Runx3 expression in lymph nodes with metastasis is associated with the outcome of gastric cancer patients

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Received April 1, 2011; Accepted August 2, 2011

DOI: 10.3892/ol.2011.381

Abstract. Accumulating evidence shows that runt-related transcription factor 3 (*Runx3*) is a putative tumor suppressor in various types of cancer, the lower levels of which are associated with a less favorable cancer outcome. However, these studies were restricted to primary cancer lesions. Lymph node metastasis (LNM) is a significant factor in determining the prognosis of patients with gastric cancer and is a frequent target of chemotherapy. In the present study, we investigated the expression of *Runx3* in the lymph nodes (LNs) of stomach carcinoma and the association of *Runx3* expression with the prognosis of patients. The expression of *Runx3* in LNs with and without metastasis was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting. The positive rate of *Runx3* mRNA in LNM specimens was significantly lower (28.4%, 21 out of 74) compared to that of the non-metastatic samples (33.3%, 9 out of 27, P<0.05). Similar findings were obtained by Western blotting. Univariate analysis revealed that the loss of *Runx3* expression in LNs was not only associated with poor clinicopathological factors, such as LNM, distant organ metastasis, later clinicopathological stages and deep infiltration, but also with a lower 5-year survival rate and poorer prognosis. These results strongly suggest a potential diagnostic value of *Runx3* expression in LNs and multiple pathways contributing to the outcome of patients with gastric cancer.

Introduction

Gastric cancer remains a major health problem and a leading cause of cancer-related death, although its incidence has decreased worldwide (1). Numerous patients are identified for gastric cancers at the advanced stage, which is associated with increased recurrence and low overall survival (OS) following potentially curative resection (2). Radical surgery, including lymph node (LN) dissection, has been the standard treatment for early gastric cancer; however, 50% of gastric cancer patients suffer from tumor relapses even after radical surgery (3,4), and their overall prognosis is suboptimal despite aggressive treatment. In recent years, much evidence has clearly demonstrated that multiple genetic changes are responsible for the development and progression of gastric cancer. Thus, it is imperative to investigate the molecular mechanism to improve the outcome of patients with gastric cancer.

Studies have demonstrated that the aggressive nature of gastric cancer is related to mutations of various oncogenes and tumor suppressor genes, as well as abnormalities in certain growth factors and their receptors (5). Recent studies have shown that runt-related transcription factor 3 (*Runx3*) gene mutation is significantly associated with primary gastric cancer progression. However, the detailed mechanism for this relationship has yet to be completely clarified (6). The *Runx3* gene encodes a protein that belongs to the runt domain family of transcription factors involved in mammalian development pathways (7). *Runx3* protein mediates the growth suppression effects of TGF-β in association with SMAD, a downstream protein in the signaling pathway (8,9). Previous studies demonstrated that *Runx3* is markedly down-regulated in gastric cancers compared to the surrounding mucosa, and that lack of *Runx3* is causally related to the growth and progression of human gastric cancer (10), indicating that *Runx3* is a novel tumor suppressor (11-14).

It is known that the down-regulation of *Runx3* in primary gastric cancer tissues is associated with poor prognosis. However, less is known about its expression in LNs, particularly the relationship between the expression of *Runx3* and the prognosis of patients. Lymph node metastasis (LNM) is a significant factor for determining the prognosis of patients with gastric cancer and is a frequent target for chemotherapy. Therefore, it is essential to analyze *Runx3* gene expression in LNs from gastric cancer. In this study, reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting were used to assess the expression level of *Runx3* in 101 LNs of stomach carcinoma, and to describe for the first time the association between the expression of *Runx3* gene in LNs and the outcome of patients with gastric cancer.

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Key words: stomach neoplasms, lymph node metastasis, *Runx3*, prognosis

Materials and methods

Tissue samples. LN specimens were obtained from 101 patients who were diagnosed with primary gastric cancer and underwent gastrectomy and LN dissection in the Department of Surgery, the Tenth People's Hospital of Shanghai, Tongji University, China, between October 2000 and October 2002. The samples were rapidly frozen in liquid nitrogen and stored at -80˚C until being used for the extraction of RNA and protein. The gastric cancer patients had well-documented clinical histories and follow-up information. Clinicopathological data were obtained from a retrospectively constructed medical database, which had been reviewed and confirmed by two pathologists. Patients who had been preoperatively treated with radiation and/or chemotherapy were excluded. Informed consent was obtained from each of the patients and the study protocol was approved by the Ethics Committee of the Tenth People's Hospital of Shanghai, China. Details of the patient characteristics and *Runx3* expression are provided in Table I. Following radical gastrectomy, patients were followed up until death or October 31, 2009, as appropriate. The median follow-up duration was 35.6 months. At the last follow-up examination, 22 (21.8%) patients were still alive, whereas 79 (78.2%) patients had succumbed.

RNA extraction and RT-PCR. LN tissues were homogenized with an ultrasound homogenizer. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (1 μg) was reversely transcribed into cDNA using dNTPs (1 mM), 5X reverse transcription buffer (500 mM Tris-HCL pH 8.3, 250 mM KCL, 50 mM $MgCl_2$ and 50 mM DTT), 16 units RNasin and 2.5 units AMV reverse transcriptase (Gibco BRL, Life Technologies, Carlsbad, CA, USA). PCR conditions were: pre-heating at 95˚C for 5 min followed by 35 cycles of denaturation for 30 sec at 95˚C, annealing for 1 min at 55˚C and extension for 1 min at 72˚C, with a final extension for 5 min at 72˚C. PCR products were separated on 1.5% agarose gel and saved as digital images (Perkin-Elmer, Wellesley, MA, USA). These experiments were performed in triplicate and the mean value was calculated. The value was normalized as the target gene divided by β-actin. The primers used were: *Runx3* gene, forward 5'-ATGACGAGAACTACTCCGCT-3' and reverse 5'-GGTCGGAGAATGGGTTCAGT-3' (PCR product, 396 bp).

Western blotting. The LN homogenates were heated in boiling water for 5 min. Protein concentrations were measured using Bradford's method (Bio-Rad, Hercules, CA, USA). Protein (50 μg) was loaded, separated by 10% SDS-polyacrylamide gel electrophoresis under reducing conditions, and transferred onto equilibrated polyvinylidene difluoride membrane (PVDF; Amersham) by electronic transfer. Membranes were blocked by 5% non-fat dried milk and then incubated with an antibody against *Runx3* (dilution 1:200) overnight at 4˚C; incubation with the secondary antibody was 1 h at room temperature, with three washes after each incubation. ECL reagents were used to show the positive bands on the membrane.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software. Continuous variables were determined as the means ± SD and compared using the two-tailed version of Fisher's exact test. The correlation was evaluated using regression analysis. Survival was analyzed by the Kaplan-Meier method, and differences in the distribution were evaluated using the log-rank test. P<0.05 was considered to be statistically significant.

Table I. Association between the expression of *Runx3* and clinicopathological characteristics of patients with gastric cancer.

Clinicopathological parameters	No.	*Runx3* mRNA		P-value
		≤0.362	>0.362	
Gender				>0.050
Male	69	38	31	
Female	32	12	20	
Age (years)				>0.050
<60	46	21	25	
≥60	55	29	29	
Growth pattern				<0.050
Expansive	39	24	15	
Infiltrative	62	41	21	
Histological grade				>0.050
WD and MD	55	31	24	
PD	46	20	26	
Infiltrative depth				<0.050
T1+T2	44	29	15	
T3+T4	56	32	24	
LN metastasis				<0.001
Absence	27	18	9	
Presence	74	53	21	
Distant metastasis				<0.001
Absence	73	42	31	
Presence	28	17	11	
TNM stage				<0.001
I and II	24	19	5	
III and IV	77	55	22	

LN, lymph node; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated.

Results

Expression of Runx3 in LN specimens. As shown in Fig. 1, *Runx3* mRNA was examined in 74 LNs with metastasis and 27 LNs without metastasis. RT-PCR results revealed that *Runx3* was positively expressed in 28.4% (21 out of 74) LNs with metastasis and 33.3% (9 out of 27) without metastasis (P<0.001). The expression of *Runx3* protein in LNs was further confirmed by immunoblotting, which revealed a 44-kDa protein band (Fig. 2). Similarly, the results also revealed that expression of the *Runx3* protein was significantly lower in LNM tissues than in those without metastasis. A consistent correlation was found between the protein and mRNA expression of *Runx3*.

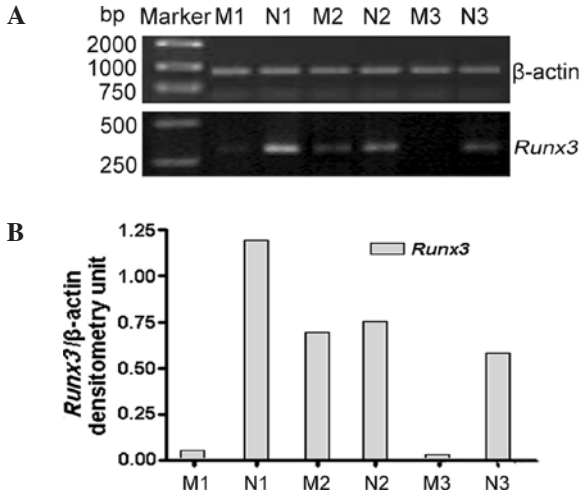

Figure 1. (A) Expression of *Runx3* mRNA in LN specimens with metastasis (M) and without metastasis (N). The 396-bp human *Runx3*-specific sequence and a 906-bp β-actin sequence were amplified from the cDNA of gastric cancer LN tissues, separated by agarose gel electrophoresis and visualized by ethidium bromide staining. (B) Densitometry of *Runx3* transcripts standardized to β-actin, for the conditions listed in (A).

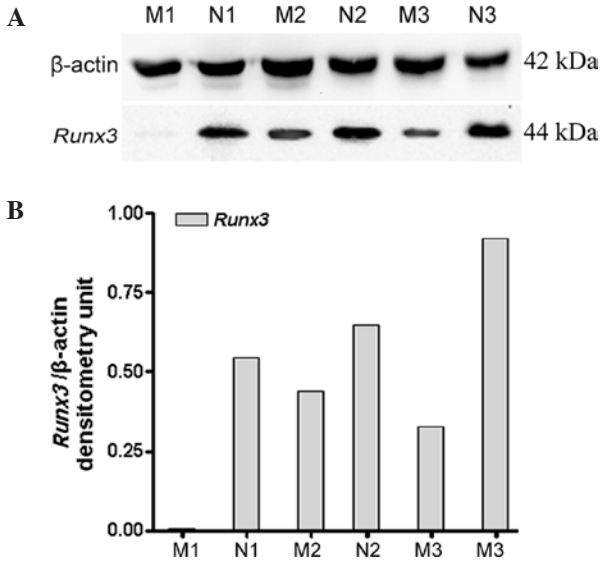

Figure 2. (A) Representative samples of Western blotting *Runx3* proteins in human gastric cancer LNs, LN specimens with metastasis (M) and without metastasis (N). The upper panel shows the β-actin levels as the internal control. (B) Densitometry of *Runx3* proteins standardized to β-actin, for the conditions listed in (A).

Relationship between the expression levels of Runx3 and clinicopathological parameters. The clinicopathological characteristics of the patients and associations with *Runx3* mRNA expression in LNs are shown in Table I. The gastric cancer patients (69 males and 32 females with a median age of 68.6 years; range 45-88) had undergone gastrectomy and lymphadenectomy. Seventy-four patients were diagnosed with LNM. Thirty-nine patients had an expansive growth pattern, whereas 62 had an infiltrative classification. With respect to TNM tumor staging, gastric cancer LNs with a higher expression of *Runx3* were 20.8% (5 out of 24) at stages I and II, and 28.6% (22 out of 77) at stages III and IV, respectively. Univariate analysis showed that the level of *Runx3* mRNA expression was inversely correlated with advanced clinical stage (P<0.001), distant metastasis (P<0.05), LNM (P<0.001), infiltrative depth (P<0.05) and histological grade (P<0.001). However, no significant associations were observed with patient age or gender (P>0.05), or with the growth pattern of the tumor (P>0.05) (Table I).

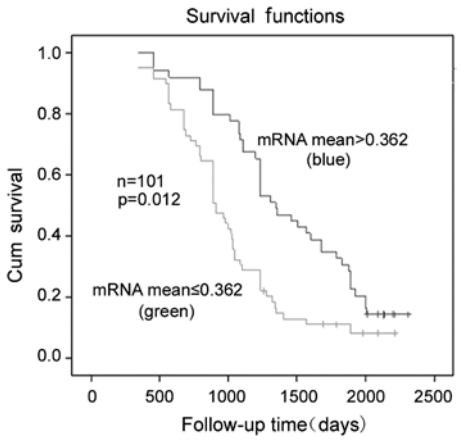

Figure 3. Kaplan-Meier survival analysis for gastric cancer patients according to the expression of *Runx3*. The y-axis shows the percentage of patients; the x-axis, their survival in days. The green line shows LN specimens with a lower (≤0.362) *Runx3* mRNA expression, with a trend of poorer survival than the blue line, indicating specimens with a higher (>0.362) *Runx3* mRNA expression. Expression in LN specimens (log-rank test, P=0.012). Mean survival time was 1,021 days for the lower expression group and 1,234 days for the higher expression group.

Survival analysis. The patients had a median follow-up duration of 35.6 months (range 3-84) following radical gastrectomy. Seventy-nine patients succumbed due to recurrence or metastasis of the tumor and 22 patients survived. According to the median of the gray scale bands (0.362; range 0.088-1.016) for *Runx3* mRNA expression in LNs, the 101 cases were separated into two groups: the lower-expressing group (≤0.362) and the over-expressing group (>0.362). Kaplan-Meier analysis confirmed that patients with a higher *Runx3* expression (>0.362) had significantly better survival outcomes compared to the lower group (≤0.362). The median OS for the high and low *Runx3* mRNA expression groups were 1,234 and 1,021 days, with a 5-year survival rate of 16 and 28%, respectively (log-rank test, P=0.012; Fig. 3).

Discussion

In the present study, *Runx3* expression was first examined at the RNA and protein levels in the LN tissues of gastric cancer using RT-PCR and Western blotting. The results indicate that the expression of *Runx3* mRNA was more frequent in the LNs without metastasis than in the metastatic regions. Western blotting demonstrated similar findings. Loss of *Runx3* expression was associated with a lower 5-year survival rate and poorer prognosis of patients.

Runx3 is a transcription factor that regulates lineage-specific gene expression in developmental processes and is involved in the formation of a variety of cancers. *Runx3* elicits its tumor suppressor role by controlling the expression of numerous genes involved in the growth, apoptosis and

differentiation of gastric epithelial cells (15-19), as well as genes involved in angiogenesis and cell junction formation (20,21). Mounting evidence suggests that *Runx3* is a tumor suppressor. *Runx3* is expressed in glandular stomach epithelial cells, and loss of *Runx3* expression is causally related to the genesis and progression of gastric cancer and correlates with differentiation, metastasis and poor prognosis of gastric cancer (22).

In primary gastric cancer, much is known about the expression of *Runx3*, but less is known about the relationship between *Runx3* expression in LNs and the prognosis of patients. In this study, various clinicopathological factors were analyzed for *Runx3* expression in LNs. A low proportion of *Runx3* mRNA expression in LNM was found to be significantly associated with poor clinicopathological factors, such as deep infiltration, distant organ metastasis, poor differentiation, LNM and later clinicopathological stages. However, no significant associations were observed with age, gender and the growth pattern of the tumor (Table I). *Runx3* expression was also associated with a lower 5-year survival rate and poorer prognosis of patients (Fig. 3). These results support our previous studies (23), which revealed that the low expression of *Runx3* in primary gastric cancer was associated with a significantly shorter survival.

LNM is one of the most significant factors predicting recurrence in patients who have undergone gastrectomy for stomach carcinoma (24,25). In China, a gastrectomy along with an extended LN dissection is an established procedure and widely accepted as the standard for the surgical treatment of gastric cancer. However, certain patients develop local or distant tumor recurrence even if a curative resection of the primary tumor is performed. In our studies, the rate of LNM in gastric cancer was 73.7%, which is higher than that previously reported in Japan and similar to reports from China (26,27). This finding may be explained due to the bias of histological criteria employed in our study and those used in Japan. We also found that the presence of metastasis is significantly correlated with the postoperative prognosis of patients with gastric cancer (data not shown).

Concerning LNM, Park *et al* (28) reported MGMT expression to be significantly associated with LNM in patients with gastric cancer. Gene analysis along with a protein examination is recommended to increase the positive detection of LNM and prognosis, leading to a more accurate diagnosis and therapy of the tumor in gastric cancer. Furthermore, such an analysis may positively contribute to the selection of optimal chemotherapeutic regimens based on the gene. Compared to the data generated in this study, *Runx3* gene expression in the LNs was significantly associated with poor clinicopathological factors and poorer prognosis of patients. Consequently, a gene expression analysis using LN tissue specimens may aid in the detection of the survival rate and prognosis of stomach carcinoma. However, the precise link between *Runx3* expression in LNs and LNM remains unclear and further biological studies are required to explain this effect (29).

In conclusion, a loss or substantial decrease of *Runx3* expression was observed in the group presenting with LN with metastasis as compared to the group without metastasis, and a low expression of *Runx3* was significantly associated with unfavorable clinicopathological variables and a shorter survival in gastric carcinoma patients. LNM is also associated with a poorer prognosis of gastric cancer. It may contribute to the detection of gene expression and gastric cancer metastasis as a potential molecular marker and a potential target in the therapy for gastric cancer.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (no. 30171039) and the Natural Science Foundation of Jiangsu Province (no. BK2008115).

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