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Expressions and clinical significances of angiopoietin-1, -2 and Tie2 in human gastric cancer ☆

Jun Wang, Kaichun Wu *, Dexin Zhang, Hongwei Tang, Huahong Xie, Liu Hong, Yanglin Pan, Mei Lan, Shengjuan Hu, Xiaoxuan Ning, Daiming Fan

State Key Laboratory of Cancer Biology and Institute of Digestive Disease, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710032, China

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Abstract

The roles of angiopoietins in gastric cancer progression are still not fully understood. In this study the expressions of angiopoietin-1 (Ang-1), -2 (Ang-2) were compared by immunohistochemistry in 53 gastric cancer and 23 normal gastric mucosa samples. Results revealed that Ang-2 expression was significantly increased in gastric cancer tissues (74%) and was correlated with higher TNM stage, lymph node metastasis as well as distance metastasis. The expression of Ang-1 was also elevated in cancerous tissues (66%) and significantly associated with differentiation degree. In addition, Ang-2 as well as its receptor Tie2 expressions were higher in 12 pairs of gastric cancer tissue samples than those in corresponding adjacent samples by Western blot, while Ang-1 expression showed great heterogeneity. Furthermore, the expressions of Ang-1 and Ang-2 were almost positive in eight gastric cancer cell lines. Among them, AGS expressed both Ang-2 and a relatively moderate amount of Ang-2_{443}, a novel splice form of Ang-2, while others showed only Ang-2 mRNA expression.

Keywords: Angiogenesis; Angiopoietin; Carcinogenesis; Gastric cancer

Gastric cancer is a common malignancy in many countries of the world, especially in Asia [1]. Conventional treatments for gastric cancer include surgery, radiotherapy, and chemotherapy. Nowadays, increasing evidence shows that angiogenesis is necessary for tumor growth and studies on anti-angiogenesis have become one of the most promising and active fields in anticancer research [2].

Recently, the Angs (Ang-1 to Ang-4) have been shown to be important mediators of angiogenesis by regulation of endothelial cell survival in malignant and non-malignant tissues [3]. Among them, Ang-1 has been identified as a major activator of the tyrosine kinase receptor Tie2, leading to receptor autophosphorylation on binding. Ang-1 also stimulates endothelial cell migration in vitro [4,5]. Ang-2 is the naturally occurring antagonist to Ang-1 and inhibits Ang-1-mediated Tie2 phosphorylation; this effect leads to vessel destabilization, a necessary step in the initiation of angiogenesis [6,7].

Growing studies have shown that VEGF, another key angiogenesis regulator, is involved in the angiogensis and progression of gastric cancer [8,9]; however, the expressions and roles of Ang-1 and Ang-2 in human gastric cancer have not been fully understood [10–12].

To further investigate the possible roles of Ang-1 and Ang-2 in human gastric cancer progression, we compared mRNA and protein expressions of Ang-1, Ang-2, and Tie2 in gastric cancer specimens as well as several gastric cancer cell lines and analyzed the relationships between the expression levels and clinical features of the patients. Since a novel splice form of Ang-2 named as Ang-2_{443}

☆ *Abbreviations:* Angs, angiopoietins; Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; RT-PCR, reverse transcriptase-polymerase chain reaction; VEGF, vascular endothelial growth factor.

* Corresponding author. Fax: +86 29 82539041.
E-mail address: kaicwu@fmmu.edu.cn (K. Wu).

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was found positively expressed in some tumor cells including breast carcinoma, cervical carcinoma, etc. and may play a important role in tumor progression [13], we also detected Ang-2_{443} mRNA expression in eight gastric cancer cell lines. Our results revealed significant Ang-1, Ang-2, and Tie2 expressions in gastric cancer tissues and cell lines, which may contribute to the progression of gastric cancer.

Materials and methods

Tissue specimens and cell lines. Serial sections of paraffin-embedded tissue from 53 patients with gastric cancer who underwent surgery in our hospital from 2000 to 2003 were selected. Twenty-three normal gastric mucosa specimens were collected from routine upper gastrointestinal endoscopy. None of the patients had received preoperative radiotherapy or chemotherapy. The patient's sex, age, tumor size, histological type of the neoplasm, and TNM stage were obtained from surgical and pathological records.

For Western blot examination, freshly resected tumor and adjacent non-tumorous tissue specimens (>5 cm from the margin of the tumor) from 12 patients with gastric cancer were immediately frozen in liquid nitrogen and stored at −70 °C until use. Histopathological analyses confirmed the malignant and surrounding normal tissues.

Human gastric cancer cell lines MKN28, MKN45, SGC7901, AGS, KATOIII, and MGC803, with various differentiation degrees, respectively, were all preserved in our institute; Gastric cancer cell line XGC9811 was established and kept in our institute [14]. The immortalized human gastric epithelial mucosa epithelial cell line GES-1 was established by the Beijing Cancer Institute [15]. NIH3T3 fibroblast cells were taken as negative control for Ang-1, Ang-2, and Tie2 expressions.

Immunohistochemical staining. The immunohistochemical study of Ang-1 and Ang-2 expressions in gastric cancer and normal mucosa was performed by the avidin–biotin peroxidase staining technique as usual. Briefly, after paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in alcohol, sections were incubated in 3 mL/L H_2O_2 to block endogenous peroxidase activity. Each slide was incubated with normal rabbit serum for 20 min at room temperature, then anti-Ang-1(SC-6319, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Ang-2 antibody (SC-7017, Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 1:100 was applied on sections and incubated overnight at 4 °C. After incubation with biotinylated mouse anti-goat IgG (dilution 1:200) for 30 min at 37 °C, each slide was rinsed in phosphate-buffered saline and was incubated in the avidin–biotin peroxidase complex for 30 min at 37 °C. The peroxidase was visualized with 3,3′-diaminobenzidinetetrahydrochloride (DAB) solution and then counterstained with hematoxylin. Sections incubated with PBS instead of the primary antibody served as a negative control. All sections were examined microscopically and scored by two independent pathologists in a blinded fashion without knowledge of clinical and pathological information. Expressions of Ang-1 and -2 were evaluated according to the ratio of positive cells per specimen and staining intensity as described by Maaser et al. [16]. The ratio of positive cells per specimen was evaluated quantitatively and scored 0 for staining of <1%, 1 for staining of 2–25%, 2 for staining of 26–50%, 3 for staining of 51–75%, and 4 for staining >75% of the cells examined. Intensity was graded as follows: 0, no signal; 1, weak; 2, moderate; and 3, strong staining. A total score of 0–12 was finally calculated and graded as negative (I; score: 0–1), weak (II; 2–4), moderate (III; 5–8), and strong (IV; 9–12).

Western blot analysis. Equal amounts of the extracted gastric tissues protein or gastric cancer cellular protein were separated by 10% SDS–PAGE. The protein bands were electro-transferred to nitrocellulose membrane. Expressions of Ang-1, -2 and Tie2 were analyzed using corresponding specific primary antibodies (rabbit anti-Tie2 polyantibody was kindly provided by Micheal Hanner from Renegeron Pharmaceuticals, USA), followed by incubation with horseradish peroxidase-conjugated anti-goat IgG for Ang-1 and Ang-2 antibodies, anti-mouse IgG for β-actin antibody, and anti-rabbit IgG for Tie2, respectively. The specific protein band was visualized by enhanced chemiluminescence (ECL, Amersham–Pharmacia Biotech, Beijing, China). Autoradiograms were quantified by densitometry (software: Bio Image IQ). The same membrane was reprobed with β-actin specific antibody to ensure equal control. Relative protein levels were calculated compared to β-actin standard.

RNA extraction and semi-quantitative RT-PCR. Total RNA of cells was extracted with Trizol (Life Technologies, Carlsbad, USA) according to the manufacturer's protocol. Appropriate cycles were chosen to assure the termination of PCR amplification before reaching stable stage in each reaction. Gene expression was presented by the relative yield of the PCR product from target sequences to that from GAPDH gene. Primers that could detect both Ang-2 and Ang-2_{443} mRNA expressions and reaction condition of Ang-2_{443} were selected from [17]. PCR primers and reaction parameters for other molecules were chosen according to the literature [18] and are listed as follows (Table 1).

Statistical analysis. Statistical analysis was performed using SPSS software (version 10.0, SPSS, Chicago). The χ^2 test and Fisher exact test were used to test significance of the difference in frequency of Ang-1 and Ang-2 between normal sample and tumors. Mann–Whitney U test for two groups and Kruskal–Wallis H test for multi-groups were used to compare the differences of groups for immunohistochemistry of Ang-1 or Ang-2 with various clinical pathological parameters. The Spearman correlation coefficient was calculated to assess the association between Ang-1 and Ang-2. Differences were considered statistically significant at $p < 0.05$.

Results

Immunohistochemical analysis of Ang-1 and Ang-2 expression in human gastric cancer specimen and normal mucosa

Ang-1 and Ang-2 expressions were found identified in the cytoplasm of epithelium of normal tissues if there were any (Figs. 1A and B). Conversely, the immnoreactive patterns were predominantly identified positive in the cytoplasm of variously differentiated cancerous tissues, 66.0% for Ang-1 (35/53, $p < 0.05$) and 74.0% for Ang-2 (39/53, $p < 0.01$), respectively (Table 2, Figs. 1C–N). Some microvessels also showed positive staining for Ang-1 or Ang-2

Table 1
Primers and reaction parameters for RT-PCR of Angs and Tie2

Products	Sequence	Annealing temperature	No. of cycles	Size (bp)
Ang-1	Forward: 5′-ACTGTGCAGATGTATATCAAGC-3′ Reverse: 5′-GTGGAATCTGTCATACTGTGAA-3′	60	32	326
Ang-2	Forward: 5′-AGCTGTGATCTTGTCTTGGC-3′ Reverse: 5′-AGTAAGCCTCATTCCCTTCC-3′	62	30	444 288
Tie2	Forward: 5′-TCTGTGCTGTTCCTTCTTGC-3′ Reverse: 5′-CTTGA GTAAC TTCCA GCGGA -3′	55	32	375
GAPDH	Forward: 5′-TGGGTGTGAACCATGAGAAGTA-3′ Reverse: 5′-CGCTGTTGAAGTCAGAGGAGA-3′	52	26	469

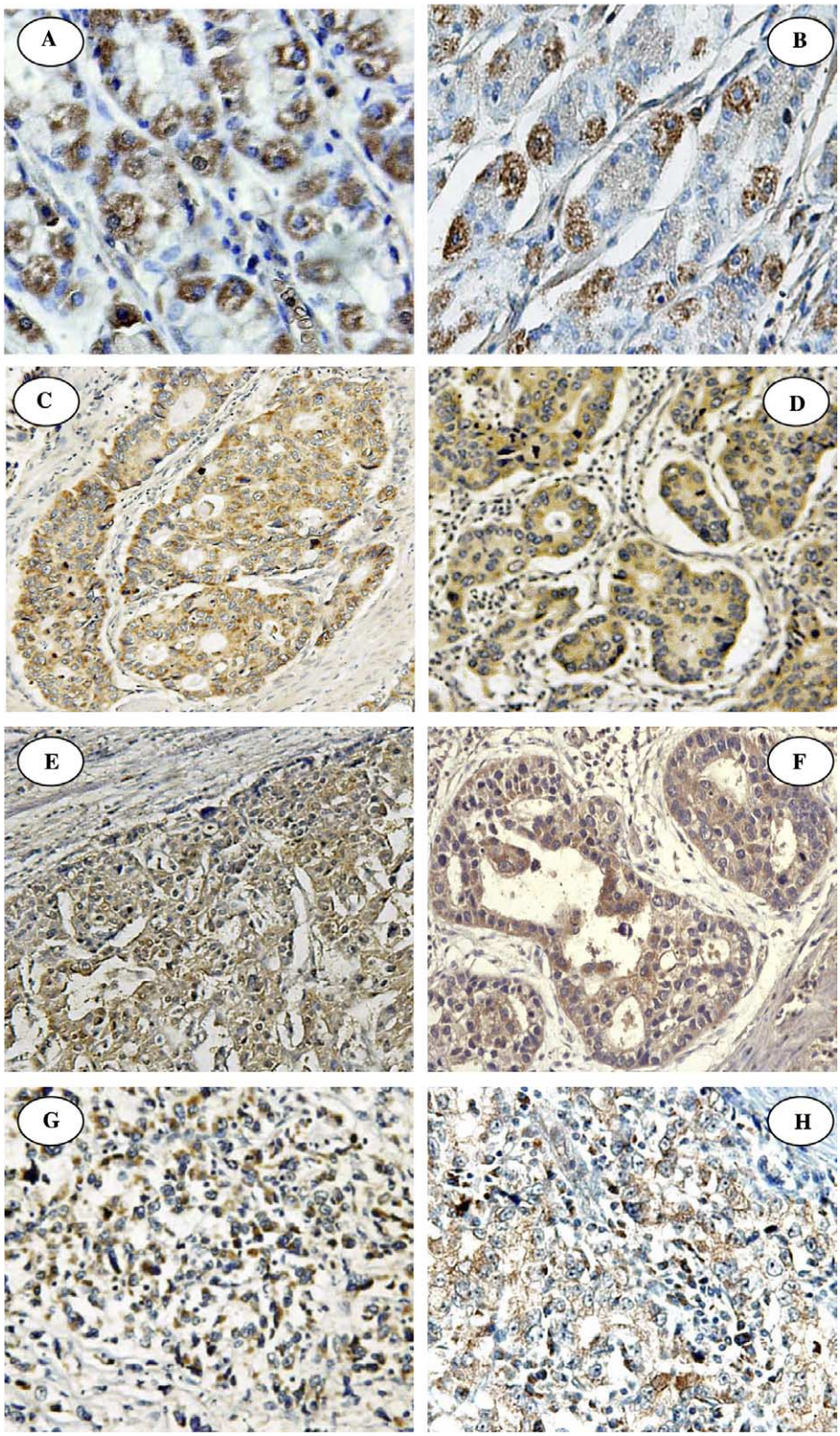

Fig. 1. Immunohistochemical staining of Ang-1, -2 in normal gastric tissue and in gastric cancer at different stages of differentiation. Paraffin sections were stained using the Ang-1 or Ang-2 specific antibodies. Ang-1 (left panels) or Ang-2 (right panels) immunostaining in the cytoplasm of normal epithelium and gastric adenocarcinoma cells. (A,B) Normal epithelium; (C,D) well-differentiated gastric carcinoma; (E,F) moderately differentiated; (G,H) poorly differentiated; (I,J) mucinous carcinoma; and (K,L) Signet-ring cell carcinoma (arrow, microvessel; triangle, poorly differentiated; arrowhead, signet-ring cells). (M,N) Positive expression in microvessels (arrow, microvessel; arrowhead, mucinous cancer cells). Original magnifications: 200× for (A), (B), (G), (H), (J), (L), and (M); 100× for (C), (D), (E), (F), (K), (N), and (I); 400× for (K).

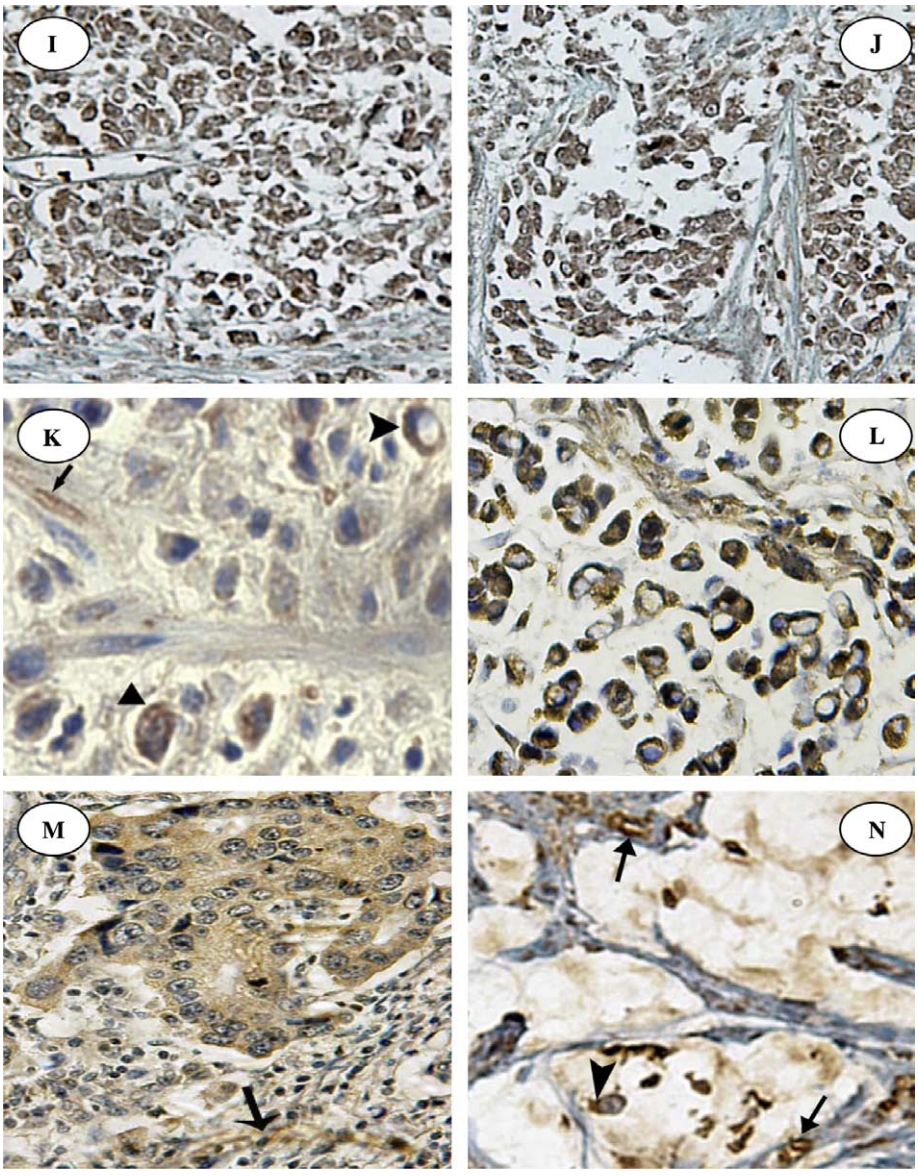

Fig. 1. (*continued*)

Table 2
Expressions of Ang-1 and Ang-2 in gastric tissues by immunohistochemical method

	Total	Ang-1 expression		*P* value	Ang-2 expression		*P* value
		Negative	Positive		Negative	Positive	
Normal mucosa	23	15	8	<0.05	18	5	<0.01
Gastric cancer	53	18	35		14	39	

(Figs. 1M and N). Meanwhile, χ^2 test showed no significant association between expression of Ang-1 and Ang-2 was found ($p > 0.05$) (Table 2).

The Ang-1 expression was correlated with differentiation grade for it was significantly higher in poorly differentiated tumors than that in well-differentiated ones. It was found that Ang-1 expression was not correlated with TNM stage, depth of tumor invasion or metastasis with a statistic $p > 0.05$ in each parameter. These results provide evidence that Ang-1 expression level may positively correlate with gastric cancer onset but not associated with the progression of the cancer. In contrast, the abundance of Ang-2 was significantly associated with TNM stage ($p < 0.01$), lymph node metastasis ($p < 0.05$), and distance metastasis ($p < 0.01$) (Table 3). No significant correlation was found between Ang-1, Ang-2 abundance, and TNM stage or the presence of lymph node metastasis ($p > 0.05$).

Table 3
Clinicopathological associations of Ang-1 and Ang-2 expressions in patients with gastric cancer

Category	*n*	Ang-1 (*n*)				*P* value	Ang-2 (*n*)				*P* value
		I	II	III	IV		I	II	III	IV	
Age (years)						NS					NS
<50	15	5	4	4	2		5	4	4	2	
⩾50	38	13	11	10	4		9	11	13	5	
Gender						NS					NS
Male	38	13	12	9	4		10	11	12	5	
Female	15	5	3	5	2		4	4	5	2	
Differentiation						<0.05					NS
Well-differentiated	9	6	2	1	0		2	3	3	1	
Moderately differentiated	13	6	3	2	2		4	3	4	2	
Poorly differentiated	31	7	10	11	4		8	9	10	4	
TNM stage						NS					<0.01
I	10	2	3	3	2		4	4	2	0	
II	11	4	2	3	2		5	5	1	0	
III	20	8	5	6	1		5	4	9	2	
IV	12	4	5	2	1		0	2	5	5	
Depth of tumor invasion (T)						NS					NS
T_1	3	1	1	1	0		0	2	0	1	
T_2	14	5	4	4	1		4	4	4	2	
T_3	23	8	5	7	3		6	5	9	3	
T_4	13	4	5	2	2		4	4	4	1	
Lymph node metastasis (N)						NS					<0.05
0	21	8	4	6	3		7	10	3	1	
⩾1	32	10	11	8	3		7	5	14	6	
Distance metastasis (M)						NS					<0.01
M_0	42	14	13	10	5		13	13	14	2	
M_1	11	4	2	4	1		1	2	3	5	

Note. NS, not significant.

Expressions of Ang-1, -2 and Tie2 in gastric cancer tissues by Western blot

Expressions of Ang-1, -2 and Tie2 were examined by Western blot in the gastric cancer and adjacent normal tissues taken from 12 patients. Four representative pairs of tissues are presented in Fig. 2. In 10 out of the 12 cases or 83% cancerous tissues, Ang-2 was overexpressed. In contrast, the expression of Ang-1 showed great heterogeneity for overexpressing in 7 cancerous samples and with less or almost equal in other 5 cases. In addition, Tie2 expressions were elevated in almost all gastric cancer tissues compared to those in adjacent normal ones. Combined with the data from immunohistological study, it indicated that Ang-1 with (or) Ang-2 accompanying Tie2 receptor might be involved in the process of gastric cancer progression or initiation.

Analysis of Ang-1, -2 and Tie2 expressions in gastric cancer cells

Moreover, we examined both mRNA and protein expressions of Ang-1, -2 and Tie2 in eight gastric cancer cell lines. Fibroblast cell line 3T3 as negative control showed almost no expressions of Ang-1, Ang-2, and Tie2. In contrast, all eight cell lines showed Ang-1, Ang-2, and Tie2 mRNA expressions (Fig. 3A). In addition, only AGS showed both Ang-2 and relatively moderate Ang-2_{443} mRNA expression, 444 and 288 bp, respectively, while other cell lines showed only Ang-2 expression. According to Western blot (Fig. 3B), Ang-1 and Ang-2 were expressed at a higher level in most gastric cancer cell lines with various differentiation degrees than in GES-1 cells. Among them, AGS and SGC7901 showed the highest expression level. Tie2 as coreceptor of Ang-1 and Ang-2 showed an almost equal expression level in all gastric cell lines. The protein data were consistent with the result from RT-PCR analysis in most cases.

Discussion

Emerging evidence showed that angiopoietin–Tie2 interaction, particularly Ang-2, played a critical role in the vascularization and progression of hepatocellular carcinoma [19], colon cancer [20], astrocytoma [21], etc. In the present study, expressions of Ang-1, -2 and Tie2 in gastric cancer at transcriptional and translational levels were studied by semi-quantitative RT-PCR, immunohistochemistry, and Western blot methods, respectively. We clearly demonstrated that expressions of Ang-1 and Ang-2 in gastric can-

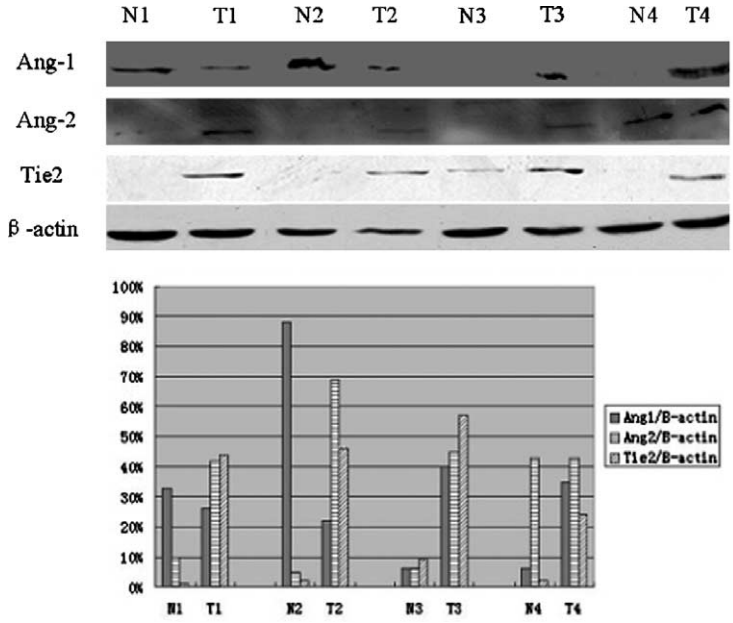

Fig. 2. Western blot analysis of Ang-1, Ang-2, and Tie2 protein in gastric tissues. Protein isolated from resected tumor and adjacent non-tumorous tissue specimens of 12 patients was separated by 10% SDS–PAGE and subjected to Western blot analysis using anti-Ang-1, anti-Ang-2, anti-Tie2, and anti-β-actin specific antibodies. Four representative pairs of tissues are presented. Relative expression levels were calculated by comparing them to the amount of internal control. N, normal tissues; T, gastric cancer tissues.

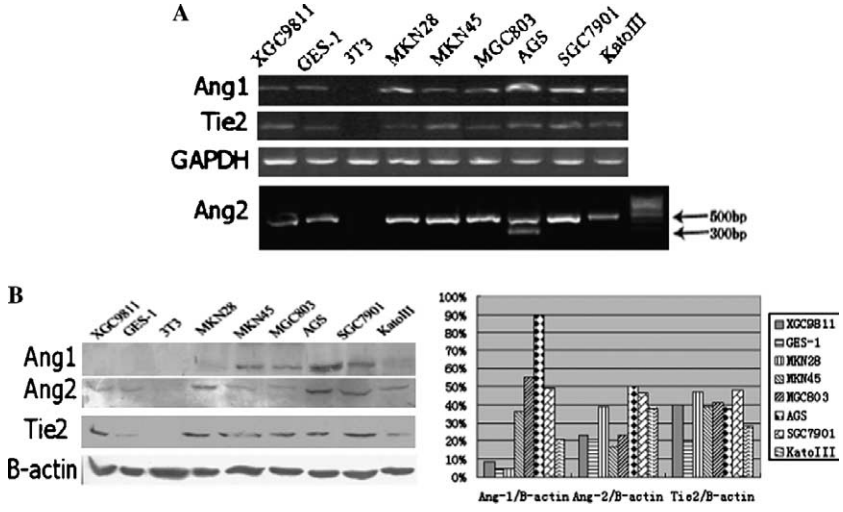

Fig. 3. Expressions of Ang-1, -2, and Tie2 in gastric cancer cell lines. (A) mRNA expressions by RT-PCR; (B) protein expressions by Western blot analysis. GAPDH or β-actin was taken as internal controls for RT-PCR and Western blot, respectively. Relative expression levels were calculated by comparing them to the amount of internal control. All results are representative of three independent experiments.

cer cell lines and tissues were increased. There were significant differences in Ang-1 and Ang-2 expressions between primary tumor and adjacent normal tissue samples. The results show that Ang-1 and -2 were mainly expressed in tumor cells as well as tumor microvessels in some cases. Further evaluation revealed several significant associations of high level Ang-1 and -2 expressions with histopathological and other features of the tumors. Ang-2 expression was significantly correlated with TNM stage, lymph node metastasis, and distance metastasis, while Ang-1 expression was significantly higher in poorly differentiated tumors than in well-differentiated ones, consistent with published findings in human glioblastoma [22].

Ang-2_{443}, absent of amino acids 96–148 of Ang-2 as a result of alternative splicing, is a secreted glycosylated dimeric protein and binds to the Tie2 receptor as an antagonist of Ang-1-induced phosphorylation. Several types of tumor cell lines have shown various patterns of Ang-2 and Ang-2_{443} mRNA expressions previously [13]; to our best knowledge, our data are first evidence of positive

Ang-2_{443} expression in gastric cancer cells, although its role in gastric cancer progression requires further investigation.

A previous report by Nakayama et al. [10] also showed the significant expression of Ang-1 (77.5%) and Ang-2 (84.3%) in Japanese with gastric cancer. Our data are consistent with their report in some cases, but their data did not include the Angs expression in normal tissues and gastric cancer cell lines. Recently, Chen et al. [11] and Sun et al. [12] also from China claimed that angiopoietin-2 may be regulating tumor angiogenesis of gastric cancer by analyzing Ang-2 mRNA in 36 cases and 72 cases of gastric cancer tissues and their paired adjacent gastric mucosa by RT-PCR, respectively. The discrepancy of Ang-2 expression between our study and theirs may be due to the limited case number and the methodology difference.

Several reports claimed that significant expressions of Tie2 in breast cancer [23], myeloid leukemia [24], and so on. An additional important point of our study is that Tie2 as coreceptor of Ang-1 and Ang-2 also showed significant expressions in gastric cell lines and cancerous tissues. To our knowledge, this is the first report of positive Tie2 expression in gastric cancer cell lines in addition to the Ang-1, Ang-2 expressions in gastric tumor endothelium. Since Ang-1 and Ang-2 were produced by gastric cancer cells and endothelial cells as well as Tie2 expressed by gastric cancer cells themselves, our results may suggest a possible autocrine and paracrine pathway of Angs/Tie2 system in gastric cancer cells, which awaits further investigation.

Previously, Ang-2 produced by gastric cancer cells was thought to be implicated in tumor development in human gastric cancers by induction of proteases such as MMP-1 and -9, thus contributing to tumor angiogenesis [25]. Our serial studies revealed that Ang-1 derived from gastric cancer cells also contributed to the gastric angiogenesis and progression [26]. Combined with our present results, it infers that both Ang-1 and -2 participate in the angiogenesis and progression of gastric cancer.

In conclusion, Angs may contribute to the progression of gastric cancers and especially Ang-2 may be a useful molecular marker for gastric cancer and also be a useful therapeutic target to prevent gastric cancer or inhibit its malignant progression.

Acknowledgments

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