 3-60 months). This study was approved by the Ethics Committee of China Medical University and informed consent was obtained from all patients before enrollment.

Immunohistochemical Staining

Immunostains were performed by the routine Streptavidin-peroxidase (S-P) method. Formalin fixed, paraffin embedded tissues were cut into sections of 4 m. Sections were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide for 10 min to quench endogenous peroxidase activity. Nonspecific bindings were blocked by treating slides with normal rabbit serum for 15 min. Tissue sections were incubated with monoclonal mouse anti-Nogo antibody (dilution 1:200, ab32298, Abcam, Cambridge, UK) overnight at 4 C. After washing, the sections were incubated with a biotin-marked secondary antibody (working concentration, PV9000, Beijing fir Jinqiao) was applied for 10 min in room temperature and subsequently incubated with streptavidin conjugated horseradish peroxidase (HRP) for 10 minutes at room temperature. Sections were colorized with 3, 3-diaminobenzidine (DAB) chromogen solution and counterstained with hematoxylin.

All sections were immunostained at the same time and under the same conditions. Results were examined by two investigators in blinded manner. In the case that the two investigators disagreed, related results were evaluated by the third party; if any two of the three investigators could not come to an agreement, related sections were excluded.

Negative controls were treated in a similar manner with the exception of primary antibodies.

Evaluation of Staining

For RTN-4 assessment, staining intensity was scored as 0 for negative staining, 1 for yellow color staining, 2 for light brown color staining, and 3 for brown color staining. Extent of staining was scored as 0 for 0%, 1 for 1-33%, 2 for 34-66%, and 3 for 67-100% according to the percentages of the positive staining areas in relation to the whole carcinoma area. Samples with a sum score of 0-2 were regarded to have low expression of RTN4, and > 2 were regarded to have high expression of RTN4.

Statistical Analysis

The software of SAS 9.2 (SAS Inc., Cary, NC, USA) was used for statistical analysis and a p value of less than 0.05 was determined to be statistically significant. The chi-square statistics and Fisher's extract test were applied to assess the relationship between RTN4 expression and clinicopathological data. Survival curves was obtained by the Kaplan-Meier method and compared by the log-rank test. Cox's proportional hazards regression models was used for multivariate analysis of prognostic factors.

Results

RTN4 Expression in Gastric Cancer Tissues

RTN4 expression was detected in 95 gastric cancer tissues and 72 matched paraneoplastic tissues by immunohistochemical staining. The results showed that high RTN4 expression was observed in 57.9% (55/95) of gastric cancer tissues, while in 16.7% (12/72) of para-cancer tissues (Figure 1). RTN4 was predominantly observed in the cytoplasm, and a statistical significance was observed that RTN4 expression was remarkably up-regulated ($p < 0.0001$, Chi-square test) in gastric cancer tissues compared with normal gastric mucosa (Table I). Among the clinicopathological variables (Table II), RTN4 expressions were significantly associated with both TNM-stage ($p = 0.0377$) and histology ($p = 0.0030$).

Relationship Between RTN4 Expression and Survival

In Kaplan-Meier survival analysis and log-rank test, high expression of RTN4 was significantly associated with poor prognosis (Figure 2). Furthermore, univariate and multivariate analyses were conducted to assess the prognostic value of RTN4 expression as a predictor of overall survival in patients with gastric cancer. Using univariate analysis, our data indicated that the survival rates had close relationship with differentia-

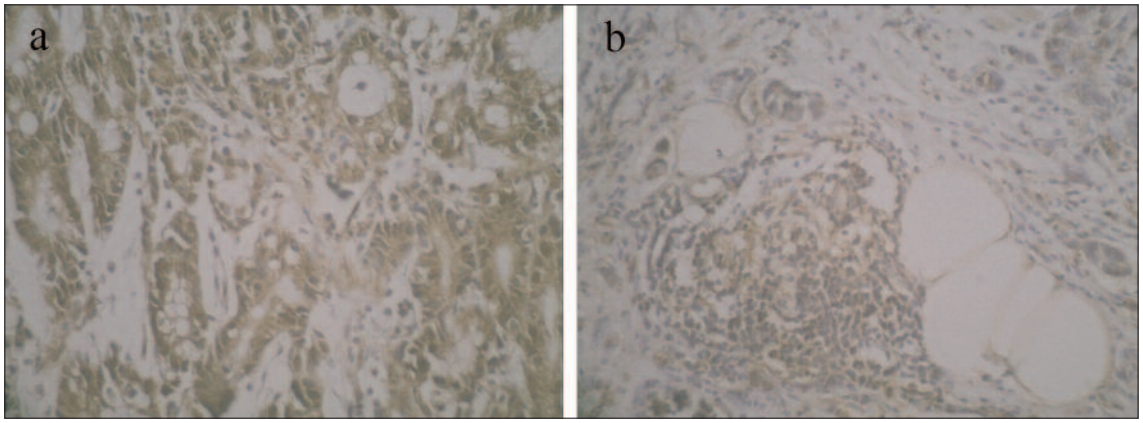

Figure 1. Immunohistochemical staining of RTN4 protein expression in gastric cancer tissues. Representative images show high RTN4 expression ***(a)*** and low RTN4 expression ***(b)***. Original magnification, ×400.

tion (p = 0.0448), Lymph node metastasis (p = 0.0429), TNM-stage (p = 0.0101) and RTN4 expression (p = 0.0119) (Table III). Multivariate analyses showed that RTN4 expression (hazard ratio, 1.961; 95% confidence interval, 1.134-3.392; p = 0.0160) was an independent risk factor in the prognosis of gastric cancer patients (Table I). Besides, TNM stage (p = 0.0135) is also an independent risk factors based on statistical data analysis. Our findings indicate that RTN4 may be a useful predictor of the survival of GC patients.

Table I. Relationship between RTN4 expression and clinicopathological features of GC.

Characteristic	RTN4				*p* value
	Negative (n=40)		Positive (n=55)		
Age					
< 60 y	45	18	27		0.6934
≥ 60 y	50	22	28		
Gender					
Male	67	29	38		0.7190
Female	28	11	17		
Tumor size					
< 4 cm	48	21	27		0.4864
≥ 4 cm	37	19	18		
Tumor site					
Gastric fundus	6	2	4		0.2145
Gastric corpus	31	17	14		
Pylorus	58	21	37		
Histology					
Adenocarcinoma	65	34	31		0.0030
Other types	30	6	24		
Differentiation					
Well/moderate	17	8	9		0.6480
Poor	78	32	46		
TNM stage					
I + II	23	14	9		0.0377
III + IV	72	26	46		
Lymph node metastasis					
Present	78	21	33		0.4662
Absent	17	19	22		
Distant metastasis					
Present	5	1	4		0.2355
Absent	90	39	51		

Table III. RTN4 expression in human gastric cancer tissues and para-cancer tissues.

		RTN4			
	Cases	Positive	Negative	χ^2	*p* value
Gastric cancer	95	55	40	28.9790	< 0.0001
Paraneoplastic tissues	72	12	60		

Discussion

In our study, RTN4 expression in 95 gastric cancer tissues and 72 matched adjacent normal gastric mucosa by immunohistochemical staining. The results showed that high RTN4 expression was observed in 57.9% (55/95) of gastric cancer tissues, but only in 16.7% (12/72) of normal gastric mucosa, indicating up-regulation of RTN4 expression in gastric cancer tissues. More importantly, statistical analysis revealed that high RTN4 expression was significantly related with clinicopathological parameters of gastric cancer patients, such as tumor differentiation, TNM stage, tumor invasion, and lymph node metastasis. In addition, the RTN4 expression was related with more advanced clinical stage and poorer survival rate, and this result is consistent with that of other studies with various human cancers including gastric cancer. This is the first study in which the relationship between the RTN4 expression and the clinicopathological features, with special attention given to the prognostic significance of GC, has been investigated. Our findings suggest that RTN4 is a useful predictor for clinical outcome of patients with gastric cancer.

RTN4/Nogo, a central nervous system myelin-associated neurite growth inhibitory protein and also recognized as an apoptosis-inducing gene, which retain a common C-terminal domain containing two trans-membrane domains and an endoplasmic reticulum (ER)-retrieval motif[18], has been reported to be highly expressed in many human malignancies. However, whether RTN4 is an oncogene or a tumor suppressor remains currently under debate. In the present study, our immunohistochemical results showed high RTN expression in 57.9% (55/95) of gastric cancer tissues, but only in 16.7% (12/72) of normal gastric mucosa, suggesting up-regulation of RTN4 expression in gastric cancer tissues. Furthermore, we also correlated RTN4 protein expression with various clinicopathological factors in gastric cancer patients. The results showed that high RTN4 expression was associated with poorly differentiated carcinoma, indicating that RTN4 may induce the differentiation of gastric cancer. Similar

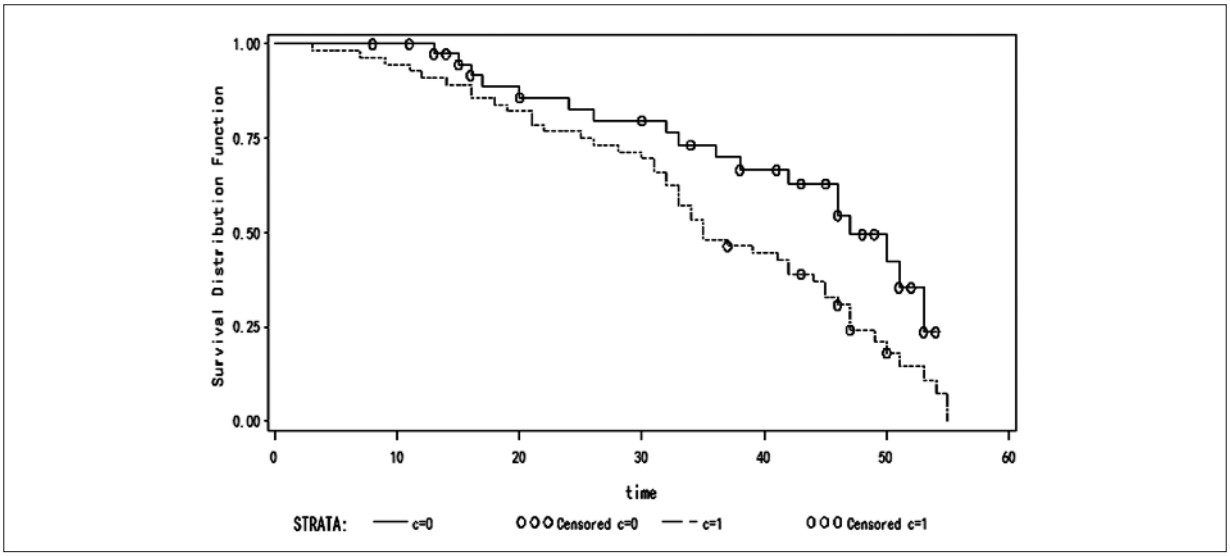

Figure 2. Kaplan-Meier curves for overall survival according to high versus low RTN4 expression. Patients with high RTN4 expression have a significantly shorter survival. The p value was calculated by the log-rank test.

Table II. Univariate and multivariate analysis of various prognostic factors in 95 gastric cancer patients.

Viariables	Univariate anlysis		Multivariate analysis	
	χ^2	*p* value	HR (95% CI)	*p* value
Age (< 60/≥ 60)	0.5475	0.4593		
Gender (male/female)	0.0002	0.9877		
Tumor site (gastric fundus/gastric corpus/pylorus)	3.7667	0.2878		
Differentiation (well and moderate/poor)	4.0269	0.0448*	2.081 (0.983-4.404)	0.0553
TNM stage (I + II/ III + IV)	6.6196	0.0101*	0.483 (0.272-0.860)	0.0135*
Lymph node metastasis (present/absent)	4.1006	0.0429*	1.685 (0.998-2.845)	0.0507
Distant metastasis (present/absent)	0.3590	0.5491		
RTN4 expression (low/high)	6.3235	0.0119*	1.961 (1.134-3.392)	0.0160*

findings have also been reported in breast cancer, gloma and cervical cancer[14-17]. We also observed a significant correlation of high RTN4 expression with advanced tumor invasion, implying that RTN4 may promote tumor growth in human gastric cancer.

In accordance with our data, Wang et al[19] reported that Nogo-B activates both PI3K-Akt and Raf-MEK-ERK pathways in human breast cancer cells and in this network may play a role in the development of cancer. Moreover, *in vitro* study[20], that Nogo-B may induce angiogenesis via downstream Akt activation in endothelial cells in zebrafish. Liu et al[16] reported that Nogo was observed to be highly expressed in liver cancer cell lines. Voeltz et al[21] have also highlighted yeast RTN and Rtn4A/B (Nogo-A/B) as ER membrane-bending proteins that shape the tubular ER, and mediate de novo nuclear pore formation[18]. And it has also been reported that over-expression of RTNs could induce ER stress that in some cases could lead to cell death[22-24]. Moreover, Watari et al[9] reported that the Nogo protein induces apoptosis in various cancer cells when overexpressed, whereas normal cells are relatively resistant to Nogo-dependent apoptosis. Furthermore, transcription of this gene is suppressed in certain types of cancers, suggesting that RTN4 may act to suppress tumor development. In addition, RTN4 expression was increased significantly in malignant tumors compared with the expression in adjacent noncancerous tissues and normal tissues. This significant difference of RTN4 expression in GC and normal tissues suggested that RTN4 might be a prognostic factor in GC.

The Kaplan-Meier survival analysis shows that high expression of RTN4 was significantly associated with a poor prognosis. Univariate analyses revealed that differentiation, lymph node metastasis, TNM sage, and RTN4 expression were significant risk factors affecting overall survival of GC patients. Multivariate analyses showed that besides TNM stage, RTN4 expression was also an independent risk factor predicting survival of GC patients. Overall, our findings indicate that RTN4 may be a useful predictor to the survival of GC patients.

We would like to place some considerations. The follow-up period was relatively short, and our cases size is small. Furthermore, our immunohistochemical-based studies may have observer bias in the process of evaluation the staining. Finally, to assessed gastric cancer tissues after surgery without previous therapy, we omitted individuals with complete response following neoadjuvant chemotherapy and radiotherapy. Therefore, it is necessary to plan and perform a larger prospective further validated study with more sensibility and accuracy.

Conclusions

We demonstrated that high expression of RTN4 was frequently observed in gastric cancer progression and its expression is directly related to more advanced stage and a poor survival rate. Though further studies are needed to explore the mechanism in detail. Therefore, RTN4 may serve as a useful prognostic factor and therapeutic target in gastric cancer in the near future, if its potential clinical utilization is confirmed.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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