

# The regulatory mechanism of CCR7 gene expression and its involvement in the metastasis and progression of gastric cancer

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Received: 11 January 2013 / Accepted: 27 February 2013 / Published online: 22 March 2013  
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**Abstract** Gastric cancer is the second leading cause of cancer mortality, but the molecular mechanisms underlying its progression and metastasis remain unclear. CCR7 and Dicer 1 protein expression in 80 gastric adenocarcinomas and 40 peritumoral tissues were measured by immunohistochemical staining. The expression of let-7a miRNA in serum, tumor tissues, and peritumoral tissues was measured by real-time PCR. The role of let-7a in CCR7 protein expression, migration, and invasion of gastric cancer cells was tested in vitro. Dicer 1 protein expression was found to be significantly reduced, whereas CCR7 protein expression was significantly increased in gastric adenocarcinomas compared to peritumoral tissues. The let-7a miRNA levels in the serum and tumor tissues of gastric adenocarcinoma patients were significantly lower than in the serum of healthy controls and peritumoral tissues, respectively. Dicer 1 protein positively correlated with let-7a miRNA level, but negatively correlated with CCR7 protein level in gastric adenocarcinoma. Negative Dicer 1 protein and let-7a miRNA expression and positive CCR7 protein expression significantly correlated with lymph node metastasis, depth of invasion, high clinical TNM stage, and larger tumor size. *Let-7a* transfection significantly inhibited CCR7 protein expression, migration, and invasion of MNK-45 cells in vitro. High expression of CCR7 protein and low expression of Dicer 1 protein and let-7a miRNA are significantly associated with the metastasis and progression of gastric cancer. High CCR7 protein expression may be caused by the loss of Dicer 1 protein expression and reduced let-7a miRNA level in gastric cancer. The serum let-7a level might be a marker for the diagnosis of gastric cancer.

**Keywords** CCR7 · Dicer 1 · Let-7a · miRNA · Gastric cancer · Metastasis

## Introduction

Chemokines comprise a family of basic chemotactic proteins that exert their effects by binding to G-protein-coupled receptors [1]. Many chemokine receptors are involved in the regulation of cytoskeletal rearrangement, adhesion, and directional migration [2]. C-C chemokine receptor type 7 (CCR7) is a newly identified chemokine receptor that is mainly expressed in differentiated lymphocytes and on the cell surface of dendritic cells. The interaction of CCL19/21 ligand and CCR7 receptor is thought to mediate lymphocyte migration [1, 3]. Recent studies in gastric cancer cells have revealed that high chemokine receptor expression is closely related to the degree of malignancy [1, 3]. Studies have shown that CCR7 is highly expressed in lymph nodes, while high CCR7 expression in gastric cancer cells is related to tumor lymphatic metastasis and prognosis [4–6]. However, the regulation of CCR7 expression in gastric cancer cells remains unexplored.

miRNAs are small (~22 nucleotides), non-coding RNA molecules whose primary function is to repress gene expression by targeting the 3'-untranslated region (3'-UTR) of mRNAs to inhibit their translation and/or promote their degradation [7, 8]. Recent studies demonstrated that miRNAs function as tumor suppressors to modulate multiple oncogenic cellular processes, including cell proliferation, apoptosis, invasion, and metastasis [9]. The expression of many miRNAs, such as miR-7, -10a, -15, -21, -23a, -27a, -29b, -34a, and -125b, has been reported to be downregulated in gastric cancer cells and tumor tissues [10]. The loss of expression of these miRNAs was associated with the metastasis, invasion, and poor prognosis of gastric cancers [11]. Let-7a is a 22-nt non-coding miRNA that functions

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as a tumor suppressor in many tumors. In breast cancer cells, let-7a miRNA has been found to suppress breast cancer cell migration and invasion through downregulation of CCR7 expression [12]. However, whether the downregulation of let-7a miRNA expression is responsible for the overexpression of CCR7 expression in gastric cancer tissues has not been reported.

Although global downregulation of miRNAs in gastric cancer cells has been observed, how were these miRNAs regulated remains unclear. Dicer is widely recognized as a key enzyme that controls miRNA production during miRNA genesis. Dicer's primary role is to cut pre-miRNA into mature double-stranded miRNA fragments of approximately 15–30 nucleotides in length [13, 14]. Given the roles played by Dicer, the level or activity of Dicer determines cellular miRNA levels. We therefore hypothesized that changes in the expression or activity of Dicer may be responsible for the global downregulation of miRNA expression in GC tumors [15]. However, whether the expression of Dicer is downregulated in gastric cancers has not been reported.

In this study, we investigated CCR7, let-7a, and Dicer 1 expression in 80 gastric cancer tissues compared with peritumoral tissues and further analyzed the correlation of serum let-7a levels with its levels in tumor tissues. Our study highlighted the key role of CCR7 protein in the clinicopathology of gastric cancer and the regulatory mechanism of CCR7 gene expression.

## Materials and methods

### Subjects

This study was approved by The Ethics Committee for Human Research, Xiangya Hospital, Central South University. Written consent has been obtained from all patients. Eighty gastric adenocarcinoma specimens were collected at Xiangya Hospital from February 2008 to December 2010. The gastric adenocarcinomas were diagnosed by clinical findings, histology, and immunostaining. The 80 gastric adenocarcinoma patients consisted of 52 males and 18 females with an average age of 54.6 years (range, 38 to 74 years). Among the 80 gastric adenocarcinomas, 37 were well-differentiated adenocarcinoma, 27 were moderately differentiated adenocarcinoma, while 16 were poorly differentiated adenocarcinoma. Lymph node metastasis was found in 56 cases. Among the 80 cases, 24 cases were TNM stage I+II and 56 were stage III+IV; 24 cases had T1+T2 invasion and 56 cases had T3+T4 invasion. No patient was given preoperative chemotherapy, radiotherapy, or steroids and hormone therapy. Peritumoral tissues were collected from 24 cases with stage I+II tumors and 16 cases with stage III tumors. Peritumoral tissues were collected at 5–7 cm from the tumor

mass. At the same time, blood was collected from all patients and 45 healthy adult individuals. Serum miRNA was isolated immediately. Partial tumor tissues and peritumoral tissues were snap frozen in liquid nitrogen for RNA isolation, while partial tissues were fixed in 10 % formalin and then embedded in paraffin.

### Immunohistochemical staining

Four-micrometer-thick sections were cut from routinely paraffin-embedded tumor tissues and peritumoral tissues. Staining was conducted with streptavidin–peroxidase immunohistochemistry kit (Wuhan Boster Biological Engineering Co., Ltd, China). The rabbit anti-Dicer 1 and anti-CCR7 antibodies were purchased from Santa Cruz Company (Santa Cruz, CA, USA). The positive control was a positive biopsy provided by Beijing Zhongshan Biotechnology Company (Beijing, China), while the negative control was created by replacing the primary antibody with 5 % fetal bovine serum. Briefly, sections were deparaffinized and then incubated with 3 % H<sub>2</sub>O<sub>2</sub> in the dark for 15 min. Antigen retrieval was performed by placing sections in 0.01 % citric acid (pH 6.0) and microwaving for 10 min. After blocking with goat serum, sections were incubated with primary antibody (1:100) at 4 °C for overnight. After washing with 1× PBS for 3×5 min, sections were incubated with biotin-labeled secondary antibody at room temperature for 10 min, incubated with streptomycin avidin–peroxidase solution for 10 min, stained with DAB, and counterstained with hematoxylin. The slides were dehydrated with different concentrations (70–100 %) of alcohol and soaked in xylene for 3×5 min. The percentage of positive cells was calculated from ten random fields. Cases with positive cells ≥25 % were considered positive, while cases with positive cells <25 % were considered negative. The positive expression was further scored for correlation analysis: no positive cells was given a score of 1; 1–25 % of positive cells was given a score of 2; 26–50 % positive cells was given a score of 3; 51–76 % of positive cells was given a score of 4; and 76–100 % of positive cells was given a score of 5.

### Real-time PCR

Quantitative real-time PCR was used to detect miRNA let-7a expression in the serum, tumor tissues, and peritumoral tissues. Total RNA from whole blood, tumor tissues, and peritumoral tissues was extracted using Trizol reagent, (Invitrogen, Carlsbad, CA, USA), and reverse transcription was performed using One Step PrimeScript<sup>®</sup> miRNA cDNA Synthesis Kit. Quantitative PCR reactions were carried out using the SYBR Premix Ex Taq<sup>™</sup> kit (TaKaRa, Shiga, Japan). Relative quantification (RQ) of gene mRNA and miRNA expression was determined by comparative CT

method ( $RQ=2^{-\Delta\Delta CT}$ ) and normalized to U6 level, respectively. The let-7a miRNA was amplified using forward primers: 5'-TGAGGTAGTAGGTTGTATAGTT-3' and reverse primers provided with the kit. U6 was amplified using forward primer: 5'-GCAAGGATGACACGCAAATTC-3' and reverse primers provided with the kit.

#### Cell culture

MNK-45, a human gastric cancer cell line, was obtained from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 with 10 % fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37 °C, 5 % CO<sub>2</sub>.

#### Testing the role of let-7a miRNA in cultured MNK-45 cells

The miRNA of matured let-7a was synthesized by Invitrogen (Grand Island, NY, USA). MNK-45 cells were passaged 24 h before transfection. Cells in 10-cm dishes were transfected with a control oligonucleotide with scrambled sequence or let-7a mRNA using Lipfectamine 2000 following the user manual (Invitrogen). Forty-two hours later, the cells were harvested for Western blot of CCR7 expression.

#### Western blot

The cells were homogenized and Western blot was performed as previously described [12]. The anti-CCR7 antibody was purchased from Santa Cruz Company. To control for loading efficiency, the blots were stripped and reprobed with α-tubulin antibody (Sigma-Aldrich). The images were scanned with Adobe photoshop (Adobe, San Jose, CA). Expression of CCR7 protein was evaluated relative to α-tubulin expression (i.e., relative density=subunit/α-tubulin levels).

#### Cell migration assay

The migration experiment was performed using a modified Boyden chamber apparatus as previously described [16]. Briefly, MNK-45 cells were transfected with a oligonucleotide with scrambled sequence or let-7a miRNA as described above for 24 h. Cells were then harvested and resuspended with RPMI 1640 containing 0.1 % BSA. The cells ( $8 \times 10^4/100 \mu\text{l}$ ) were loaded into the upper chamber. The membrane filter (8 µm pores) was coated with 100 µg/ml of rat tail collagen overnight at 4 °C. The number of cells that migrated to the lower surface in 5 h was determined by counting the cells in four fields.

#### Matrigel invasion assay

The invasion activity of MNK-45 cells was assessed using a Matrigel invasion chamber (BD Biosciences, San Jose, CA)

according to the instructions provided by the manufacturer. The scrambled oligonucleotide or let-7a mRNA transfected cells ( $8 \times 10^4/800 \mu\text{l}$ ) were loaded on the transwell chamber (8 µm pore) covered with growth factor-reduced Matrigel. The number of cells that migrated to the lower surface during a 24-h incubation period was determined by counting the cells in four fields [17].

#### Statistical methods

All statistical analyses were performed using SPSS v17.0 (SPSS Inc., Chicago, IL, USA). Gene expression was assessed using the Chi-squared test or *t* test. A  $p < 0.05$  was considered statistically significant.

## Results

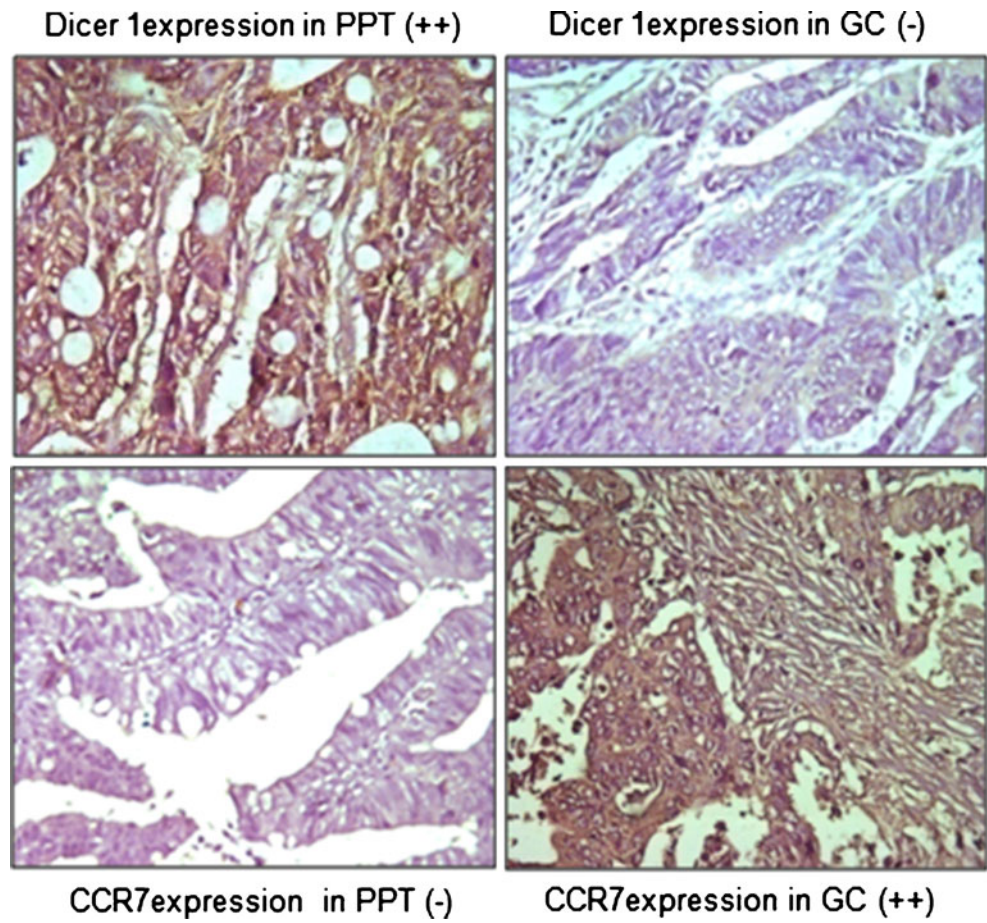
### Dicer 1 and CCR7 expression in gastric cancer and peripheral tissues

Immunohistochemical staining showed that positive Dicer 1 protein expression was mainly located in the cytoplasm and some in the nucleus and cell membrane (Fig. 1a, b). In 80 gastric adenocarcinomas, 27.5 % were Dicer 1 protein positive, while 72.5 % showed negative Dicer 1 protein expression. In contrast, 5 % of 40 peritumoral tissues were Dicer 1 protein negative, while 95 % were Dicer 1 protein positive expression (Table 1). The Dicer 1 protein expression was significantly decreased in adenocarcinomas than in peritumoral tissues ( $p < 0.001$ ). The percentage of cases with high let-7a miRNA (higher than the medium of 80 tumor tissues and 40 peritumoral tissues) and low let-7a miRNA (lower than the medium) was the same as Dicer 1 protein in adenocarcinomas (Table 1). In peritumoral tissues, 97.5 % showed high let-7i expression. The let-7i miRNA expression was significantly decreased in adenocarcinomas ( $p < 0.001$ ). CCR7 protein expression was mainly located in the nucleus and cell membrane, and some in the cytoplasm (Fig. 1c, d). Among the 80 adenocarcinomas, 67.5 % were CCR7 protein positive expression, while 32.5 % were negative expression. In 40 peritumoral tissues, no positive CCR7 protein expression was detected (Table 1). In adenocarcinomas, CCR7 protein expression was significantly higher in tumor tissues than that in peritumoral tissues ( $p < 0.001$ ).

Let-7a miRNA expression in gastric adenocarcinomas, peritumoral tissues, and serum as well as its correlation with Dicer 1 and CCR7 protein expression in adenocarcinomas

We measured *let-7a* miRNA expression in the serum of 80 gastric adenocarcinoma patients, 45 healthy adult

**Fig. 1** Dicer 1 and CCR7 immunohistochemistry. Original magnification  $\times 400$ . Dicer 1 and CCR7 protein positive staining was mainly located in the cytoplasm and some in the nucleus and cell membrane. *PPT* peritumoral tissue, *GC* gastric cancer tissue



individuals, 80 gastric adenocarcinoma tumor tissues, and 40 peritumoral tissues by real-time PCR (Fig. 2a). *Let-7a* miRNA expression was significantly lower in the serum of cancer patients than in the serum of healthy individuals. *Let-7a* miRNA expression was significantly lower in gastric adenocarcinoma tissues than in peritumoral tissues (Fig. 2a). To investigate whether *let-7a* miRNA expression could be a diagnostic marker for gastric cancer, we performed correlation analysis of the *let-7a* levels in serum and adenocarcinoma tissues (Fig. 2b). The serum *let-7a* level significantly correlated with its level in tumor tissues ( $p < 0.001$ ). We further analyzed the correlations of Dicer 1 protein and *let-7a* (Fig. 2c) or *let-7a* and CCR7 (Fig. 2d) levels in adenocarcinomas. Dicer 1 levels positively correlated with *let-7a* levels ( $p < 0.001$ ), while *let-7a* mRNA levels negatively correlated with CCR7 protein levels in adenocarcinomas ( $p < 0.001$ ).

Relationship between Dicer 1, *let-7a*, and CCR7 expression and clinicopathological characteristics in gastric cancer

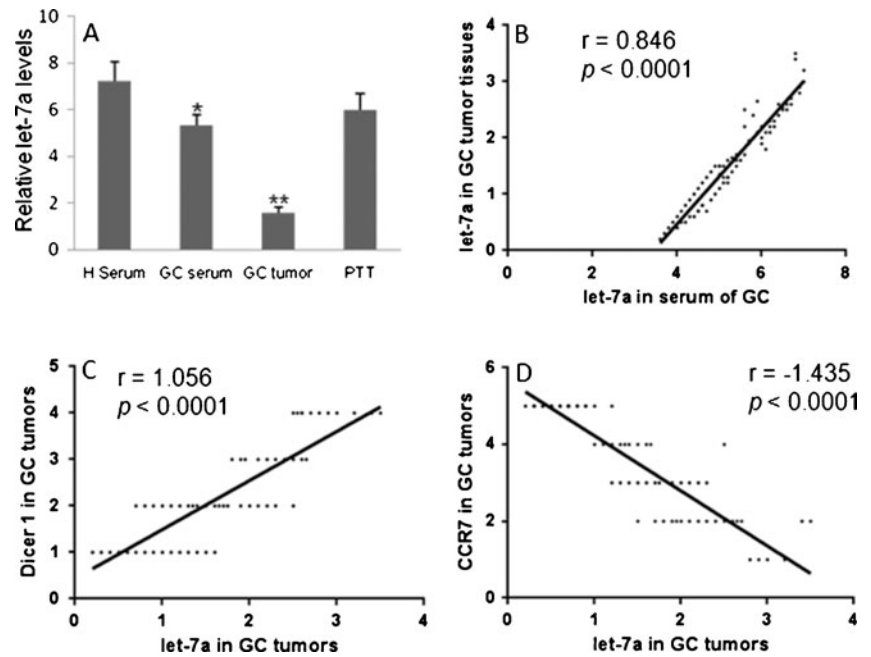
Negative Dicer 1 protein expression was significantly related to the lymph node metastasis ( $p < 0.001$ ), depth of invasion ( $p < 0.001$ ), higher clinical TNM stage ( $p < 0.005$ ), and larger tumor size ( $p = 0.002$ ). Dicer 1 expression had no association with age, sex, or tumor differentiation (Table 2). The relationship of *let-7a* miRNA expression with clinicopathological characteristics was highly similar to Dicer 1. CCR7 protein expression was significantly increased in adenocarcinomas compared to peritumoral tissues. Positive CCR7 protein expression was significantly associated with lymph node metastasis ( $p < 0.001$ ), depth of invasion ( $p = 0.001$ ), higher clinical TNM stage ( $p < 0.001$ ), and larger tumor size ( $p = 0.039$ ). CCR7 protein expression had no association with age, sex, or tumor differentiation (Table 2).

**Table 1** Positive rate of Dicer 1, *let-7a*, and CCR7 in gastric tumor tissue cancer and peritumoral tissue

*GCT* gastric cancer tissue, *PPT* peritumoral tissue

Tissue	Dicer 1		<i>p</i> value	CCR7		<i>p</i> value	Let-7 a		<i>p</i> value
	+	-		+	-		+	-	
GCT	22	58	<.001	54	26	<.001	22	58	<.001
PPT	38	2		0	40		39	1	

**Fig. 2** Let-7a miRNA expression and correlations with Dicer 1 and CCR7 levels. **a** Let-7a miRNA expression in serum, tumor tissues, and peritumoral tissues. **b** Correlation of let-7a levels between serum and tumor tissues. **c** Correlation of let-7a levels with Dicer 1 protein levels in gastric adenocarcinoma. **d** Correlation of Let-7a levels with CCR7 protein levels in gastric adenocarcinoma



Let-7a inhibited CCR7 expression, MNK-45 cell migration and invasion

The regulatory effect of let-7a on CCR7 gene expression in gastric cancer cells was analyzed by transfection of let-7a miRNA into MNK-45 cells. Western blot showed that transfection of let-7a miRNA significantly inhibited CCR7 protein level (Fig 3a, b). The role of let-7a miRNA on gastric cancer cell invasion and metastasis was tested in vitro by its effect on the migration and invasion of MNK-45 cells. MNK-45 cells

were transfected with scrambled sequence or let-7a sequence for 24 h and then subjected to migration (Fig. 3c) and invasion (Fig. 3d) assay. Let-7a miRNA transfection significantly inhibited the migration and invasion in MNK-45 cells.

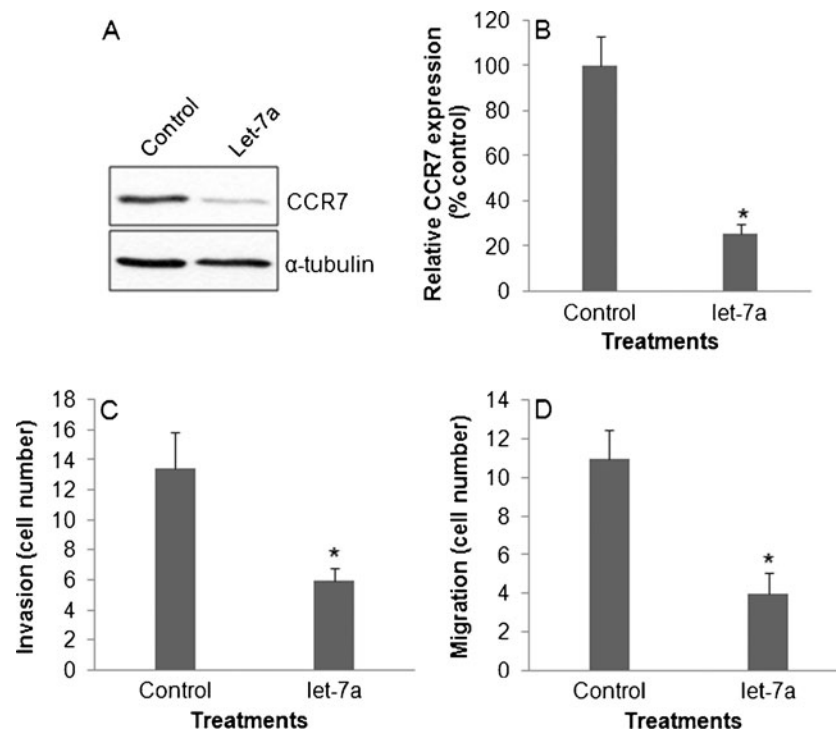
**Discussion**

Gastric cancer is the fourth most common cancer and the second leading cause of cancer mortality worldwide [18].

**Table 2** Relationship between clinicopathological parameters of gastric cancer and Dicer 1, let-7a, and CCR7 protein expression

Factors		Dicer 1			p value	Let-7a			p value	CCR7			p value
		Case											
		+	-		+	-		+	-				
Age	>60	48	13	35	0.919	14	34	0.683	33	15	0.770		
	<60	32	9	23		8	24		21	11			
Gender	Male	52	15	37	0.713	15	37	0.713	35	17	0.960		
	Female	28	7	21		7	21		19	9			
TD	High	37	11	26	0.993 <sup>a</sup>	12	25	0.811 <sup>a</sup>	23	14	0.814 <sup>a</sup>		
	Middle	27	8	19	0.494 <sup>b</sup>	8	19	1.000 <sup>b</sup>	16	11	0.017 <sup>b</sup>		
	Low	16	3	13	0.510 <sup>c</sup>	4	12	0.748 <sup>c</sup>	15	1	0.022 <sup>c</sup>		
LNM	N <sup>0</sup>	24	14	10	<.001	14	10	<.001	9	15	<.001		
	N <sup>+</sup>	56	8	48		9	47		45	11			
Invasive depth	T1+T2	24	13	11	<.001	12	12	0.001	10	14	0.001		
	T3+T4	56	9	47		8	48		44	12			
TNM stage	I+II	24	14	10	<.001	14	10	<.001	9	15	<.001		
	III+IV	56	8	48		8	48		45	11			
Tumor size	>5 cm	44	6	38	0.002	5	39	0.001	34	10	0.039		
	<5 cm	36	16	20		16	20		20	16			
Tumor location	Colon	48	17	31	0.052	18	30	0.034	28	20	0.032		
	Rectal	32	5	27		5	27		26	6			

TD tissue differentiation, LNM lymph node metastasis  
<sup>a</sup>Compared with middle  
<sup>b</sup>Compared with low  
<sup>c</sup>Compared with high



**Fig. 3** Let-7a inhibited CCR7 protein expression, migration, and invasion of MNK-45 cells. **a** Representative Western blot. MNK-45 cells were transfected with let-7a or scrambled sequence (control) for 42 h. The cells were harvested for Western blot of CCR7 protein expression. **b** Relative CCR7 protein expression. The blots were scanned with Adobe photoshop. Expression of CCR7 protein was evaluated relative to  $\alpha$ -tubulin expression and then normalized to

control. **c** let-7a-mediated inhibition of invasion. **d** let-7a transfection-mediated inhibition of migration. MNK-45 cells were transfected with let-7a or scrambled sequence (control) for 24 h. The cells were then loaded into the upper chamber. The results are expressed as migrated cell numbers in four fields under microscopy at  $\times 400$  magnification. Data were presented as the means  $\pm$  SEM of five separate experiments

However, the molecular mechanisms driving the progression, metastasis, and poor prognosis of gastric cancer are not fully elucidated. In this study, we demonstrated that positive CCR7 protein expression is significantly associated with lymph node metastasis, invasion, and progression of gastric cancer. The overexpression of CCR7 protein is associated with reduced let-7a miRNA expression caused by the downregulation of Dicer 1 protein expression. Our study first provided link between downregulation of Dicer 1 protein expression and global miRNA deficiency in gastric cancer. Although downregulation of let-7a miRNA expression and overexpression of CCR7 protein were previously observed in gastric cancer, the correlation between let-7a levels and CCR7 protein expression has not been established in gastric cancer. In this study, we observed a significant correlation between let-7a miRNA and CCR7 protein levels in gastric adenocarcinomas.

Overexpression of CCR7 protein in gastric cancer has been associated with preferential lymph node metastasis of gastric carcinoma [1, 4]. Ishigami et al [19] study demonstrated that CCR7-positive gastric cancer patients had significantly poorer surgical outcomes than CCR7-negative patients. However, CCR7 protein was not an independent prognostic factor, although it can be used to predict lymph node metastasis because of the close correlation with lymphatic factors.

Besides confirming the effect of CCR7 in lymph node metastasis, we also demonstrated that positive CCR7 protein expression was significantly related to the depth of invasion ( $p=0.001$ ), higher clinical TNM stage ( $p<0.001$ ), larger tumor size ( $p=0.039$ ), and tumor differentiation ( $p<0.05$ ). A previous study revealed that low levels of let-7a miRNA are involved in the tumorigenesis of gastric cancer [18] and are associated with shorter overall survival and relapse-free survival of gastric cancer patients [20]. Overexpression of let-7a miRNA expression inhibited gastric cancer cell growth in vitro and in vivo [21]. However, no study clarified the relationship between let-7a and CCR7 protein expression in gastric cancer. In contrast, a study in breast cancer showed that let-7a miRNA suppresses breast cancer cell migration and invasion through downregulation of CCR7 protein expression [12]. Our study found that let-7a expression was significantly reduced, while CCR7 protein expression was significantly increased in gastric adenocarcinoma tissues compared with peritumoral tissues. Correlation analysis revealed that let-7a miRNA levels negatively correlated with CCR7 protein levels in gastric adenocarcinoma ( $p<0.001$ ). Let-7a is currently the only miRNA that was experimentally identified to target CCR7. We therefore conclude that overexpression of CCR7 gene is due to the deficiency of let-7a miRNA expression.

The expression of many miRNAs has been found to be downregulated in gastric cancers and have been associated with metastasis, invasion, and poor prognosis of gastric cancers [10, 11]. However, the mechanism responsible for global downregulation of miRNAs expression in gastric cancers has not been identified. In this study, we demonstrated that Dicer 1 protein expression was significantly reduced in gastric adenocarcinomas, which significantly correlated with the level of let-7a miRNA ( $p < 0.001$ ). Dicer's cleavage of pre-miRNA into mature miRNA is a key process in the synthesis of mature miRNA [13, 14]. The deficiency of Dicer expression or activity is therefore proposed to result in abnormalities in global miRNA processing. A previous study revealed that abrogation of global miRNA processing promotes tumorigenesis [22]. We therefore hypothesized that loss of Dicer 1 protein expression may be responsible for the global downregulation of miRNAs in gastric cancer. It has been demonstrated that *DICER1* gene mutant tumors eventually underwent complete inactivation of *DICER1* gene via an alternative mechanism like epigenetic silencing [23]. Further studies are needed to determine whether loss of Dicer 1 protein expression in gastric cancer is due to the mutation of *DICER1* gene or hypermethylation of *DICER1* gene promoter.

In conclusion, CCR7 protein plays a key role in the metastasis and progression of gastric cancer. The elevated CCR7 protein expression may be caused by the deficiency of let-7a miRNA expression due to the downregulation of *DICER1* gene expression. The mechanism for the silencing of *DICER1* gene expression requires further studies.

**Acknowledgments** We acknowledge the financial support of the National Hi-tech Program (863 Project) of China (no. 2007AA021804, 2007AA021809).

**Conflict of interest** All authors declared no conflict of interest.

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