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ORIGINAL ARTICLE

Relationship between PRRX1, circulating tumor cells, and clinicopathological parameter in patients with gastric cancer

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Summary

Purpose: *Paired related homoeobox 1 (PRRX1) has been identified as a new epithelial-mesenchymal transition (EMT) inducer in gastric cancer and that PRRX1 upregulation is closely correlated with gastric cancer metastasis. In addition, circulating tumor cells (CTCs) play an important role in the process of gastric cancer's distant metastasis. Our study aimed to correlate Prrx1, CTCs and the clinicopathological parameters in primary gastric cancer patients.*

Methods: *Expressions of PRRX1 in a sample of 95 gastric carcinoma and adjacent nontumorous tissues were detected by immunohistochemistry. Then the integrated subtraction enrichment and immunostaining fluorescence in situ hybridization (SET-imFISH) platform were applied to detect and characterize CTCs in patients with gastric cancer. Finally, their correlations with clinicopathological parameters could be analyzed.*

Results: *The positive rate of PRRX1in gastric cancer was 56.84% and the rate was 36.84% in adjacent normal gastric mucosa, which was confirmed to be statistically significant. In the meantime, both the expression of PRRX1 and the positive rate of CTCs did not significantly correlate with age, gender or histologic type ($p>0.05$) but significantly related to tumor size, grade of differentiation, lymph node invasion, vascular invasion, metastasis status, depth of tumor invasion, lymph node metastasis and TNM stage ($p<0.05$). Besides, there was a close relationship between the PRRX1 of gastric cancer and the CTCs of peripheral blood specimens of cancer patients with the correlation coefficient 0.322.*

Conclusion: *Gastric cancer tissues showed that the level of PRRX1 expression was higher compared to the adjacent normal gastric mucosa. Both the expression of PRRX1 and the positive rate of CTCs significantly correlated with clinicopathological parameters. In addition, there was a positive correlation relationship between the PRRX1 of gastric cancer and the CTCs of peripheral blood specimens of cancer patients. These findings demonstrate that higher-level expression of PRRX1 in gastric cancer tissues increased the amount of CTCs in peripheral blood and facilitated the invasion and metastasis in patients with gastric cancer. Meanwhile, it gave some clues to clinical treatment. CTCs may contribute to promotion in diagnosis, therapy monitoring and prognosis of gastric cancer*

Key words: *gastric cancer, paired related homoeobox1 (prrx1), circulating tumor cells, clinicopathological parameter, epithelial–mesenchymal transition, metastasis*

Introduction

Epidemiological statistics confirm that gastric cancer (GC) is the fourth most common cancer and the second most common cause of cancer death around the world. There is a 10-fold international variation in the occurrence of GC, with rates in men double those of women. The high occurrence

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rate is particularly present in East Asia, Eastern Europe, and parts of Central and South America. This malignancy holds a rather poor prognosis, with 5-year relative survival below 30% in most countries [1].

Invasion and metastasis are the signs of malignant tumor's evolution [2]. Sufficient evidence proves that EMT is the initial stage before metastasis of primary carcinomas [3]. Furthermore, some of EMT inducers, such as TWIST1 [4], SNAI1 [5] are verified to be related to EMT in cancer. According to current research, PRRX1 has been reported as another new EMT inducer. Moreover, there are papers providing corroborative evidence for the overexpressing PRRX1 assists in EMT of breast cancer and colorectal cancers [6]. Further studies confirmed that PRRX1 facilitates EMT process in GC cells by activated signal pathway of Wnt/b-catenin and that the increased expression of PRRX1 in GC closely correlates with tumor invasion and metastasis [7].

There are several common ways for metastasis, such as direct invasion, lymphatic spread, metastasis by bloodstream, and implantation metastasis. The cells that derived from the primary solid tumor and have gone through EMT process and finally involved into the blood circulation system are defined as circulating tumor cells (CTCs). It is by this way that the metastasis take place. According to the current study, the CTCs have been detected in the blood from patients with gastrointestinal cancers for obtaining useful information before the start of therapy, including surgery, chemotherapy and comprehensive treatment [8-11]. Some authors also confirmed that CTCs in peripheral blood could be used for predicting tumor progression, prognosis, and that it could be a useful tool to monitor the effect of chemotherapy dynamically in patients with GC [12]. What is more, American Society of Clinical Oncology (ASCO) has proved the CTC as tumor markers for breast cancer. In addition, there are papers that reported that CTC count has some degree of correlation with the clinical features of NSCLC and reflects the status of the disease to certain extent[13]. Since we know that PRRX1 has close link to invasion and metastasis of GC, the CTC act as a medium that play an important role in the process of patients with tumor metastases, and it could be applied to predict tumor progression, prognosis and dynamically monitor the effect of chemotherapy in patients with GC [12,14-17], then what is the relationship between PRRX1 and CTCs and what is the relationship between PRRX1, CTCs, and clinical features in patients with GC? At present, they are still unclear. In our current study, the relationship between PRRX1, CTCs and clinical features in patients with GC were analyzed.

In the present study, a method of EpCAM independent subtraction enrichment (SET) integrating with immunostaining-fluorescence in situ hybridization (imFISH) was performed to isolate and determine CTCs in patients with GC [18,19]. Precisely speaking, aneuploidy of chromosome 8 and 17 in CTCs enriched from the patients was examined by SET-imFISH.

Methods

Patients' selection and sample collection

From January 2014 to February 2016, 95 patients definitely diagnosed with GC in the Department of General Surgery of the Gansu Province People's Hospital (Lanzhou, China) were recruited for study, including 57 male and 38 female (Table 1). Peripheral blood samples (3.2 ml) were collected before gastrectomy and drawn into an ACD anticoagulative tube. All blood samples were processed within 24 h after collection. The collection, enrichment and result reading of all blood samples were blindly operated by different professionals. All tumor specimens and corresponding normal tissues from gastrectomy were placed into 10% neutral-buffered formalin, embedded in paraffin blocks, and then made into continuous 4μm tissue sections for immunohistochemical identification. The corresponding normal tissues were defined as those isolated≥5 cm away from the edge of cancer lesions. All patients had been histologically diagnosed without preoperative radiotherapy, chemotherapy or other anti-cancer therapy. Postoperative adjuvant chemotherapy was administered to each individual, and all the removed samples were confirmed by more than three leading pathology experts. The staging of GC was according to the American Joint Committee on Cancer (ACJJ, 7th edition) [20]. All the tissue samples for experimental research were obtained with each patient's approval. This study was approved by the Ethics Committee of Gansu Province People's Hospital.

Table 1. Expression of PRRX1 in 95 paired samples of GC and adjacent normal gastric mucosa

Proteins	*Gastric cancer tissues*	*Normal gastric mucosa*	*x^2 test*	*p value*
PRRX1			7.63	0.006
+	54	35		
-	41	60		

Enrichment and identification of GCs CTCs

The strategy of enrichment and isolation of GC CTCs was basically similar to the one that was previously published [21] except that the blood volume was modified to 3.2 ml. In brief, 3.2 ml of peripheral blood was firstly collected by ACD Anticoagulant tube (Becton, Dickinson and Company, Franklin Lake, NJ, USA) and detected within 24 h. Then, it was centrifuged to separate plasma from blood cells, processed by lysis of red blood cells. Next, the surplus cell pellet was resuspended in phosphate buffered saline (PBS) and subsequently incubated with anti-CD45 monoclonal antibody-coated magnetic beads for 30 min to filter out white cells, followed by separation of magnetic beads using a magnetic stand (Promega, Madison, WI, USA). Supernatants were subsequently subjected to identification.

The identification of enriched GC CTCs was performed by CD45-FISH, which combined the FISH with chromosome 8 (orange) and 17 (green) centromere probes (Abbott Molecular Diagnostics, Des Plaines, IL, USA) and anti-CD45 monoclonal antibody (red). In brief, the probes CEP8, CEP17 and specimen were hybridized at 37°C for 20 min in hybridizer (DAKO, California, USA). Subsequently, they were washed in 50% formamide at 43°C for 15 min, and immersed into saline-sodium citrate (SSE) and gradient alcohol again. Then, the specimens were washed twice with 0.2 % bovine serum albumin (BSA) and incubated with CD45 mixture/2% BSA conjugated to Alexa Fluor 594 (Invitrogen, Carlsbad, USA) for 1 h. Afterward, they were washed again with 0.2 % BSA. Finally, the specimens were covered with DAPI which contained Vectashield mounting medium. The area of the fixed sample should be observed entirely along "S" track with a microscope (Olympus, Tokyo, Japan). Positive CTCs must meet hyperdiploid CEP8+/DAPI+/CD45-, hyperdiploid CEP17+/DAPI+/CD45- or hyperdiploid CEP8+,17+/DAPI+/CD45-. DAPI labels nucleus, CD45 labels leucocyte, CEP8 17 labels centromere of chromosomes 8 and 17, respectively. All the normal cells keep being diploid. The count of CTCs ≥2 were recognized as meaningful and considered positive to final data analysis.

Immunohistochemistry (IHC)

The rabbit anti-human PRRX1 antibody and immunohistochemical SP kit (Sigma-Aldrich, St.Louis, Missouri, USA) were used to detect the expression PRRX1 in GC tissue and corresponding normal tissues. Slides were deparaffinized and antigen was retrieved by heating in a microwave oven for 15 min at 90°C in citrate buffer, incubating in 3% hydrogen peroxide for 20 min and blocking with normal goat serum for 30 min at room temperature. Slides were incubated with primary antibody overnight at 4°C and then incubated with secondary antibodies at 37°C for 30 min. Next, the slides were incubated with streptavidin–HRP for 30 min at 37°C, rinsed with PBS, incubated for 15 min with the chromogen 3,30-diaminobenzidine and then counterstained with hematoxylin. The staining results of targeted proteins were observed under microscope. Negative controls were prepared by substituting primary antibody with nonimmune rabbit serum. Each section was evaluated and scored in 10 random visual fields by two independent pathologists (double-blinded). The expression intensity scores (0 points for 0-5%; 1 point for 6-25%; 2 points for 26-50%; 3 points for ≥50 %) and staining intensity score (1 point=weak intensity; 2 points=moderate intensity; 3 points=strong intensity) were summed. A total score >3 was believed to represent significant overexpression and considered positive to simplify data analysis.

Statistics

Statistical analyses were carried out by SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The protein expression levels of PRRX1 and the status of CTCs with the clinicopathological parameters were compared by the chi-square test. Spearman's rank correlation coefficient was used for the comparison between the expression levels of PRRX1and the positive rate of CTCs. $P<0.05$ was considered to be statistically significant.

The depth of tumor invasion, lymph node metastasis and the TNM stage were classified according to the 2010 TNM classification of malignant tumors by the International Union Against Cancer.

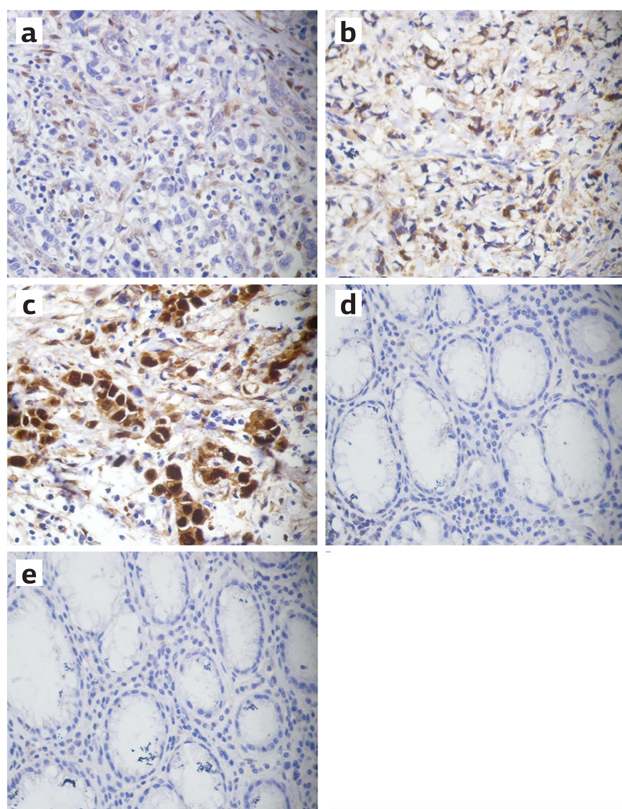

Figure 1. Typical expression levels of PRRX1 in gastric cancer and adjacent normal gastric mucosa by immunohistochemistry (×400). Immunohistochemical results show that the expression of PRRX1 in poorly and undifferentiated tissue **(c)** is higher than well and moderately well differentiated tissue **(a, b)** ($p<0.05$). Moreover, the expression of PRRX1 in gastric cancer tissue **(a, b, c)** is higher than the adjacent normal gastric mucosa tissue **(d, e)** ($p<0.05$).

Results

The expression of PRRX1 in cancer tissue and adjacent normal gastric mucosa of GC patients

The immunohistochemical method was used for detecting the protein levels. The expression levels of PRRX1 in 95 GC specimens is presented in Table 1 Relative to adjacent normal gastric mucosa, GC tissues showed higher levels of PRRX1 expression (Figure 1). The positive rate of PRRX1 in GC was 56.84% and the rate was 36.84% in adjacent normal gastric mucosa ($p<0.05$).

Circulating tumor cells and clinical correlation

Ninety-five patients with GC had CTCs detected. CTCs detected with hyperdiploid CEP8+/DAPI+/CD45, hyperdiploid CEP17+/DAPI+/CD45- or hyperdiploid CEP8+,17+/DAPI+/CD45- were considered positive in 67 patients (70.53%). In all patient with GC, the positive rate of CTCs did not significantly correlate with age, gender, histologic type ($p>0.05$), however, it was significantly related to tumor size, grade of differentiation, lymph node invasion, vascular invasion, metastasis status, depth of tumor invasion, lymph node metastasis, and TNM stage ($p<0.05$) (Table 2).

Table 2. Correlation between PRRX1, CTCs and clinicopathological features in 95 GC cases (x^2 test)

Parameters	*n*	*CTCs*				*PRRX1*			
		+	-	x^2	*p value*	+	-	x^2	*p value*
Age (years)				0.022	0.883			0.054	0.816
<60	52	37	15			29	23		
≥60	43	30	13			25	18		
Gender				0.051	0.821			0.741	0.389
Male	56	39	17			31	25		
Female	39	28	11			23	16		
Tumor size (cm)				9.576	0.002			4.876	0.027
<5	55	32	23			26	29		
≥5	40	35	5			28	12		
Differentiation grade			5.799	0.016			5.129	0.024	
Well and moderately differentiated	43	25	18			19	24		
Poorly and undifferentiated	52	42	10			35	17		
Histologic type				0.012	0.912			0.055	0.814
adenocarcinoma	82	58	24			47	35		
Signet-ring cell carcinoma	13	9	4			7	6		
Lymph node invasion				7.989	0.005			4.079	0.043
No	61	37	24			30	31		
Yes	34	30	4			24	10		
Vascular invasion				10.938	0.001			5.213	0.022
No	57	33	24			27	30		
Yes	38	34	4			27	11		
Metastasis status				8.17	0.004			5.884	0.015
M0	69	43	26			34	35		
M1	26	24	2			20	6		
Depth of tumor invasion				5.124	0.024			8.763	0.003
T1-2	58	36	22			26	32		
T3-4	37	31	6			28	9		
Lymph node metastasis				5.54	0.019			8.354	0.004
N0-1	69	44	25			33	36		
N2-3	26	23	3			21	5		
TNM stage				7.224	0.007			6.954	0.008
I-II	41	23	18			17	24		
III-IV	54	44	10			37	17		

The relationship between the clinicopathological factors of GC patients and the expression of PRRX1

The correlations between the clinicopathological features of GC patients and the expression levels of PRRX1 are summarized in Table 2. It's easy to see there were close relationships between the levels of PRRX1 and tumor size, grade of differentiation, lymph node invasion, vascular invasion, metastasis status, depth of tumor invasion, lymph node metastasis, and TNM stage ($p<0.05$) (Table 2).

The relationship between the expression of PRRX1 and CTCs in patients with gastric cancer

For the patients with the negative of PRRX1, the positive rate of CTCs was 53.66%, and for the patients with the positive of PRRX1, the positive rate of CTCs was increased to 83.33% ($p<0.05$). There were close relationships between the PRRX1 of GC and the CTCs of peripheral blood specimens of cancer patients with correlation coefficient 0.322 (Table 3).

Category and identification of CTCs

The cells isolated from the peripheral blood specimens of cancer patients were sorted as either CEP8>2 (the number of hybridization signals of CEP8≥2) or CEP8<2 (the number of hybridization signals of CEP8 <2) , CEP17≥2 (the number of hybridization signals of CEP17≥2) or CEP17<2 (the number of hybridization signals of CEP17<2). Based on the different number of CEP8,CEP17 hybridization signals and the immunostaining of the cells, abnormal cells were classified into the following four types: A, CD45- DAPI + CEP17 ≥ 2CEP8 ≥ 2; B, CD45- DAPI+ CEP17 < 2CEP8 ≥ 2; C, CD45- DAPI+ CEP17 ≥ 2CEP8 < 2; D, CD45- DAPI+ CEP17 < 2CEP8 < 2; (Figure 2). Among the four types, CD45-DAPI +CEP17≥2CEP8≥2, CD45-DAPI+ CEP17< 2CEP8 ≥ 2, CD45- DAPI + CEP17 ≥ 2CEP8 < 2 were determined as CTCs; CD45- DAPI CEP17<2CEP8<2 were considered to be indeterminate cells (Figure 2).

Table 3. Correlations between PRRX1 and CTCs in 95 cases of gastric cancer (Spearman correlation test)

PRRX1	*CTCs*		*NO. of cases*	*r*	*p value*
	+	-			
+	45	9	54	0.322	0.001
-	22	19	41		

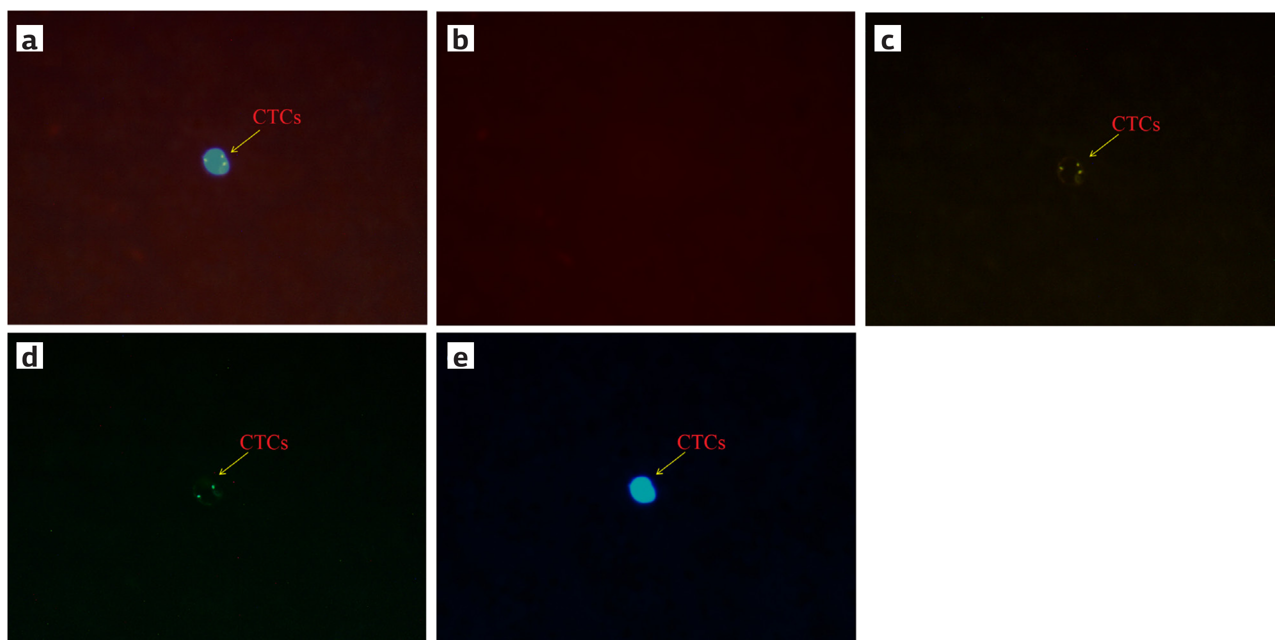

Figure 2. The identification and evaluation of CTCs. CTCs were defined as nucleated cells with hyperdiploid CEP8 (labeled with orange) or CEP17 (labeled with green) and that lacked allophycocyan (CD45, labeled with red). DAPI (labeled with blue) indicates 4,2-diamidino-2-phenylidole dihydrochloride drived from unabridged nucleus (**a:** showed figure combined with staining of anti-CD45, CEP8 probe, CEP17 probe and DAPI. **b:** showed staining of white blood cells by anti-CD45. **c:** showed staining of chromosome by CEP8 probe. **d:** showed staining of chromosome by CEP17 probe. **e:** showed staining of nucleus by DAPI).

Discussion

The incidence of GC is associated with many factors such as environment, diet, infection and the expression of genes. In short, GC is a heterogeneous disease with high mortality world over [22]. In recent years, strong evidence has been accumulated, which revealed that patient outcomes not only depend on tumor stage, but also result from the expression of some key factors and tumor-specific alteration: epithelial-mesenchymal transition (EMT). EMT is a transformation process by which epithelial cells lose their cell polarity and cell-cell adhesion and obtain some features of mesenchymal cell instead [23]. EMT is essential for organ formation, wound healing, tissue fibrosis, and cancer progression. For the cancer progression part, EMT is related to tumor invasion and dissemination and is a necessary precondition for metastatic colonization. EMT gives cells migratory and invasive abilities, spurs stem cell properties, prevents apoptosis and senescence and contributes to immunoregulation [24]. The PRRX1 works as a transcription coactivator just like any other factors, such as TWIST1. SNAI1 facilitates EMT process in carcinoma cells. But one wonders which one played a decisive effect for progression and metastasis of GC ?

First, we know from our experiments that a higher level of PRRX1 was expressed in GC relative to adjacent normal gastric mucosa. Likewise, the high level of PRRX1 expression was significantly related to decreased metastasis and better outcomes in breast cancer [6], while it had the opposite association with colorectal cancer [25]. Thus, it is reaffirmed that there is a close relationship between the incidence of a malignant tumor and the high expression of PRRX1. For specific cancers, the result may be different because there may be other factors involved in regulating the expression of PRRX1. For example, recent research showed that miR-124 could regulate the radiosensitivity of colorectal cancer cells by directly blocking PRRX1 [26].There is also a paper suggesting that miR-106b regulates the expression of PRRX1 by establishing a negative feedback loop with TGF-β1, but miR-106b knockdown could not efficiently achieve distant metastatic colonization [27]. Other studies found that miR-655 inhibits the EMT phenotype in triple negative breast cancer (TNBC) by downregulating PRRX1, and then decreasing cell migration and invasion during cancer progression [28]. However, miR-655 has provided a deeper understanding of the molecular mechanisms underlying of PRRX1 and tumor metastasis but also provided an additional target for therapeutic intervention. In addition, a newest study [29] reveals that the isoform-specific roles of PRRX1 and PRRX1 play an opposite effect in primary pancreatic duct adenocarcinoma (PDAC) formation, dissemination, and metastatic colonization. Specifically, PRRX1b facilitates invasion, tumor dedifferentiation, and EMT. In contrast, PRRX1 promotes metastasis in the liver, cancer differentiation, and EMT in both mouse models of PDAC and human PDAC [30], but for patients with GC, whether it will be happened is worth to conduct further research by similar methods.

Patients with prostate cancer, breast cancer or colorectal cancer with metastasis had a high positive rate of CTCs according to several papers [31-33]. And that whether the incidence of CTCs can reflect the facilitation from PRRX1 to EMT? Herein, we have investigated the status of CTCs from the peripheral blood of patients before operation. Because of the minor amount of CTCs uncontinuous released from the primary tumor and sneak into the blood stream, the method to enrich and identify CTCs has been continually developed [34,35].The CellSearch system is based on the theory to detect the expression of epithelial cell adhesion molecule (EpCAM) and cytokeratins (CKs) on cancer cells. Nevertheless, the published works reveal that most of CTCs may have lost both EpCAM and CKs phenotype after EMT transition [36-39]. Certainly, the detection of CTCs by EpCAM and CK-dependent strategy results in inevitable failure. So, in the present study, we performed EpCAM independent subtraction enrichment [18,19] integrating with imFISH to isolate and determine CTCs in patients with GC. Because several studies have reported multiple centromere of chromosome probes application that did improve positive detection rate of CTCs , we first applied both CEP8, CEP17 probes of FISH technique and CD45 subtractive enrichment to identify CTCs from patients with GC. Of course, this identification approach of CTCs with CEP 8 and CEP17 still results in inevitable loss of CTCs count, so the detection of CTCs derived from negative enrichment and FISH technique still need to be improved continuously in terms of sensitivity and specificity.

As expected, the detection result of CTCs confirmed a statistically positive correlation with PRRX1. With that, we gathered clinical features in GC patients and analyzed the relationships between those and PRRX1, CTCs respectively. Meanwhile, both of them are significantly relate to tumor size, grade of differentiation, lymph node invasion, vascular invasion, metastasis status, depth of tumor invasion, lymph node metastasis, and TNM stage. Then, the PRRX1 facilitates EMT process in GC

cells by the activated signal pathway of Wnt/b-catenin, further caused dissemination of cancer cells into the blood stream, and finally formed a successful metastasis, which is thought to be logical relation. Review of the literature revealed that the TWIST1 reported before, acts as another inducer of EMT, whose positive expression is also significantly correlated with lymph node metastasis in GC [40]. According to the expression of PRRX1, we have reasons to doubt whether PRRX1 acts as a primary control factor and regulates the expression of TWIST1 from downstream in metastasis and prognosis of GC. However, further studies are needed to see whether a dual directional regulation mechanism is existing between PRRX1 and TWIST1.

In summary, our research reports that different expression of PRRX1 in cancer tissue and adjacent normal gastric mucosa of GC patients and found that there is a positive correlation relationship between PRRX1 and CTCs. Meanwhile, both the expression of PRRX1 and positive rate of CTCs have close relationships with clinicopathological parameters in patient with GC. PRRX1 facilitates EMT in the part of invasion and metastasis, finally contributing to poor prognosis. The expression of PRRX1 is consistent with different phenotypes in different cancers. Now we know that multiple factors, for example, transforming growth factor-b [6], miR [40],miR-124 [26], miR-106b [27], miR-655 [28] can regulate the expression of PRRX1. Therefore, the specific and comprehensive regulation of PRRX1 still needs to be studied in further researches. The detection of CTCs acts as a new generation testing method and it has more irreplaceable advantages than imaging studies, such as ultrasonography, computed tomography, and positron emission tomography. Thus, CTCs may contribute to promotion in diagnosis, therapy monitoring and prognosis of GC. As the patients' follow-up time was too short, there was not sufficient effective evidence to predict their outcome. Our follow-up research will focus on relationship between PRRX1, CTCs and survival time. In addition, the clinical significance of CTCs in diagnosis, chemotherapy response monitoring and evaluation of prognosis of primary GC will be elaborated in the follow-up study.

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Conflict of interests

The authors declare no conflict of interests.

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