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• BRIEF REPORTS •

Expression and significance of angiopoietin-2 in gastric cancer

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Abstract

AIM: To investigate the expression and pathological factors of Angiopoietin-2 (Ang-2) in primary gastric cancers and adjacent normal tissues.

METHODS: The expression of Angiopoietin-2 and VEGF were studied in 72 primary gastric cancers and adjacent normal tissues from the same patients by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and immumohistochemistry.

RESULTS: Ang-2 was mainly expression in tumor cells. There were significantly difference between expression of Ang-2 in primary gastric cancer and in adjacent normal tissue samples (P=0.003). It was statistically correlation between Ang-2 and VEGF expression in tumors (P=0.0055). With regard to Ang-2 expression in tumors, there were significant difference between early stage and advanced stage (P=0.017), and significant difference between positive vascular involvement and negative vascular involvement (P=0.032). However, there was no significant difference between moderate-poor differential type and high differential type (P=0.908), between positive lymph node metastasis and negative lymph node metastasis (P=0.752), between positive serosal invasion and negative serosal invasion (P=0.764). The cases with expression of Ang-2 were increasing with advanced stage and vascular involvement.

CONCLUSION: The results manifested that Angiopoietin-2, coordinated with VEGF, play role in regulating tumor angiogenesis of gastric cancer.

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INTRODUCTION

Angiogenesis is required for the growth and metastasis of malignant tumor, and is defined as the sprouting of blood vessels from pre-existing vessels that migrate into the tumor and form a new vascular network. Many factors attend the process of angiogenesis. Recently, several studies have shown that, the expression of angiopoietin-2 (Ang-2) probably correlates to tumor angiogenesis[1,2]. However, the role and mechanism of Ang-2 in tumor angiogenesis still remain to be determined. Here, we investigate the expression and significance of angiopoietin-2 in gastric cancer.

MATERIALS AND METHODS

Materials

Reagents Trizol liquid, AMV reverse transcriptase, Oligd(T)$_{14}$, RNAsin, dNTP, Taq DNA polymerase were purchased from shanghai sangon biological engineering technology and service co.Ltd, PCR primers were synthesized by shanghai sangon biological engineering technology and service co.Ltd. N-18 and P-20, the monoclonal antibodies of Ang-2 and VEGF respectively, were purchased from santa cruz company.
Clinical data Total of 72 patients with respectable primary gastric cancer were analysed. There were 46 males and 26 females with primary gastric cancer. Age varied from 38 to 72 years, with a mean age of 53.5 years. Routine pathological diagnosis showed that 53 cases were adenocarcinoma, 19 cases were signet carcinoma. Of these 72 patients, 48 individuals had lymph nodes metastasis, and 26 others had no lymph node metastasis.

Methods

Detection of expression of Ang-2 AND VEGF Expressions of Ang-2 and VEGF were assessed in every gastric cancer sample and its adjacent normal tissue by semi-quantitative RT-PCR.
RNA extraction Total RNA was extracted by Trizol one step procedure, and suspended in DEPC-treated reverse osmosis-H_2O, and conserved at -70 ℃ for reverse transcription. RNA yield and purity were determined by standard UV spectrophotometric assay. The ratio of A_{260}/A_{280} is 1.80.
First strand cDNA synthesis A 5 μg of the total RNA was dissolved in 20 μL of a mixture containing 2 μL of 10× first-strand buffer, 20 μL of AMV reverse transcriptase, 2 μL of dNTP, 20 μL of RNAsin, 500 ng of Oligd(T)$_{14}$, and DEPC-treated reverse osmosis-H_2O. The reaction conditions were as following: at 42 ℃ for 60 min, at 95 ℃ for 5 min. The first strand cDNA was stored at -20 ℃ until use.
PCR amplification Primers of Ang-2, VEGF and β-actin were synthesized according to primer design principles, all primer sets used span an intron to control amplification of genomic DNA sequences. A 3 μL of the first strand cDNA were amplified in 20 μL volume. The primers of Ang-2 were yielded 921-bp product and as following: 5' -end primer: 5' -GGGGGAGGACTG GTGACAGCCACGG-3' ,3' -endprimer:5' -GAAATCTGCTGGC CGGATCATCAT-3' . Following an initial denaturation at 94 ℃ for 5 min, the samples were amplified by 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 30 s, extension at 72 ℃ for 30 s, and ended by extension at 72 ℃ for 10 min. The primers of VEGF were yielded 356-bp product and as following: 5' -end primer: 5' -ACCATGAACTTTCTGCTCTCTTGG-3' , 3' -end primer:5' -CCGCCTTGGCTTGTCACATCTGCA-3' .Following an initial denaturation at 94 ℃ for 5 min, the samples were amplified by 28 cycles of denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 1 min, extension at 72 ℃ for 1 min, and ended by extension at 72 ℃ for 10 min. The primers of β-actin, which was

amplified with Ang-2 and VEGF as internal control, were yielded 644 bp product and as following: 5' -end primer: 5' -ACGTTATG GATGATGATATCGC-3' ,3' -end primer: 5' -CTTAATGTCACG CACGATTTCC-3' . PCR products were separated on 1.7% agarose gel , stained with ethidium bromide, and analysed with Quantity one 4.1.0 software. The ratios of Ang-2/β-actin, AEGF/ β-actin were used to semiquantify the levels of Ang-2 and VEGF.

Immunohistochemical staining The immunohistochemical study of expression of Ang-2 and VEGF in gastric cancer and adjacent normal tissue was performed by the avidin-biotin-peroxidase technique using monoclonal antibody N-18 and P-20, as previously described[3,4]. Briefly, after formaldehyde-fixed paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in alcohol, they were incubated in 3 mL/L H_2O_2 to block endogeneous peroxidase activity. Each slide was incubated with normal horse serum for 20 min at room temperature, and then monoclonal antibody N-18 or P-20, the working dilution were 1:100 and 1:200 respectively, was incubated on the tissue section overnight at 4 ℃. After incubated in biotinylated mouse anti-goat IgG (the working dilution were 1:200) for 30 min at room temperature, each slide was rinsed in phosphate-buffered saline and was incubated in the avidin-biotin peroxidase complex for 40 min at 37 ℃. The peroxidase was visualized with 3-3' -diamino-benzidine-tetrahydrochloride solution and then counterstained with methyl green.

Expressions of Ang-2 and VEGF in primary gastric cancers and adjacent normal tissues were detected under microscopy.

Statistical analysis Results are calculated as mean±SD for each group. *t* test groups or *chisq* test was used to analyze the variables. Statistical analyses were performed with SPSS software, version 10.0.

RESULTS

Results of RT-PCR

Seventy-two primary gastric cancers and adjacent normal tissues from the same patients were examined for the expression of Ang-2 and VEGF by RT-PCR. In 72 cases of primary tumors, Ang-2 and VEGF were expressed in 46 (63.9%) and 48 cases (66.7%) respectively. However , in 72 adjacent normal samples, Ang-2 and VEGF were expressed in 10 (13.9%) and 16 (22.2%) respectively. The expression of Ang-2 in primary gastric cancer has significant differences from that in adjacent normal tissues (*P*=0.003), (Figure 1, Table 1).

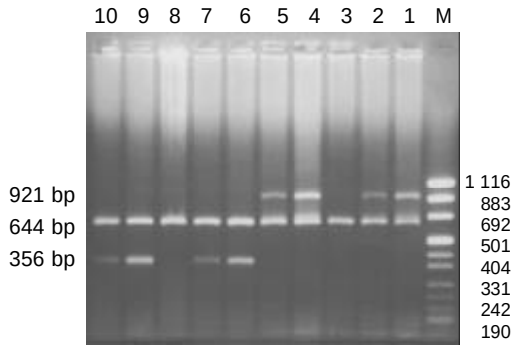

Figure 1 Semi-quantitative RT-PCR amplified products of Ang-2 and VEGF in primary gastric cancer and adjacent normal tissue 644 bp: internal standards, 921 bp: Ang-2 expression, 365 bp: VEGF expression, Lane-M: puc mix marker 8 (1116, 883, 692, 501, 404, 331, 242, 190, 147, 110, 67, 34, 26, 19) bp, Lanes 1-5: Ang-2 expression, Lanes 6-10: VEGF expression.

Meanwhile, in 46 cases of gastric cancers with Ang-2 positive expression, VEGF was coexpressed in 36 cases (78.26%), but 26 cases of gastric cancers with Ang-2 negative expression showed VEGF expression in 12 cases (46.15%). The expressions of Ang-2 and VEGF in the primary tumors were significantly correlated (*P*=0.0055), (Table 2).

Table 1 Expression of Ang-2 in primary gastric cancers and adjacent normal tissues detected by semi-quantitative RT-PCR

	Cases	Ang-2 (mean±SD)
Primary gastric cancers	72	0.497±0.393[a]
Adjacent normal tissues	72	0.088±0.224

a: *P*=0.003 *vs* adjacent normal tissues.

Table 2 Correlation between expression of Ang-2 and VEGF in 72 primary gastric cancer detected by semi-quantitative RT-PCR

Ang-2 expression (Cases)	VEGF expression (Cases)		*P*
	Positive	Negative	
Positive	36	10	0.0055
Negative	12	14	

Result of immunohistochemistry

Positive control included human placenta. Positive expression of Ang-2 and VEGF show brown staining in the cytoplasm of tumor or normal cells, Ang-2 was mainly expression in tumor cells (Figures 2,3).

Pathologic factors affecting expression of VEGF-C

Several pathological factors, including tumor stage, histological type, lymph node metastasis, serosal invasion and vascular involvement, were investigated to predicting expression of Ang-2 in gastric cancer. The results show that, in expression of Ang-2, there were significant difference between early stage and advanced stage (*P*=0.017), and significant difference between positive vascular involvement and negative vascular involvement (*P*=0.032). However, there was no significant difference between moderate-poor differential type and high differential type (*P*=0.908), no significant difference between between positive lymph node metastasis and negative lymph node metastasis (*P*=0.752), and no significant difference between positive serosal invasion and negative serosal invasion (*P*=0.764), (Table 3).

Table 3 Correlation between pathological factors and Ang-2 expression in 72 primary gastric cancer

Pathological factors	No. of cases	Ang-2 mRNA (mean±SD)	*P* value
Tumor stage			
Early stage	26	0.222±0.310	
Advanced stage	46	0.593±0.318	0.017
Histological type			
Moderate-Poor differential type	45	0.425±0.350	0.908
High differential type	27	0.203±0.290	
Lymph node metastasis			
Positive	48	0.413±0.346	0.752
Negative	24	0.490±0.450	
Serosal invasion			
Positive	43	0.404±0.327	0.764
Negative	29	0.334±0.459	
Vascular involvement			
Positive	46	0.640±0.335	0.032
Negative	26	0.272±0.298	

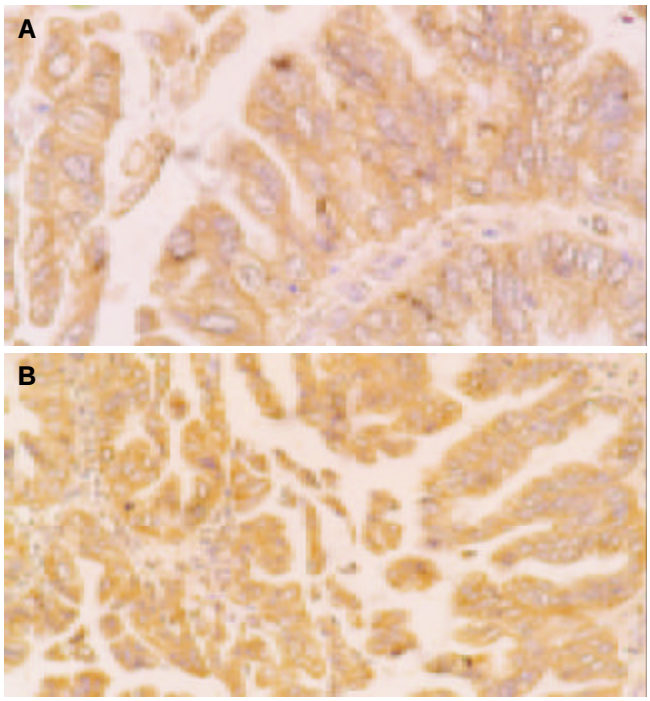

Figure 2 Expression of Ang-2 and VEGF in gastric cancer, A: Ang-2 positive expression in gastric cancer (×400), B: VEGF positive expression in gastric cancer (×400).

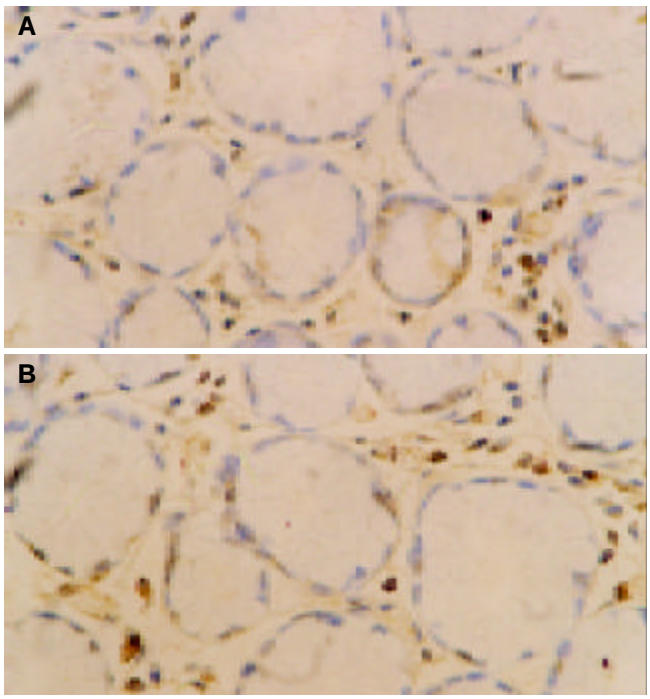

Figure 3 Expression of Ang-2 and VEGF in adjacent normal tissue, A: Ang-2 negative expression in adjacent normal tissue (×400), B: VEGF negative expression in adjacent normal tissue (×400).

DISCUSSION

Solid tumors could recruit blood vessels from the neighboring tissue by angiogenesis, and adequate blood supply could promote solid tumor growth to a clinically relevant size[5]. The ability of a tumor to induce angiogenesis could determine its rate of growth and its likelihood of metastasis[6-8]. It has been found angiogenesis is dependent on a tightly regulated balance between angiogenic promoters and inhibitors[9]. Numerous factors have been implicated in regulate angiogenesis, including growth factors and their receptors, a variety of proteases, adhesion receptors and ECM component[10,11].

Angiopoietins, novel endothelial factors, were found to be ligands for the endothelium-specific tyrosin kinase receptor Tie-2[12]. Angiopoietins (Ang) included Ang-1, Ang-2, Ang-3 and Ang-4, the best characterized were Ang-1 and Ang-2. Ang-1 and Ang-2 were soluble 70-ku factors, which consist of an amino-terminal coiled-coil domain and a carboxy-terminal fibrinogen-like domain[13,14].

Both of Ang-1 and Ang-2 could bind to the Tie-2 receptors. Ang-1 could bind to the Tie-2 receptor and activate it by inducing phosphorylation, and help to maintain and stabilize mature vessels by promoting interactions between endothelial cells and supporting cells[15-17]. Ang-2 could also bind to the Tie-2, but not activate phosphorylation. Ang-2 could block the action of Ang-1[18]. That is to say, Ang-2 was an antagonist of Ang-1 and induces the loosening of the interactions between endothelial and perivascular support cells and ECM, reducing vascular integrity and facilitating access to angiogenic induces[19,20]. Recent studies have shown that the expression pattern of Ang-2 is strongly associated with that of VEGF in the process of tumor angiogenesis, VEGF and Ang-2 seemed to play complementary and coordinated roles in the development of new blood vessels[21,22]. Angiopoietins were mainly produced by endothelial cells and pericytes, and their receptor Tie-2 was also expressed in endothelial cells and pertly in hematopoietic cells[23]. Ang-2 was selectively expressed in endothelial cells of tumors, ovaries, uterus and placenta, which are known to have extensive vascularization patterns[24-26].

The role and mechanism of Ang-2 in tumor angiogenesis have not been clarified. Some studies suggested that the production of VEGF and Ang-2 must be coordinated in development of tumor angiogenesis[27,28]. Ang-2 could produce destabilization and induce angiogenic response in the presence of VEGF, but lead to vessel regression in the absence of VEGF[13,29]. While other studies manifested that VEGF upregulates expression of Ang-1, but not Ang-2[30]. The expression of Ang-2 correlated with tumor size, but had no correlation with expression of VEGF[31]. Kuroda's results showed that upregulation of Ang-1, Ang-2 and Tie-2 is closely associated with the development of microvascular proliferation in psoriasis, and the angiopoietin-Tie-2 system might act coordinately with VEGF to promote angiogenesis[32]. Hatanaka's results suggested that tumor-produced IL-10 promotes stromal vascularization through expression of Ang-1, Ang-2 and Tie-2[33]. Angiopoietin-Tie-2 system, particularly Ang-2, played critical role in the vascularization of prostate carcinoma, breast cancer, colon cancer, astrocytoma, gastric carcinoma, *etc*[23,34-37]. Ang-2 expression was highest during the early stages of angiogenesis, perhaps reducing Tie-2 activity to allow the established vasculature to respond to angiogenic stimuli, consequently, Ang-2 expression was decreased and superseded by Ang-1 expression, perhaps activating Tie-2 and resulting in the stabilization and maturation of neovessel[13].

We studied the expression of Ang-2 in 72 primary gastric cancer and adjacent normal tissues by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and immumohisto-chemistry. The results showed that Ang-2 was mainly expression in tumor cells, and there were significantly difference in Ang-2 expression between primary tumor and adjacent normal tissue samples. The present study also clearly manifested that it was statistically correlated between Ang-2 and VEGF expression in tumors. The expression of Ang-2 was related with tumor stage and vascular involvement. Ang-2 overexpression by newly formed tumor blood vessels may lead to vessel destabilization and relative hypoxia, which could drive the release of VEGF, leading to robust angiogenesis[37].

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