Combination of circulating *CXCR4* and *Bmi-1* mRNA in plasma: A potential novel tumor marker for gastric cancer

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Received January 20, 2009; Accepted May 4, 2009

DOI: 10.3892/mmr_00000170

Abstract. Cell-free nucleic acids in plasma have been reported to be a novel tumor marker. However, their exact significance in gastric cancer is unclear. In the present study, we focused on circulating *CXCR4* and *Bmi-1* mRNA in plasma and evaluated their diagnostic values for patients with gastric cancer. *CXCR4* and *Bmi-1* mRNA levels, as well as levels of CEA and CA19-9 proteins, were determined in the plasma of 89 gastric cancer patients. The levels of these markers were significantly higher in cancer patients than in healthy subjects; however, there was a decrease in their expression after surgery. The sensitivity of detection for the combination of circulating *CXCR4* and *Bmi-1* mRNA was higher than the sensitivities of detection for CEA and CA19-9. In patients expressing both genes, *CXCR4* and *Bmi-1* mRNA levels were strongly correlated. Expression of the two circulating genes was also strongly correlated with the CEA level, and was significantly associated with the age of the patient, histological type, clinical stage, extent of cell differentiation and gastric cancer metastasis. Our results indicate that the combined expression of circulating *CXCR4* and *Bmi-1* mRNA represents a novel tumor marker, superior to traditional markers such as CEA and CA19-9, for the diagnosis and monitoring of gastric cancer.

Introduction

Gastric cancer is one of the most lethal malignant tumors worldwide, with a 5-year survival rate of only 30-40% in patients with aggressive-phase disease. Lymph node metastases are found in most patients with advanced gastric cancer. However, treatment of early-stage disease results in higher survival rates (1). Thus, the early detection of gastric cancer and prediction of the metastatic potential of the tumor are necessary to improve the survival rate.

It has been reported that the chemokine CXC receptor 4 (CXCR4) and the CXCL12 ligand are involved in the metastasis of various types of cancer, including gastric carcinoma (2). The *CXCR4* gene is located on the long arm of chromosome 2 and encodes 352 amino acids. The protein is a seven-transmembrane domain-containing G protein-coupled receptor, and is the unique high-affinity receptor for CXCL12 (3). It has been demonstrated that cancer cells express chemokine receptors, and that their corresponding ligands are frequently expressed at the site of tumor metastasis (4,5). Muller *et al* revealed that *CXCR4* was highly expressed in human breast cancer cells and their distal metastases, while *CXCL12* expression was increased at the common sites of breast cancer metastasis, such as the lymph nodes, lungs and liver (6). This indicates that *CXCR4* is a potential tumor marker for predicting the locations and probability of tumor metastasis.

The *Bmi-1* gene is a member of the polycomb family that is located on the short arm of chromosome 10 and encodes a 326-amino acid protein. By deregulating two tumor suppressors, p16INK4a and p19ARF (p14ARF in humans), Bmi-1 can inhibit the apoptosis or senescence induced by p53 and pRb (7,8). Furthermore, Bmi-1 plays a role in the activation of human telomerase reverse transcriptase (*hTERT*), which extends the replicative lifespan and immortalizes cells (9). Some studies have indicated that *Bmi-1* is important for the self-renewal of stem cells and cancer stem cells and for tumorigenesis by cancer stem cells (10-12). In gastroenteric tumor cells, upregulated expression of Bmi-1 has previously been demonstrated (13).

Previous studies have focused on diagnosing and determining the optimum treatment strategy for gastric carcinoma based on tumor specimens collected during surgery. However, not every patient is capable of undergoing surgery, and even in cases where the primary tumor is resected, it is difficult to obtain and apply early screening or consecutive monitoring via biopsy. Serum tumor markers, such as carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9, have been widely used as convenient diagnostic assays, but their lack of sufficient sensitivity and specificity for the early detection of

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Key words: gastric cancer, chemokine CXC receptor 4, *Bmi-1*

Table I. Primers for the *CXCR4*, *Bmi-1* and *β-actin* genes.

Gene	Primer sequence		Annealing temperature (˚C)	Product length (bp)
CXCR4	Forward:	5'-TCATCAGTCTGGACCGCTACCT-3'	54	637
	Reverse:	5'-GAAACAGATGAATGTCCACCTCG-3'		
	Nested Forward:	5'-CTGCCCTCCTGCTGACTATTC-3'	56	200
	Nested Reverse:	5'-GGAGTGTGACAGCTTGGAGATG-3'		
Bmi-1				
	Forward:	5'-GCTGCCAATGGCTCTAATG-3'	60	531
	Reverse:	5'-GGAGACTGCACTGGAGTACTG-3'		
	Nested Forward:	5'-AATCTAAGGAGGAGGTGA-3'	59	359
	Nested Reverse:	5'-CAAACAAGAAGAGGTGGA-3'		
β-actin				
	Forward:	5'-CGTCTGGACCTGGCTGGCCGGGACC-3'	60	600
	Reverse:	5'-CTAGAAGCATTTGCGGTGGACGATG-3'		
	Nested Forward:	5'-CACGAAACTACCTTCAACTCC-3'	56	265
	Nested Reverse:	5'-CATACTCCTGCTTGCTGATC-3'		

gastric cancer has limited their application (14,15). Several studies have determined that tumor-associated circulating cell-free mRNA in the plasma or serum is increased in cancer patients (16-20). At least some of these nucleic acids are released from tumor cells and can be considered new non-invasive biomarkers for diagnosing disease or predicting patient prognosis (21). It has also been reported that these nucleic acids are protected in vesicle-like structures, have the characteristic of anti-degradation and are stable in plasma (22-24). This indicates that circulating mRNA is suitable for screening for primary tumors and for monitoring patients for the progression or response of tumors. Unlike cellular RNA, the expression levels of some genes are not high enough for auxiliary diagnosis. We hypothesize that the detection of multiple genes can be applied to overcome this issue.

To date, there have been few studies focusing on the detection of circulating mRNA in patients with gastric carcinoma. We previously reported that the concentration of supernatant RNA decreases when human gastric carcinoma cells (SGC7901) are exposed to anti-cancer drugs *in vitro* (25). In the present study, we evaluated the expression of *CXCR4* and *Bmi-1* mRNA in the plasma of gastric cancer patients, and assessed their potential utility in diagnosis, the prediction of prognosis and the monitoring of response to treatment in gastric cancer.

Materials and methods

Subjects. After obtaining informed consent, peripheral blood samples were collected between August 2006 and March 2008 from 89 untreated patients with primary gastric cancer at the First People's Hospital of Zhenjiang and the Affiliated Hospital of Jiangsu University (Zhenjiang, P.R. China). There were 64 male and 25 female patients with ages ranging from 35 to 86 years (median 60 years). All patients were diagnosed by pathological analysis based on the International Union Against Cancer (UICC) criteria. Sixty-nine of the patients consented to a second collection of blood 30 days after surgery. Forty-two healthy subjects, including 28 males and 14 females with a mean age of 52 years, were recruited as controls.

Processing of blood samples. Blood samples were collected in EDTA-containing tubes and centrifuged at 4000 rpm for 20 min at 4˚C (Centrifuge 5810R, Eppendorf, Hamburg, Germany). The plasma supernatant (200 µl) was mixed with 800 µl Trizol Reagent (Invitrogen, Carlsbad, CA). All samples were stored at -80˚C until RNA extraction. CEA and CA19-9 were detected in the residual plasma.

RNA extraction. Isolation of cell-free RNA was derived using the optimal method previously reported (26). To each plasma-Trizol mixture, 200 µl chloroform was added. The mixture was then centrifuged at 12000 x g for 15 min at 4˚C (Centrifuge 5417R, Eppendorf). The aqueous layer (450 µl) was transferred to fresh Eppendorf tubes, and 1 volume of isopropanol was added and mixed thoroughly. The mixture was applied to an RNAfasta200 Minicolumn (Fastagen Biotech, Shanghai, China) and processed according to the manufacturer's protocol. Finally, total RNA was dissolved in 30 µl RNase-free water and stored at -80˚C.

Reverse transcription. A reverse transcription kit (Toyobo, Osaka, Japan) was used to generate cDNA. Each reaction contained a total volume of 20 µl comprised of 4 µl 5X RT-Buffer, 2 µl 10 mmol/l each dNTPs, 1 µl 10 U/µl RNase inhibitor, 1 µl 10 pmol/µl oligo(dT), 1 µl 5U/µl ReverTraAce and 11 µl purified RNA. As suggested by the manufacturer, the profile was 42˚C for 20 min, 99˚C for 5 min and 4˚C for 5 min. Subsequently, the samples were briefly centrifuged. The cDNA was stored at -20˚C until real-time PCR was carried out.

Nested real-time quantitative PCR. To eliminate the effects of plasma DNA, the primers for the human *CXCR4*, *Bmi-1* and *β-actin* genes were designed to be intron-spanning (Table I). All reagents were purchased from Takara Bio Inc. (Shiga,

Table II. Plasma *CXCR4* and *Bmi-1* mRNA and CEA and CA19-9 proteins in cancer patients and healthy subjects.

	Healthy (42)	Preoperative (89)	Postoperative (69)
CXCR4	21.4% (9)	41.6% (37)[a]	23.2% (16)[b]
Bmi-1	28.6% (12)	57.3% (51)[a]	43.5% (30)[a]
CXCR4+Bmi-1	35.7% (15)	73.0% (65)[a,c,d]	52.2% (36)[a,b,d]
CEA	14.3% (6)	36.2% (32)[a]	17.4% (12)[b]
CA19-9	9.5% (4)	23.6% (21)[a]	13.0% (9)
CEA+CA19-9	19.0% (8)	44.9% (40)[a]	24.6% (17)[b]

Significant difference compared to the [a]healthy subjects (P<0.05), and [b]preoperative group (P<0.05). [c]Compared with the single gene test, P<0.05. [d]Compared with the CEA+CA19-9 group, P<0.01.

Japan). The PCR process was similar to one we previously described (25). The reaction mixture consisted of 2.5 μl 10X PCR Buffer, 2.0 μl 2.5 mmol/l of each dNTP, 2.5 μl 25 mmol/l $MgCl_2$, 0.5 μl 10 pmol/μl of each of the primers, 0.2 μl 5 U/μl Taq polymerase, and 5.0 μl cDNA template and distilled water for a total volume of 25 μl. The reaction was carried out in an ABI 2720 thermal cycler (Applied Biosystems, CA, USA) as follows: 94˚C for 10 min, then amplification for 20 cycles with denaturation at 94˚C for 30 sec, annealing for 30 sec, and elongation at 72˚C for 30 sec. The second amplification with nested primers was accomplished in Rotor-gene 6000 real-time fluorescence thermal cycler (Corbett Ltd., Sydney, Australia) in which the first amplification product was diluted 1:50 in distilled water, and 5.0 μl of the dilution was used as the template for the second amplification step. SYBR Green I (10X, 1.5 μl; Invitrogen) was added in this step. The other components were the same as those used in the first amplification step, and distilled water was added for a total final volume of 25 μl. This mixture was cycled for 30 cycles as follows: 94˚C for 30 sec, annealing for 30 sec, and 72˚C for 30 sec. After all cycles were completed, a final extension at 72˚C was performed for 10 min, and then the samples were subjected to a final melting from 72 to 99˚C. In order to obtain a standard curve, the PCR products were subcloned into the pGEM-T vector (Promega, Madison, WI, USA) following the manufacturer's instructions. After a serial dilution, the standard data were analyzed by real-time analytical software (Rotor-gene). Both the standards and samples were processed in duplicate. To confirm the identity of the specific amplicons, melting curve analysis and agarose gel electrophoresis of the PCR products were conducted, followed by staining with ethidium bromide.

Detection of CEA and CA19-9. CEA and CA19-9 were detected in a chemiluminescence analyzer (Elecsys 2010, Roche) following the manufacturer's instructions.

Statistical analysis. The Mann-Whitney U test was applied to compare the gene (*CXCR4* and *Bmi-1*) and protein (CEA and CA19-9) expression levels. Differences in positive rates in the different groups were analyzed by the χ^2 test. Two-tailed P<0.05 values were considered significant. Statistical analysis was managed using SPSS 11.0 software.

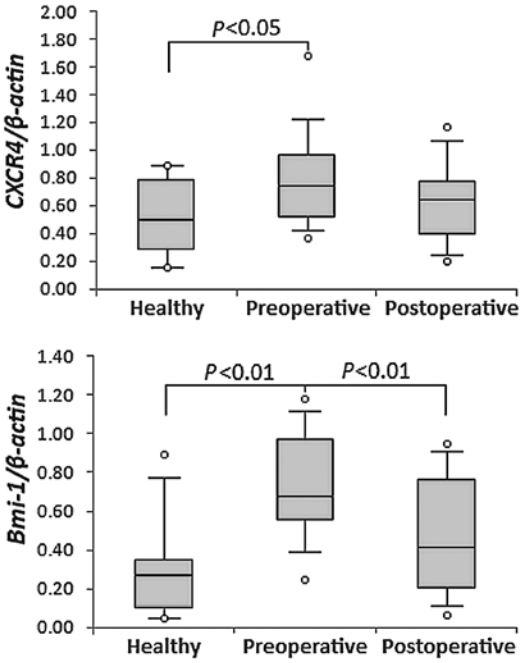

Figure 1. *CXCR4* and *Bmi-1* mRNA expression levels in the plasma of patients with gastric cancer. In the box plots, the blank dots indicate the maximum and minimum, the whiskers indicate the interval between the 10th and 90th percentiles, the boxes denote the interval between the 25th and 75th percentiles, and the lines inside the boxes mark the medians.

Results

CXCR4 and Bmi-1 expression levels are upregulated in patients with gastric cancer. CXCR4 and *Bmi-1* were amplified by nested real-time PCR from the plasma of 89 patients with gastric carcinoma prior to surgery and the plasma of 42 healthy subjects. Of the cancer patients, 69 were again evaluated for gene expression after surgery. A signal appearing within the first 25 cycles was regarded as positive for the existence of the target cell-free mRNA in the plasma samples. *CXCR4* or *Bmi-1* mRNA expression and the combined expression of both mRNAs were found to be higher in the cancer patients than in the controls (P<0.05). Moreover, detection using two genes improved the detection rate compared to the single-gene tests (P<0.05; Table II). Among the plasma samples where both genes were detected, the expression levels of each gene were significantly higher in tumor patients compared to the controls (P<0.05; Fig. 1). After surgery, the levels of both *Bmi-1* and *CXCR4* gene expression in plasma were decreased.

Increased levels of CEA and CA19-9 in gastric cancer patients. Expression of the traditional tumor markers CEA and CA19-9 was also examined using electrochemiluminescence. When the expression levels were >5 ng/ml (CEA) and 39 U/ml (CA19-9), they were regarded as positive. Similar to previous studies, a significant increase in the expression of CEA was found in the plasma of preoperative patients with gastric cancer. Following surgery, CEA expression was reduced (P<0.05; Table II; Fig. 2). However, the mean level of CA19-9 in preoperative patients did not show a significant increase compared to the healthy subjects.

Table III. Diagnostic values of tumor markers in gastric cancer patients.

	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy
CXCR4	41.6%	78.6%	80.4%	38.8%	53.4%
Bmi-1	57.3%	71.4%	80.9%	44.1%	61.8%
CXCR4+Bmi-1	73.0%[a,c]	64.3%	81.3%	52.9%	70.2%[b,c]
CEA	36.2%	85.7%	84.2%	38.7%	51.9%
CA19-9	23.6%	90.5%	84.0%	35.8%	45.0%
CEA+CA19-9	44.9%	81.0%	83.3%	41.0%	56.5%

[a]Compared with a single-gene test, P<0.05. [b]Compared with the *CXCR4* group, P<0.05. [c]Compared with the CEA+CA19-9 group, P<0.05.

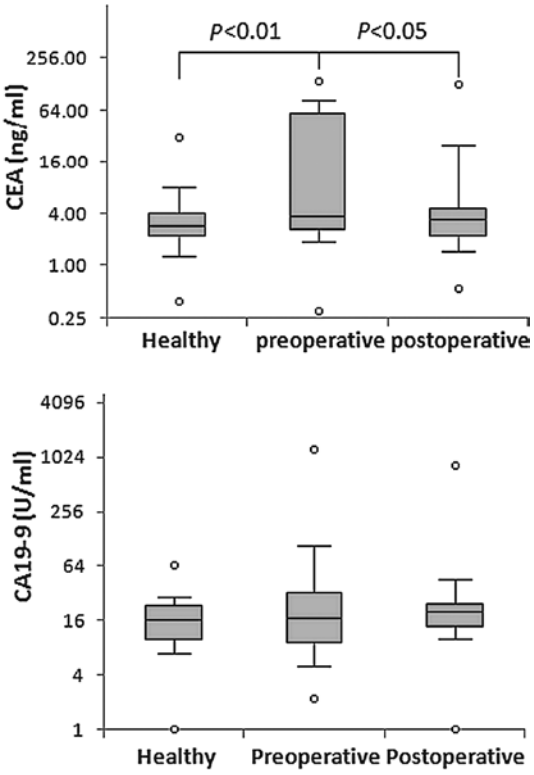

Figure 2. Quantification of CEA and CA19-9 in the plasma of gastric cancer patients and healthy subjects. In the box plots, the blank dots indicate the maximum and minimum, the whiskers indicate the interval between the 10th and 90th percentiles, the boxes denote the interval between the 25th and 75th percentiles, and the lines inside the boxes mark the medians.

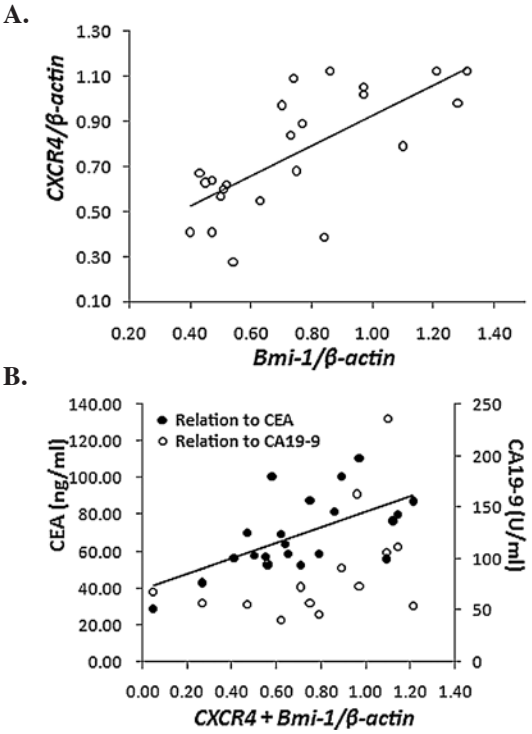

Figure 3. (A) Correlation between plasma *CXCR4* and *Bmi-1* expression levels. The line indicates that there is a strict correlation between the expression of the two genes. (B) Correlation between *CXCR4* or *Bmi-1* expression and CEA or CA19-9. The black dots indicate the relationship with CEA expression, and the open dots denote the relationship with CA19-9 expression. The line indicates that there is a strong association. Data for *CXCR4*/*β-actin* or *Bmi-1*/*β-actin* in the *CXCR4+Bmi-1* group are all distributed on the X axis, indicating that both of the genes are positively correlated with CEA expression.

Diagnostic values of the two genes in combination. The results regarding the diagnostic values of plasma *CXCR4* and *Bmi-1* are shown in Table III. By combining the two genes for diagnosis, the sensitivity was improved, though there was no significant decrease in the specificity. Compared to the use of *CXCR4* alone, the accuracy also increased (P=0.0051). Although the negative predictive value exhibited an increasing trend, there was no significant difference between using single genes and the combination. The sensitivity and accuracy of *CXCR4* and *Bmi-1* mRNA in combination were significantly higher than the sensitivity and accuracy of the combination of plasma CEA and CA19-9 (P<0.05).

Correlation between CXCR4 and Bmi-1 mRNA in plasma. In preoperative patients, plasma samples positive for both *CXCR4* and *Bmi-1* mRNA were found to be strictly correlated (r=0.713, P=0.0001; Fig. 3A).

Relationship between plasma mRNA and traditional tumor markers. In the preoperative *CXCR4+Bmi-1*-positive group, samples positive for CEA or CA19-9 were examined, and the relationship between plasma gene and tumor protein marker expression was analyzed. Tumor gene expression levels were found to be correlated with the expression of CEA (r=0.615, P=0.003), but were not related to CA19-9 expression (Fig. 3B).

Table IV. Relationship between circulating *CXCR4* and *Bmi-1* mRNA and clinicopathologic characteristics.

	CXCR4			*Bmi-1*			*CXCR4+Bmi-1*		
Patients (no.)	+	%	P-value	+	%	P-value	+	%	P-value
Gender									
Male (64)	26	40.6	0.959	34	53.1%	0.299	44	68.8	0.233
Female (25)	11	44.0		17	68.0%		21	84.0	
Age									
≤60 (34)	10	29.4	0.108	15	44.1%	0.079	20	58.8	0.033
>60 (55)	27	49.1		36	65.5%		45	81.8	
Clinical stage									
Early (29)	8	27.6	0.103	13	44.8%	0.154	18	62.1	0.172
Advanced (60)	29	48.3		38	63.3%		47	78.3	
Histological type									
Tubular adenocarcinoma (56)	24	42.9	0.109	35	62.5%	0.175	44	78.6	0.017
Mucinous adenocarcinoma (16)	9	56.3		9	56.3%		13	81.3	
Signet ring cell carcinoma (10)	2	20.0		4	40.0%		5	50.0	
Malignant change of ulcer (7)	2	28.6		3	42.9%		3	42.9	
Tumor size									
≤5 cm (51)	20	39.2	0.760	31	60.8%	0.581	40	78.4	0.277
>5 cm (38)	17	44.7		20	52.6%		25	65.8	
Extent of cell differentiation									
Well differentiated (53)	19	35.8	0.184	27	50.9%	0.141	35	66.0	0.033
Poorly differentiated (36)	18	50.0		24	66.7%		31	86.1	
Lymph node metastasis									
Yes (55)	28	50.9	0.023	35	63.6%	0.125	46	83.6	0.004
No (34)	9	26.5		16	47.1%		19	55.9	

+, number of positive subjects; %, percentage of positive subjects.

CXCR4 and Bmi-1 mRNA in plasma and clinicopathologic characteristics. The possible relationship between the expression of the two genes in plasma and a series of preoperative tumor clinicopathologic parameters was analyzed (Table IV). A significant correlation between plasma *CXCR4* mRNA and lymph node metastasis was found (P=0.023). In the *CXCR4+Bmi-1* group, there were significant associations with the age of the patient and with cancer histologic type, extent of differentiation and the presence of lymph node metastasis (P<0.05).

Discussion

In patients with various types of cancer, including lung, breast and colon cancer as well as leukemia, CXCR4 is overexpressed on the surface of tumor cells. This expression has been shown to be related to the presence of malignancy and metastases (6,27-30). Yasumoto *et al* found that the CXCR4 protein and its ligand, CXCL12, were highly expressed in gastric cancer cells that transferred to the peritoneum. This suggests that the CXCL12/CXCR4 axis is associated with tumorigenesis, metastasis, prognosis and the response to treatment of gastric carcinoma (31).

Bmi-1 is a protooncogene that inhibits tumor suppressor genes. Overexpression of *Bmi-1* often leads to tumorigenesis (32-35). In the plasma of breast cancer patients, an increase in circulating *Bmi-1* was detected by Silva and co-workers. They found that the 5-year survival rate of patients whose plasma was positive for *Bmi-1* mRNA was significantly lower than that of patients without *Bmi-1* mRNA in their plasma (36). It has previously been demonstrated that *Bmi-1* mRNA is expressed in gastroenteric carcinomas (13,37).

Although the expression of the two genes has been reported in gastric tumor tissues, whether circulating cell-free *CXCR4* and *Bmi-1* mRNA are expressed in the plasma of patients with gastric cancer has not been determined. In the present study, we were able to detect the presence of both mRNAs in most of the gastric cancer patients examined. Similar to results obtained for the widely used tumor markers CEA and CA19-9, the rate of positivity for expression and the expression levels of the two genes were significantly higher in preoperative cancer patients than in healthy control subjects. After surgical resection of the tumor, the rate of positivity and the expression level of the two genes decreased. These results indicate that the

detection of circulating *CXCR4* and *Bmi-1* mRNA in plasma is useful in auxiliary diagnosis, as well as for monitoring the treatment response to gastric cancer.

The present findings demonstrate that evaluating the plasma expression of a combination of circulating *CXCR4* and *Bmi-1* mRNA improves the sensitivity and accuracy of diagnosis. Although the negative predictive value tended towards an increase, the differences were not significant. We also did not find any obvious differences in specificity among the *CXCR4*, *Bmi-1* and *CXCR4+Bmi-1* groups. Due to the low quantity of circulating cell-free RNA, the detection rate of single plasma tumor-associated mRNA is generally insufficient for diagnosing cancer. In order to resolve this issue, it is preferable to simultaneously detect multiple genes in plasma. We found that by detecting a combination of circulating *CXCR4* and *Bmi-1* mRNA, we were able to achieve better sensitivity and accuracy compared to the conventional markers, CEA and CA19-9. This indicates that circulating cell-free mRNA detection may be superior to currently used tumor markers.

In addition, a significant correlation between the expression levels of the two genes in plasma was found, as well as a relationship between the expression of the two genes and the level of CEA protein. Not only are circulating *CXCR4* and *Bmi-1* mRNA superior to CEA and CA19-9, but our results confirm that circulating mRNA represents a novel tumor marker that can be used either alone or in combination with traditional tumor markers.

We also compared the expression of plasma *CXCR4* and *Bmi-1* mRNA levels with clinicopathologic characteristics, and found that their individual expression was not significantly associated with the gender or age of the patient, or with histological type, size, clinical stage or the extent of cell differentiation of their tumors. This is in contrast to previous studies, which showed CXCR4 or Bmi-1 expression in tumor tissues to be associated with tumor size, the extent of differentiation and clinical stage (by immunohistochemistry or RT-PCR) (37). This indicates that the method used to detect circulating mRNA to describe tumors has some limits. The drawbacks are mostly a result of the low level of total circulating RNA and the limits of detection. However, the presence of *CXCR4* mRNA alone in plasma was strongly associated with lymph node metastasis, which is consistent with observations in tumor tissue. Based on these findings, we can infer that circulating cell-free *CXCR4* mRNA is a potential marker for tumor metastasis, and can be used to monitor the metastatic progression of gastric cancer.

By analyzing *CXCR4+Bmi-1* mRNA (in combination), we not only achieved similar results to those obtained in each of the single-gene tests, but also revealed additional significant findings: i) in patients aged 60 years or older, the detection rates of *CXCR4* or *Bmi-1* mRNA in plasma were higher than they were in younger subjects; ii) patients with tubular and mucinous adenocarcinomas were found to have higher rates of expression of the two genes than patients with Signet ring cell carcinoma or malignant changes of a benign gastric ulcer; iii) expression of *CXCR4+Bmi-1* was also associated with the extent of cell differentiation. These results indicate that combining multiple genes can offset some of the limitations to using circulating RNA. Moreover, combining multiple genes can lead to the more accurate classification of diseases, which can be used to determine the course of treatment.

While the use of circulating mRNA has previously been reported for tumor diagnosis and prognosis, the present study is the first to reveal that using a combination of plasma *CXCR4* and *Bmi-1* mRNA may be a potentially useful non-invasive method for diagnosing gastric cancers and for monitoring their response to therapy. We also demonstrated that the detection of circulating mRNA is superior to the use of traditional currently used tumor markers, such as CEA and CA19-9. Finally, our study suggests that examining the expression of the circulating mRNA of multiple genes will likely provide an improved method of diagnosis, not only for gastric carcinoma, but also for other types of cancer.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (no. 30840053), Jiangsu Province's Outstanding Medical Academic Leader Program (no. LJ200614), the Natural Science Foundation of Jiangsu Province (nos. BK2007705, BK2007092 and BK2008232) and Sci-tech innovation team and talents of Jiangsu University (no. 2008-018-02).

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