Research Article

Integrative Gastroenterology and Hepatology

Expression of Angiogenin is inversely correlated to Progression of Gastric Tumors

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#Equal contribution

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Abstract

Objective: Angiogenin (ANG) is upregulated in a variety of cancers including those of prostate, cervix, pancreas, liver, oral cavity, skin, and etc., however, the role of ANG in gastric cancer has not been fully elucidated yet. We use Tissue Microarray (TMA) to examine ANG expression to investigate the role of ANG in the progression of gastric cancer.

Method: Immunohistochemistry was used to evaluate ANG expression in TMA with 208 spots from 104 patients diagnosed with gastric cancer and the corresponding adjacent tissue.

Results: In normal adjacent tissue, ANG was expressed mainly in cytoplasm at basal gland of the gastric mucus where gastric stem cells are reserved, and also sparsely expressed in the nucleus of gastric mucosal gland cells at isthmus where gastric stem cells are gathered. In cancer tissues, ANG was very sparsely expressed in the nucleus of gastric glandular cells. ANG expression in the cytoplasm was found to be significantly associated with pathological types ($p < 0.001$) and malignancy ($p < 0.001$). ANG expression in the nucleus was inversely correlated with malignancy ($p = 0.019$), differentiation status ($p < 0.001$), and tumor stage ($p = 0.048$).

Conclusion: ANG might play an important role in gastric cancer development.

Introduction

Angiogenin (ANG) was originally isolated as the most potent angiogenic protein based on chicken embryo Chorioallantoic Membrane (CAM) angiogenesis assay [1]. ANG has a dual role in cancer progression: it stimulates cancer cell proliferation as well as mediates tumor angiogenesis. ANG may also have a dual role in amyotrophic lateral sclerosis (ALS) by regulating both endothelial cells and motor neurons [2]. More recently, ANG has been shown to play a role in stem cell regulation by promoting hematopoietic regeneration through

dichotomous regulation of stem cell quiescence and progenitor cell proliferation [3]. ANG was reported to be upregulated in a variety of cancers including prostate, cervical, pancreatic, liver, oral and skin cancer. It has been suggested that angiogenin may be clinically useful in diagnosis, prognosis, and therapy of cancers [4-9]. Here we report that ANG expression is downregulated in gastric cancers. We examined ANG expression in a tissue microarray containing 104 gastric cancer tissues and with the same number of corresponding normal adjacent tissues, and found that ANG expression was inversely correlated with gastric cancer.

Methods

Tissue sampling

The micro tissue array (MTA) was bought from Xi'an Elena Biotechnology Co., Ltd, China with 208 points from 104 gastric cancer patients. For each patient, a carcinoma tissue and an adjacent tissue (AT) were selected. The 104 patients are composed of 40 cardia cancer, 4 fundus cancer, 32 gastric body cancer, and 28 antrum cancer. Pathological diagnosis was made by hematoxylin and eosin staining, and the evaluation of the tissue staining was assessed according to the general role of clinical and histopathological studies for stomach disease in China.

ANG immunohistochemistry staining

MTA tissues were treated with xylene for 20, 15, 10 minutes respectively to dewax, and then washed with 100%, 95%, 90%, 80%, 70% ethanol, respectively, for 5 minutes each, to dehydrate, and then washed with phosphate buffered saline (PBS) 3 times for 5 minutes each. Endogenous peroxidase was blocked with 3% hydrogen peroxidase solution in methanol for 15 minutes. After heat retrieval in citric acid (0.01 M, $P^H = 6.1$), the MTA was incubated with 5% bovine serum albumin (BSA) at room temperature for 40 min, incubated with ANG monoclonal antibody 26-2F (10 $\mu\text{g/ml}$) at 4°C overnight and room temperature for 1 hour. Negative control was normal goat IgG (10 $\mu\text{g/ml}$). After washed three times with PBS, HRP conjugate of second antibody (1:1000) was added and incubated for 30 minutes at 37°C. The MTA was visualized by 3, 3'-diaminobenzidine (DAB) and counterstained by hematoxylin.

Evaluation of ANG immunostaining

The evaluation of ANG expression was performed under routine histological methods [10]. Each spot of the slide was examined under a microscope at low magnification (40 $\times$) to identify the strongest ANG expression according to color and area. Then, five microscopic areas at 400 fold magnification were chosen for analysis. ANG was detected in both cytoplasm and nucleus in glandular cells but not in mesenchymal cells at all. ANG staining in both nucleus and cytoplasm were categorized as 3 grades in color corresponding to (-/0) negative, (+/1) weak expression, (++)/2 strong expression (Figure 1), and 6 grades in positive area corresponding to (0, $\leq 1\%$), (1, 1%~5%), (2, 6%~10%), (3, 11%~25%), (4, 26%~50%), (5, 50%). A total score was obtained by multiplying color and area grades, and recorded as immunoreactivity score (IS) that are defined as: negative (-), score = 0; weak expression (+),

score = 1-4; moderate expression (++) , score = 5-8; and strong expression (+++) , score = 9-12.

Figure 1: Grading of representative staining of nuclear and cytoplasmic ANG. The upper panel shows nuclear ANG staining at 400 X, the bottom panel shows cytoplasmic ANG staining at 200 X.

Statistical analysis

Chi-square test and *t*-test were used to evaluate the difference; all statistical calculations were carried out using the SigmaPlot 12.5 statistical software (copyright 2011 systat software, Germany). It is considered significant when *P* value is < 0.05.

Results

Cancer characteristic and histological examination

The demographic and clinical characteristic of the patients as well as the tumor and adjacent tissues are described in table 1. The patients were 77 men and 27 woman, had a mean age of 60.3 years ranging from 33 to 81. The main three areas of stomach that were affected by cancer were cardia, body/fundus and antrum, including gastric cardia (n=40), gastric body/fundus (n=36) and antrum (n=28). The histological diagnosis were adenocarcinoma (n=102), singlet-ring cell carcinoma (n=1), and squamous cell carcinoma (n=1). The adjacent tissues were normal gastric mucosa (n=42), chronic gastritis (n=59), and atrophic gastritis (n=3).

ANG expression in malignant tumor tissue, adjacent normal gastric tissue and different histological pathology type

In adjacent normal gastric tissue, ANG was expressed mainly in the cytoplasm of gastric basal gland or in the nucleus at isthmus, but not in mesenchymal cells at all.

In cancer tissues, ANG was very sparsely expressed in nucleus as shown in figure 2. The expression score of nuclear and cytoplasmic ANG was evaluated separately and showed in tables 2 and 3, respectively. There was a significant difference both in the nucleus ($P = 0.019$) and in the cytoplasm ($P < 0.001$) in term of ANG expression between cancer and adjacent normal tissues, both showing a decreased expression in cancers as compared with normal gastric glandular tissues. These data indicate that ANG expression is negatively related with gastric malignancy. There was a progressive decrease in the cytoplasmic ANG expression score in the continuum of gastric tissue from normal gland to chronic gastritis, atrophic gastritis, and cancer, which was 4.68 ± 2.90 , 3.86 ± 3.31 , 3.33 ± 5.77 and 0.75 ± 1.24 , respectively, indicating that ANG expression in the cytoplasm is negative related with gastric cancer progression ($P < 0.001$). It is notable that no correlation of ANG expression in the nucleus with the gastric cancer progression was observed ($P = 0.084$).

Figure 3: Representative expression of ANG in gastric adenocarcinoma, signet-ring carcinoma and squamous carcinoma (400 X). A. Gastric adenocarcinoma, B. Signet-ring carcinoma and C. Squamous carcinoma. Arrowhead shows nucleus expression in cancerous cells.

Figure 2: Representative images of ANG staining in adjacent normal gastric tissues (A and B, 40 X and 400 X, respectively) and gastric cancer tissue (C and D, 40 X and 400 X, respectively). Arrows indicate positive ANG staining.

ANG expression in different types of carcinoma

The expression of ANG in gastric adenocarcinoma, signet-ring carcinoma, and squamous carcinoma was all low and mainly distributed in the nucleus, as shown in figure 3, indicating that ANG expression is universally lower in gastric cancers regardless of carcinoma types.

ANG expression in different sites of gastric carcinoma

The expression of ANG in cancer tissues at antrum, fundus body, and cardia was all lower than the adjacent normal tissues, and was mainly detected in the nucleus, less in the cytoplasm, as shown in tables 2 and 3, indicating that lower ANG expression in gastric cancer is not restricted to any particular anatomical sites in the stomach.

ANG expression with tumor grades of gastric carcinoma

Our results show that there is a significant correlation of tumor grade to ANG expression in the nucleus ($P < 0.001$), but not to that in the cytoplasm ($P = 0.3$).

ANG expression with tumor stages of gastric carcinoma

Our results also show that there is a significant correlation of gastric carcinoma stages to ANG expression in the nucleus ($P = 0.048$), but not to that in the cytoplasm ($P = 0.284$).

ANG expression with tumor metastasis of gastric carcinoma

Our results show that there is no significant correlation between tumor invasion and metastasis with ANG

expression in either nucleus or cytoplasm.

Discussion

ANG was originally isolated from the conditioned medium of HT-29 human colon adenocarcinoma cells solely based on its angiogenic activity [1]. Subsequently, ANG was found to have a wide tissue distribution with the liver to be the major source of circulating ANG [11]. ANG is the fifth member of the pancreatic ribonuclease (RNase) superfamily with 33% amino acid identity and 56% homology to RNase-A, is the prototype family member with no angiogenic activity. The ribonucleolytic activity of ANG is several orders of magnitude lower than that of RNase-A, but this unique ribonucleolytic activity is important for the biological activity [12-15]. Two important structural differences between ANG and RNase-A are responsible for this feature. The first is that ANG has a receptor binding site comprising of amino acid residues 59 to 68 [16], but RNase-A does not have this site as the corresponding amino acid sequence of this region is very different [17]. Therefore ANG binds to its target cells (including endothelial cells, cancer cells and motor neurons) but RNase-A does not. Another structural difference between ANG and RNase-A is that ANG has a Nuclear Localization Sequence (NLS), whereas RNase-A does not [18]. Therefore ANG undergoes nuclear translocation in its target cells but RNase-A does not. ANG has been shown to undergo nuclear translocation in endothelial cells [18-20], in cancer cells [5,21], and in motor neurons [5,22,23]. Upon arriving at the nucleus, ANG accumulates in the nucleolus where ribosome biogenesis takes place. Nuclear ANG has been shown to bind to the promoter region of rDNA [24] and stimulates rRNA transcription [25,26]. Thus, ANG acts as a transcription factor to promote rRNA transcription and protein synthesis directly. The rate-limiting step in ribosome biogenesis is rRNA transcription, which would be an important aspect of growth control. Ribosomes are also important for maintaining normal cell function as proteins are essentially required for all cellular activities. ANG-stimulated rRNA transcription has been shown to be permissive for other angiogenic factors to induce angiogenesis [21]. But in stress condition, ANG will accumulate in cytoplasm to produce tRNA (tRNA-derived, stress-induced small RNA) and inhibit protein translation and promotes the assembly of stress granules (SGs). ANG has been found to promote the formation of SGs when cells are subjected to stresses. ANG has been shown to be responsible for stress-induced cleavage of tRNA and as an effector of stress-induced translational repression and significantly inhibits protein synthesis

thereby promoting cell survival [27,28]. Recently, ANG has also been shown to play a role in stem cell regulation, promoting hematopoietic regeneration by dichotomously regulating quiescence of stem and progenitor cell proliferation. Niche-secreted ANG, distinctively alters the functional characteristics of primitive hematopoietic stem/progenitor cells (HSPCs) compared with lineage-committed myeloid-restricted progenitor (MyePro) cells. Specifically, ANG reduces the proliferative capacity of HSPCs while simultaneously increasing proliferation of MyePros by inducing tRNA generation in HSPCs and rRNA transcription in MyePro cells, leading to respective reduction and increase in protein synthesis. Recombinant ANG protein improves survival of irradiated animals and enhances hematopoietic regeneration of mouse and human HSPCs in transplantation. Thus, ANG plays a non-cell-autonomous role in regulate hematopoiesis by simultaneously preserving HSPC stemness and promoting MyePro proliferation [3].

Many studies suggest that ANG has an important function in tumor growth and proliferation. High levels of ANG have been found in solid tumors, and patients with higher levels of ANG have a poor prognosis [4-9]. ANG in healthy person will vary from 294 ng/mL to 496 ng/mL, but in tumor patients it was from 330 ng/mL to 600 ng/mL, and there was a decrease in the amount of ANG levels in tumors after treatment [29]. However, two studies in breast cancer showed opposite result where high angiogenin levels were associated with a good prognosis in one study [30], and were associated with a poor prognosis in the other [31]. Chen et al. [32] reported that increased expression of angiogenin in gastric carcinoma in correlation with tumor angiogenesis and proliferation, but the ANG expression level had no correlation with the age of patients, location, histological type, lymph node metastasis, and clinical stage of the tumor from 68 operated patients with gastric cancer. Shimoyama and Kaminski [33] reported that increased angiogenin expression in gastric cancer correlated with cancer progression from 21 operated patients with gastric cancer. In our study, we used TMA from 104 operated patients with gastric cancer, by TMA technology avoided dying difference and we used the monoclonal antibody 26-2F donated by Guofu Hu's Lab (whose lab discover ANG 30 years ago and concentrated in ANG research work all along) to provide a more convincing result than before, the discrepancy between ANG expression in gastric cancer tissue may also limit by earlier knowledge of ANG function.

Table 1: The characteristic of cancer and patient with histological diagnosis.

Variable		N
Gender	Male	77
	Female	27
Mean age		60.3 ± 9.85
Gastric Area	Cardia	40
	Body/Fundus	36
	Antrum	28
Histological diagnosis(104)	Adenocarcinoma	102
	Signet-ring carcinoma	1
	Squamous carcinoma	1
Cancer adjacent gastric mucosa(104)	Normal mucosa	42
	Chronic gastritis	59
	Atrophic gastritis	3

Note: Data are presented as mean ± standard deviation

Table 2: ANG expression in nucleus in patients with different clinicopathological feature.

			Nucleus score range				P
Variable		Mean ±SDV	0	1-4	5-8	9-12	
Metastasis	T1N0M0	5 ± 1.41	0 / 2	1 / 2	1 / 2	0 / 2	0.392
	T2N0M0	3.26 ± 3	7 / 19	5 / 19	7 / 19	0 / 19	
	T2N1M0	2 ± 1.67	3 / 6	2 / 6	1 / 6	0 / 6	
	T2N2M0	6 ± 0	0 / 1	0 / 1	1 / 1	0 / 1	
	T3N0M0	3.34 ± 2.64	14 / 47	12 / 47	21 / 47	0 / 47	
	T3N1M0	3.91 ± 2.43	5 / 22	7 / 22	10 / 22	0 / 22	
	T3N2M0	2 ± 0	0 / 1	1 / 1	0 / 1	0 / 1	
	T3N2M1	4 ± 0	0 / 1	1 / 1	0 / 1	0 / 1	
	T4N0M0	6 ± 0	0 / 4	0 / 4	4 / 4	0 / 4	
	T4N2M0	4 ± 0	0 / 1	1 / 1	0 / 1	0 / 1	
Benign, Malignant	Adjacent tissue	3.63 ± 2.77	34 / 104	21 / 104	49 / 104	0 / 104	0.019
	Malignant	3.49 ± 2.61	29 / 104	30 / 104	45 / 104	0 / 104	
Grade	1	4.93 ± 2.37	2 / 15	4 / 15	9 / 15	0 / 15	<0.001
	2	4.5 ± 1.94	1 / 26	10 / 26	15 / 26	0 / 26	
	3	2.84 ± 2.71	18 / 45	10 / 45	17 / 45	0 / 45	
Area	Cardia	3.33 ± 2.58	12 / 40	12 / 40	16 / 40	0 / 40	0.237
	Body/fundus	3.17 ± 2.72	13 / 36	8 / 36	15 / 36	0 / 36	
	Antrum	4.3 ± 2.4	3 / 27	10 / 27	14 / 27	0 / 27	
Sex	Male	3.53 ± 2.72	48 / 154	38 / 154	68 / 154	0 / 154	0.555
	Female	3.63 ± 2.61	15 / 54	13 / 54	26 / 54	0 / 54	
Stage	IA/IB	3.43 ± 2.91	7 / 21	6 / 21	8 / 21	0 / 21	0.048
	II/IIA	3.2 ± 2.64	17 / 54	14 / 54	23 / 54	0 / 54	
	III/IIIA/IIIB	4.07 ± 4.07	5 / 29	10 / 29	14 / 29	0 / 29	
Histological diagnosis	Adenocarcinoma	2.39 ± 2.61	29 / 102	30 / 102	45 / 102	0 / 102	0.084
	Atrophic gastritis	2.67 ± 3.06	1 / 3	1 / 3	1 / 3	0 / 3	
	Chronic gastritis	4.41 ± 2.57	13 / 59	10 / 59	36 / 59	0 / 59	
	Normal gastric tissue	2.6 ± 2.73	20 / 42	10 / 42	12 / 42	0 / 42	

Data are presented as mean ±standard deviation

Table 3: ANG expression in cytoplasm in patients with different clinicopathological feature.

			Cytoplasm score range				
Variable		Mean± DV	0	1-4	5-8	9-12	P
Metastasis	T1N0M0	5 ± 1	2 / 2	0 / 2	0 / 2	0 / 2	0.85
	T2N0M0	0.68 ± 1.95	16 / 19	2 / 19	1 / 19	0 / 19	
	T2N1M0	0 ± 0.67	5 / 6	1 / 6	0 / 6	0 / 6	
	T2N2M0	0 ± 0	1 / 1	0 / 1	0 / 1	0 / 1	
	T3N0M0	0.36 ± 1.01	41 / 47	6 / 47	0 / 47	0 / 47	
	T3N1M0	0.36 ± 0.95	19 / 22	3 / 22	0 / 22	0 / 22	
	T3N2M0	0 ± 0	1 / 1	0 / 1	0 / 1	0 / 1	
	T3N2M1	0 ± 0	1 / 1	0 / 1	0 / 1	0 / 1	
	T4N0M0	0 ± 0	4 / 4	0 / 4	0 / 4	0 / 4	
	T4N2M0	15 ± 0	0 / 1	1 / 1	0 / 1	0 / 1	
benign, Malignant	Adjacent tissue	4.31 ± 3.15	19 / 104	45 / 104	30 / 104	10 / 104	<0.001
	Malignant	0.43 ± 1.24	90 / 104	12 / 104	2 / 104	0 / 104	
Grade	1	0.93 ± 2.22	12 / 15	2 / 15	1 / 15	0 / 15	0.3
	2	0.19 ± 0.69	24 / 26	2 / 26	0 / 26	0 / 26	
	3	0.24 ± 0.83	41 / 45	4 / 45	0 / 45	0 / 45	
Area	Cardia	0.3 ± 0.94	36 / 40	4 / 40	0 / 40	0 / 40	0.415
	Body/fundus	0.25 ± 0.73	32 / 36	4 / 36	0 / 36	0 / 36	
	Antrum	0.89 ± 1.93	21 / 27	5 / 27	1 / 27	0 / 27	
Sex	Male	2.51 ± 3.17	79 / 154	40 / 154	27 / 154	8 / 154	0.275
	Female	1.96 ± 2.77	30 / 54	18 / 54	4 / 54	2 / 54	
Stage	IA/IB	0.62 ± 1.86	18 / 21	2 / 21	1 / 21	0 / 21	0.284
	II/IIA	3.90 ± 1.07	47 / 54	7 / 54	0 / 54	0 / 54	
	III/IIIA/IIIB	0.38 ± 0.98	25 / 29	4 / 29	0 / 29	0 / 29	
Different histological pathology	Adenocarcinoma	0.75 ± 1.24	90 / 102	13 / 102	1 / 102	0 / 102	<0.001
	Atrophic gastritis	3.33 ± 5.77	2 / 3	0 / 3	0 / 3	1 / 3	
	Chronic gastritis	4.68 ± 2.9	5 / 59	30 / 59	18 / 59	4 / 59	
	Normal tissue	3.86 ± 3.31	12 / 42	15 / 42	10 / 42	5 / 42	

Data are presented as mean ± standard deviation

Conclusion

Role of ANG in gastric cancer is not very clear yet. Here our results show that ANG expression in the cytoplasm and nucleus is inversely correlated with gastric malignancy and that ANG expression in the nucleus is inversely correlated with tumor progression of gastric cancer (grades and stages). But neither cytoplasm nor nucleus ANG expression is correlated with tumor metastasis. Thus, ANG may be clinically useful gastric cancer diagnosis and prognosis. An interesting finding was that ANG seems to be expressed in the area where gastric stem cells are situated (gastric basal gland and isthmus) in normal gastric glands. It is therefore plausible that a decrease in ANG expression will affect the repairing function of gastric stem cells and lead to carcinogenesis. We infer that, as ANG expression decrease, gastric stem cells at the base and isthmus undergo more robust apoptosis and exhaustion under stress conditions and therefore impair damage repair leading to cancer initiation and progression. The molecular details by Int Gast Hepatol, 2(1): 137-144 (2019)

which ANG is involved in gastric carcinogenesis need to be investigated in the future.

Conflict of interest statement

The authors declare no conflict

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