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Original Article

The overexpression membrane type 1 matrix metalloproteinase is associated with the progression and prognosis in breast cancer

Yongping Li[1*], Guohong Cai[2*], Shifang Yuan[1], Yi Jun[1], Nanlin Li[1], Ling Wang[1], Feng Chen[3], Rui Ling[1], Jun Yun[1]

[1]Department of Vascular and Endocrine Surgery, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, China; [2]Department of Anatomy, Histology and Embryology & K.K. Leung Brain Research Centre, Preclinical School of Medicine, Fourth Military Medical University, Xi'an 710032, China; [3]Division of General Surgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai 200233, China. [*]Equal contributors.

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Abstract: Membrane type 1 matrix metalloproteinase (MT1-MMP) has been demonstrated to play an important role in tumor progression. The aim of the present study was to analyze the expression of MT1-MMP in breast cancer and its correlation with clinicopathologic characteristics, including the survival of breast cancer patients. In our results, MT-MMP1 was up-expressed in breast cancer tissues compared with ductal hyperplasia tissues in microarray data (GSE2429). MT1-MMP mRNA and protein expression was markedly higher in breast cancer tissues than in normal breast tissues (P=0.005 and P=0.037, respectively). Using immunohistochemistry, high levels of MT1-MMP protein were positively correlated with the status of clinical stage (I-II vs. III-IV; P=0.043), lymph node metastasis (absence vs. presence; P=0.024), and distant metastasis (No vs. Yes; P=0.017) of breast cancer patients. Patients with higher MT1-MMP expression had a significantly shorter overall survival time than did patients with low MT1-MMP expression. Multivariate analysis indicated that the level of MT1-MMP expression was an independent prognostic indicator (P<0.001) for the survival of patients with breast cancer. In conclusions, MT1-MMP plays an important role on breast cancer aggressiveness and prognosis and may act as a promising target for prognostic prediction.

Keywords: Breast cancer, membrane type 1 matrix metalloproteinase, prognosis, immunohistochemistry

Introduction

Breast cancer is the most common cancer in females, and the second leading cause of cancer related mortality in women, accounting for approximately 29% of all new cancer cases among women and 14% cancer related mortality, representing a serious health threat to women worldwide [1, 2]. The prognosis of breast cancer is encouraging because of the advances in diagnosis and systemic therapy, including surgery, chemotherapy, hormone therapy, and radiation. However, the clinical outcome of breast cancer patients remains unsatisfactory. Similar to many other solid tumors, distant metastases account for more than 90% of breast cancer-related death [3]. This is largely because of a lack of effective and specific biomarkers that predict the risk of metastases in patients with breast cancer. Thus, new prognostic markers enabling oncologists to effectively distinguish high-risk patients with unfavorable prognosis and choose appropriate treatment strategies for breast cancer patients are urgently needed.

The matrix metalloproteinase (MMP) family has twenty-three members that differ in their substrate specificities toward varied components of the extracellular matrix. Structurally, the MMPs usually include a highly conserved propeptide domain, a zinc-binding catalytic domain, and a hemopexin-like domain [4]. MMPs are involved in many phases of cancer progression, including tumor invasion, metastasis, and angiogenesis [5, 6]. Membrane-type 1 MMP (MT1-MMP), which is a member of the MMPs family, has been implicated in multiple biological processes for its extracellular matrix degrading and accelerating angiogenesis [7]. Generally,

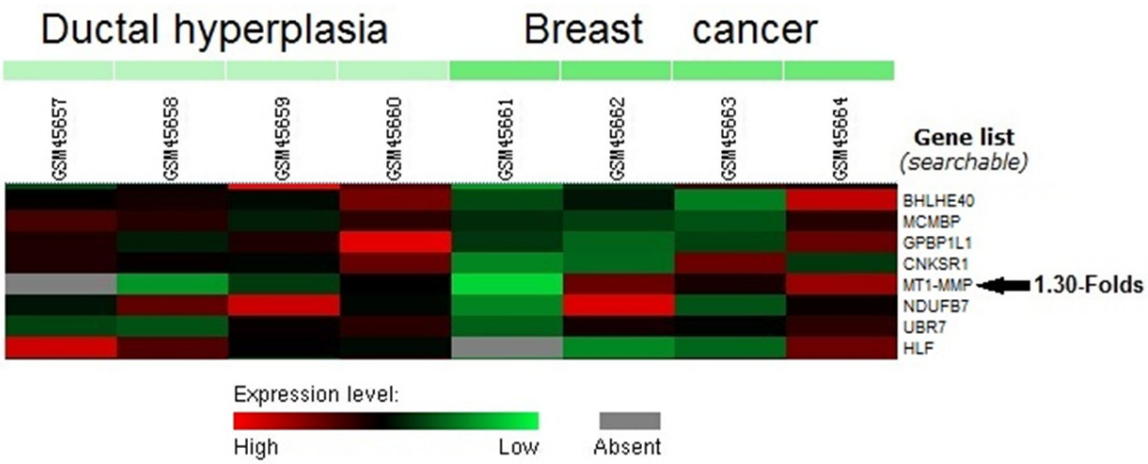

Figure 1. Increased MT1-MMP expression was shown in breast cancer by microarray data analysis of GSE2429 data set retrieved from the GEO database.

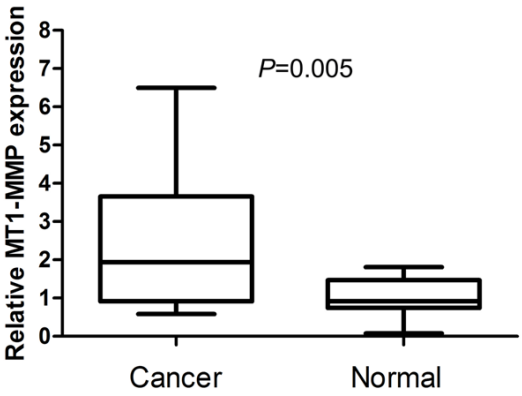

Figure 2. Expression of MT1-MMP mRNA is increased in breast cancer tissues compared with normal breast tissues by real-time PCR.

MMPs are produced by tissues as inactive zymogens and require further activation. However, MT1-MMP does not require additional activation, due to its capacity to be presented on the cell membrane in its active form [8]. Furthermore, present studies indicated that the expression of MT1-MMP was increased in tumor cells and overexpression of MT1-MMP directly correlate with accelerated cell migration [9].

The aim of this study was to identify the pathological roles of MT1-MMP in breast cancer. This study suggested that MT1-MMP was increased expression in breast cancer tissue and positively associated with clinical stage, lymph node metastasis, and distant metastasis. Furthermore, we found that overexpression of MT1-MMP was a significant predictor of poor prognosis for breast cancer patients.

Materials and methods

Analysis of microarray data

Microarray data set (GEO accession number: GSE2429) from four ductal hyperplasia tissues and four breast cancer tissues submitted by Poola was retrieved from the GEO database. Those differentially expressed genes were screened and identified by Real-time PCR for the following study.

Patients and specimens

One hundred and twenty six paraffin-embedded breast cancer samples and twenty one non-cancerous breast samples were retrieved from Xijing Hospital. Fifteen fresh breast cancer samples and fifteen non-cancerous fresh breast samples were collected from Xijing Hospital. All fresh samples were immediately preserved in liquid nitrogen. No patients had received any form of relevant tumor therapy before diagnosis. Before the use of these clinical samples, prior consents from the patients and approval from the Institutional Ethics Committee of the Xijing Hospital were obtained. The histopathological diagnosis of all samples was respectively diagnosed by two pathologists. The clinical staging was based on the 7th AJCC Cancer Staging Manual. Overall survival (OS) was defined as the interval from the date of diagnosis to breast cancer related death. In the 126 breast cancer cases, the age ranged

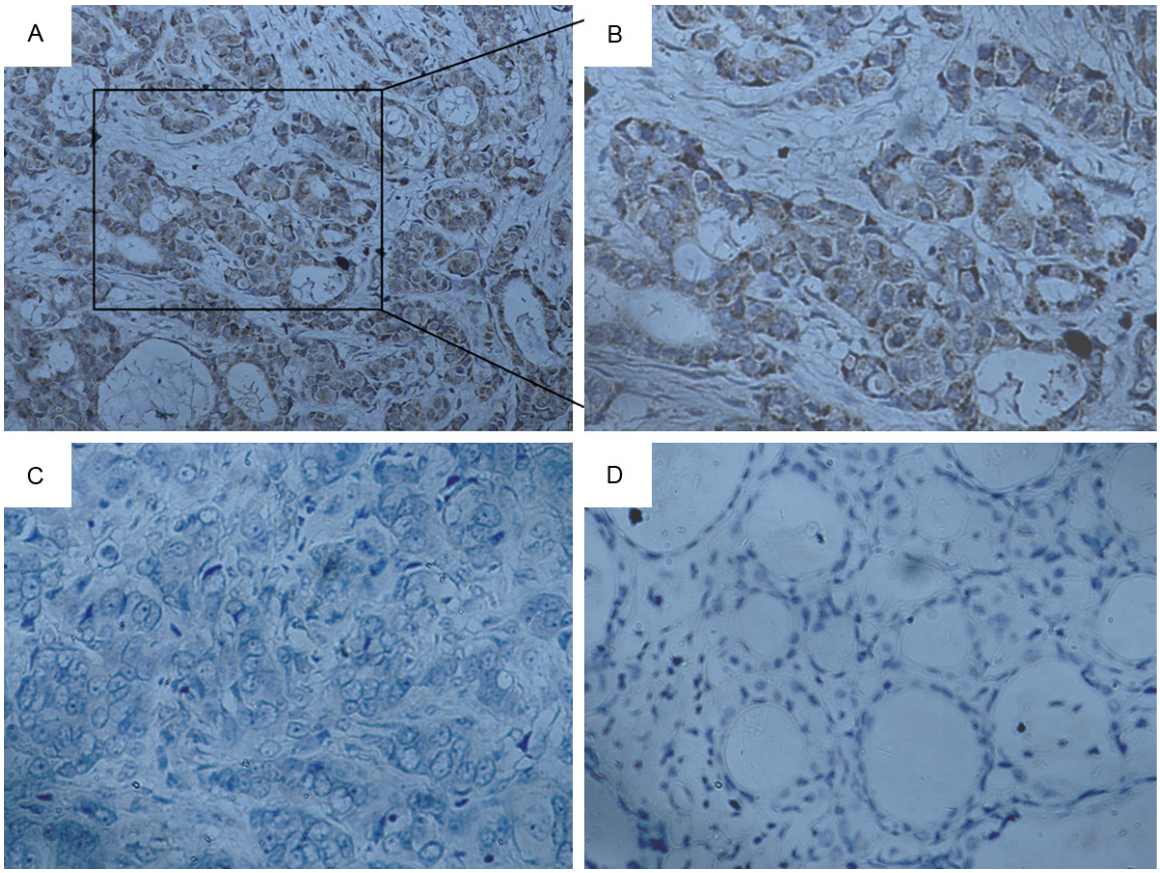

Figure 3. Immunohistochemical staining of MT1-MMP in breast cancer tissues. (A, B) Strong staining of MT1-MMP in breast cancer (A, original magnification ×200; B, original magnification ×400). (C) Negative expression of MT1-MMP in breast cancer (original magnification ×400). (D) Negative expression of MT1-MMP in normal breast tissues (original magnification ×400).

from 23 to 79 years (median, 57.18 years). The clinical follow-up time of patients ranged from 6 to 96 months.

Real-time PCR

The isolated total RNA was reversely transcribed using the PrimeScript RT Master Kit (Takara). qPCR was performed using SYBR Premix Ex TaqTM II (Takara) on a LightCycler (Roche). The sequence-specific forward and reverse primers sequences for MT1-MMP mRNA were 5'-AAGAGGAGAAGAGCAAACAG-3' and 5'-CGGTAGGCACTGAACTTG-3' respectively. Forward and reverse primers sequences for ARF5 mRNA were 5'-ATCTGTTTCACAGTCTGGGACG-3' and 5'-CCTGCTTGTTGGCAAATACC-3' respectively. Relative quantification of mRNA expression was calculated by using the $2^{-\Delta\Delta ct}$ method. The raw data were presented as the relative quantity of MT1-MMP mRNA, normalized with ARF5, and relative to a calibrator sample. All qRT-PCR reactions were performed in triplicate.

Immunohistochemistry

Paraffin sections from normal and breast cancer specimens were deparaffinized in xylene and rehydrated in a descending ethanol series (100%, 90%, 80%, 70% ethanol) and double distilled water according to standard protocols. Heat-induced antigen retrieval was performed in citrate buffer and boiled for 10 min. After antigen retrieval, sections were treated with 3% hydrogen peroxide and 1% bovine serum albumin to block the endogenous peroxidase activity and non-specific binding. The sections were incubated with MT1-MMP antibody (Chemicon, clone 114-6G6, dilution 1:100) overnight at 4°C. After phosphate buffered saline (PBS) washing, the tissue sections were incubated

Table 1. Expression of MT1-MMP protein between breast cancer and non-cancerous samples

Group	cases	MT1-MMP		*p*
		High expression	Low expression	
cancer tissue	126	67	59	0.037
non-cancerous tissues	21	6	15	

Table 2. Correlations between MT-1MMP protein expression and clinicopathological characteristics in breast cancer

Characteristics	n	High expression	Low expression	*P*
Age (y)				
<50	50	30	20	0.213
≥50	76	37	39	
Histological grade				
I	43	18	25	0.067
II-III	83	49	34	
Clinical stage				
I-II	46	19	27	0.043
III-IV	80	48	32	
Tumor size				
<5 cm	78	37	41	0.100
≥5 cm	48	30	18	
Lymph node metastasis				
absence	57	24	33	0.024
presence	69	43	26	
Distant metastasis				
No	118	59	59	0.017
Yes	8	8	0	
Triple-negative breast cancer				
No	108	54	54	0.080
Yes	18	13	5	

with the biotinylated secondary antibody and streptavidin-horseradish peroxidase complex, each for 20 min at room temperature. Diaminobenzidine (DAB) was used as the chromogen, and tissue sections were counterstained with hematoxylin and then viewed under a bright-field microscope.

Evaluation of staining

The tissue sections stained immunohistochemically for MT1-MMP were reviewed, and scored separately by two pathologists blinded to the clinical parameters. Any disagreements were arbitrated by the third pathologists. Staining intensity was graded (0, negative; 1, weak; 2, moderate; 3, strong), and percentage of positive-staining cells was counted (0, 10%; 1, 11-50%; 2, 51-75%; 3, >76%). The final score was determined by the combined staining score and proportion score (intensity score × proportion score) [10]. The final staining score ranged from 0 to 9. For statistical analysis, a final staining scores of 0-4 and 6-9 were respectively considered to be low and high expression.

Statistical analysis

All statistical analyses were performed using SPSS version 17.0 software. Data were presented as mean ± SD. The unpaired T test was applied to test the differential mRNA expression of MT1-MMP in breast cancer compared to non-cancerous breast tissues. The Chi-square test was used to examine the differences of MT1-MMP protein expression between breast cancer and non-cancerous breast tissues. The Chi-square test was applied to the examination of relationship between MT1-MMP expression levels and clinicopathologic characteristics. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. The significance of survival variables was analysed using the Cox multivariate proportional hazards model. A *P*-value of less than 0.05 was considered statistically significant.

Results

MT1-MMP mRNA and protein was overexpressed in breast tissue

From Poola et al.'s microarray data (GSE2429), MT-MMP1 was up-expressed in breast cancer tissues compared with ductal hyperplasia tissues by an average of 1.30-folds (**Figure 1**).

In order to verify the role of MT1-MMP in breast cancer, we performed real-time PCR to measure the expression of MT1-MMP mRNA transcripts in fifteen fresh breast cancer tissues and fifteen fresh normal breast tissues. This expression pattern was similar to the microarray data. Compared with normal non-cancerous

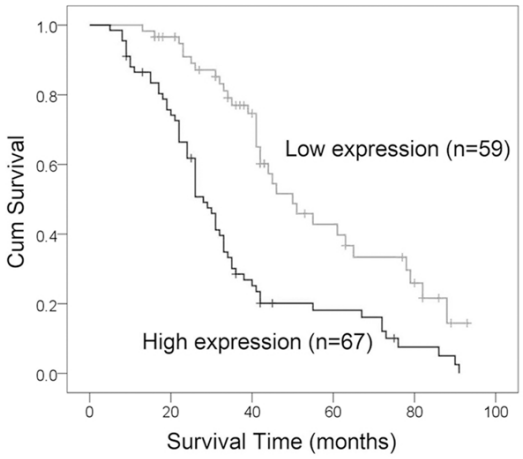

Figure 4. Increased MT1-MMP protein expression predicts an unfavorable prognosis. The association between patient survival and MT1-MMP expression was estimated using the Kaplan-Meier method and the log-rank test (P<0.001).

breast tissues, the levels of MT1-MMP mRNA was overexpressed with an average increase of 2.49-fold (P=0.005) (**Figure 2**).

We detected the expression levels of MT1-MMP protein in 126 archived paraffin-embedded breast cancer samples and 21 non-cancerous breast samples using immunohistochemical staining (**Figure 3A-D**). We observed that MT1-MMP protein was overexpressed in 53.17% (67/126) of breast cancer samples. In comparison, only 28.57% of normal breast samples had highly expressed MT1-MMP protein, significantly lower than that in the breast cancer samples (P=0.037) (**Table 1**).

Correlation between MT1-MMP and clinicopathological characteristics in breast cancer patients

We next analyzed the correlation between the expression of MT1-MMP protein and clinicopathological characteristics of breast cancer. As summarized in **Table 2**, there were no significant correlations between MT1-MMP expression and age (P=0.213), histological grade (P=0.067), tumor size (P=0.100) or triple-negative breast cancer (P=0.080). However, MT1-MMP was associated significantly with clinical stage (I-II vs. III-IV; P=0.043), lymph node metastasis (absence vs. presence; P=0.024), distant metastasis (No vs. Yes; P=0.017).

Survival analysis

To explore the prognostic value of MT1-MMP expression in patients with breast cancer, we measured the association between the levels of MT1-MMP expression and patients' survival using Kaplan-Meier analysis with the log-rank test. In 126 breast cancer patients with prognosis information, we found that the level of MT1-MMP protein expression was significantly associated with the overall survival of breast cancer patients, as patients with higher levels of MT1-MMP expression had poorer survival than those with lower levels of MT1-MMP expression (P<0.001) (**Figure 4**). Furthermore, we also found that increased expression of MT1-MMP showed poor prognosis in breast cancer patients, regardless of clinical stage, tumor size, lymph node metastasis and distant metastasis. Multivariate analysis showed that increased MT1-MMP expression was a poor independent prognostic factor for breast cancer patients (**Table 3**).

Discussion

MT1-MMP, which is a member of the membrane type MMPs, has been implicated in many biological processes [11]. MT1-MMP activity is not restricted to extracellular matrix degradation and is necessary for several processes involved in tumorigenesis, including angiogenesis, regulation of growth factor and induction of the epithelial-mesenchymal transition [12]. Generally, MMPs are produced by tissues as inactive zymogens and require further activation. However, MT1-MMP does not require additional activation, due to its capacity to be presented on the cell membrane in its active form [8]. Furthermore, present studies demonstrated that MT1-MMP played a significant role in tumor progression. MT11-MMP was originally known as a tumour specific activator of MMP2 [13] and is now identified to activate MMP13 and degrade a variety of ECM components, including fibronectin, collagens, and laminins [14]. Moreover, MT1-MMP also processes and interacts with membrane proteins such as integrins [15], CD44 [16], syndecan-1 [17] and BRCA2 [18].

MT1-MMP is highly expressed in almost all types of human cancer, such as lung cancer [10, 19], breast cancer [20], colon cancer [20], cervical cancer [21], prostate cancer [22], and

Table 3. Univariate and multivariate Cox regression analyses of overall survival in 126 breast cancer

Parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*	HR	95% CI	*P*
Age (<50 vs. years ≥50)	0.913	0.605-1.377	0.663			
Histological grade I vs. II-III	1.234	0.794-1.919	0.349			
Clinical stage I-II vs. III-IV	4.505	2.652-7.652	0.000	1.928	0.785-4.739	0.152
Tumor size (<5 cm vs. ≥5 cm)	2.592	1.681-3.997	0.000	2.473	1.555-3.934	0.000
Lymph node metastasis (absence vs. presence)	4.992	2.855-8.727	0.000	2.694	1.103-6.582	0.030
Distant metastasis (No vs. Yes)	10.716	3.392-25.564	0.000	3.187	1.294-7.845	0.012
Triple-negative breast cancer (No vs. Yes)	1.681	0.990-2.857	0.055			
MT1-MMP (Low vs. High)	2.643	1.714-4.077	0.000	1.988	1.270-3.112	0.003

HR, hazard ratio; 95% CI, 95% confidence interval

glioblastomas [23]. In a recent microarray analysis, we found significantly high levels of MT1-MMP mRNA in squamous cell lung cancer compared to normal lung tissues [24]. In addition, we also found increased level of MT1-MMP in human pleural mesotheliomas compared with normal pleural specimens in a microarray analysis for 39,000 human transcripts [25]. Similar to a microarray analysis performed by Poola et al. (GSE2429) [26], we found MT1-MMT was higher level in breast cancer samples than in ductal hyperplasia. Then, we verified the mRNA and protein expression of MT1-MMP in breast cancer and normal breast samples through Real-time PCR and immunohistochemistry. This expression pattern was similar to the microarray data.

In order to further identify the role of MT1-MMP in the development and progression of breast cancer. We analyzed the expression of MT1-MMP in 126 breast patients and found MT1-MMP overexpression was significantly associated with clinical stage, lymph node metastasis, and distant metastasis. Our study may suggest that MT1-MMP plays significant roles in breast cancer progression, including tumor invasion and metastasis. Similarly, Têtu et al. also showed that overexpressed MT1-MMP was positively correlated with involved lymph nodes of breast cancer [27]. Furthermore, the expression of MT1-MMP also showed a significant positive correlation with the expression of MMP1, MMP2, MMP3, MMP9, and MMP11 in breast cancer [28]. Inhibition of MT1-MMP significantly suppressed breast cancer cell migration [29]. These studies consistently indicated that overexpressed MT1-MMP may play an unfavorable role in breast cancer pathogenesis. However, the correlation between MT1-MMP expression and the survival of breast cancer patients has been seldom reported.

MT1-MMP overexpression in tumor cells has been shown to be an independent unfavorable prognostic factor in many types of tumors [30]. In gastric cancer, He et al. [31] and Peng et al. [32] observed that a high MT1-MMP protein level correlated with an unfavorable prognosis, which was consistent with Wang et al's. [10] and Zhou et al's. [19] reports in non-small cell lung cancer. Furthermore, a meta-analysis, which included 1,918 cases from 11 studies, showed that MT1-MMP is a potential prognostic factor in human cancers, and the pooled hazard ratio (HR) and corresponding 95% confidence interval (CI) was 2.46 (95% CI: 1.75-3.47) [30].

In this study, we first presented that MT1-MMP expression in breast cancer was inversely correlated with patient's overall survival in protein

level. The patients with higher expression of MT1-MMP protein had shorter survival time. According to multivariate analyses, overexpression of MT1-MMP protein was a significant predictor of unfavorable prognosis for breast cancer patients. These results were consistent with Têtu et al's and McGowan et al's reports. Têtu et al. [27] and McGowan et al. [28] found that high MT1-MMP mRNA predicted a significantly shorter overall survival for patients with breast cancer. Thus, the MT1-MMP mRNA and protein expression has been shown to play an important role in prognosis of breast cancer patients.

In conclusions, Our study indicated that the mRNA and protein level of MT1-MMP is significantly increased in breast cancer and correlated with clinical stage, lymph node metastasis, and distant metastasis of breast cancer patients. Moreover, our results suggested that MT1-MMP was a significant prognostic factor for breast cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Feng Chen, Division of General Surgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, 600 Yishan Road, Shanghai 200233, China. E-mail: chenfengmail@163.com; Rui Ling, Department of Vascular and Endocrine Surgery, Xijing Hospital, Fourth Military Medical University, 15 West Changle Road, Xi'an 710032, China. E-mail: lingrui0105@163.com; Jun Yun, Department of Vascular and Endocrine Surgery, Xijing Hospital, Fourth Military Medical University, 15 West Changle Road, Xi'an 710032, China. E-mail: yunjun2000@163.com

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