Expression and significance of angiopoietin-2 in 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

Primer: 5'-CCGCCTTGGCTTGTCACATCTGCA-3'. Following an initial denaturation at 94°C for 10 min, the primers of Ang-2, VEGF and β-actin were yielded 356-bp product and as following: 5’-end primer: 5’-GGGGGAGGACTGGTGACAGCCACGG-3’, 3’-end primer: 5’-GAAATCTGCTGGCGGATCATCAT-3’. Following an initial denaturation at 94°C for 30 s, and ended by extension at 72°C for 10 min. The primers 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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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 tissues (P=0.003). It was statistically correlation between Ang-2 and VEGF expression in tumors (P=0.0055).

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.

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-H2O, and conserved at -70°C for reverse transcription. RNA yield and purity were determined by standard UV spectrophotometric assay. The ratio of A260/A280 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 OligdT14, and DEPC-treated reverse osmosis-H2O. The reaction conditions were as following: at 42°C for 60 min, at 95°C for 5 min. The first strand cDNA was stored at -20°C 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: S′-GGGGGAGGACTGGTGACAGCCACGG-3’, 3’-end primer: S′-GAAATCTGCTGGCGGATCATCAT-3’. Following an initial denaturation at 94°C for 5 min, the samples were amplified by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s, and ended by extension at 72°C for 10 min. The primers of VEGF were yielded 356-bp product and as following: 5’-end primer: S′-ACCATGAACTTTCTGCTCTCTTGG-3’, 3’-end primer: S′-CGGCCTTGGCTTTGTCACATGCG-3’. Following an initial denaturation at 94°C for 5 min, the samples were amplified by 28 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 1 min, extension at 72°C for 1 min, and ended by extension at 72°C 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 CACCATTTC-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 H2O2 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 has significant differences from that in adjacent normal tissues detected under microscopy.

Expressions of Ang-2 and VEGF in primary gastric cancers and adjacent normal tissues were detected by semi-quantitative 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).

**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).

**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     |
| 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                | Positive                | 0.0055  |
| Positive                | Negative                |         |
| Negative                | Positive                |         |
| Negative                | Negative                |         |

**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 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          |         |
growth factors and their receptors, a variety of proteases, adhesion receptors and ECM components.[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 partly 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.[34-36]. 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 neovessels[37].

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 immunohisto-chemistry. The results showed that Ang-2 was mainly expression in tumor cells, and there were significant 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].

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.[39]. 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.[39]. Numerous factors have been implicated in regulate angiogenesis, including

**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).
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