Apelin Over-Expression Promotes Proliferation and Angiogenesis of Gastric Cancer Cells

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Abstract

Background: Apelin is a recently identified endogenous ligand associated with proliferation and angiogenesis of several cancers. However, only few studies have reported on the functions and the role of apelin in gastric cancer (GC). Therefore, in the present study, we investigated the association and the mechanisms underlying Apelin expression and proliferation of GC cells both *in vitro* and *in vivo*.

Methods: We enrolled 178 postoperative care GC patients to investigate clinicopathological and immunohistochemical factors associated with Apelin expression. The relationship between Survival of patients and apelin expression was evaluated using Kaplan-Meier method and Cox regression analyses. The expression of apelin mRNA and its proteins in GC tissues and cell lines were analyzed using quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR), western blot and ELISA. The role and mechanisms underlying regulation of Apelin expression in human GC cells were evaluated through several *in vitro* and *in vivo* experiments.

Results: Apelin was over expressed in human GC cells, relative to adjacent normal tissues. The over expression of apelin was associated with vessel invasion ($P < 0.01$), lymph node metastasis ($P < 0.01$), late-staged tumor (T) ($P < 0.05$), worse pathological type ($P < 0.05$), nerve invasion ($P < 0.05$). In addition, expression of apelin strongly and positively correlated with that of vascular endothelial growth factor (VEGF). Over-expression of apelin promoted proliferation and invasion of MGC-803 cell via the ERK/Cyclin D1/MMP-9 signaling pathway. Apelin over-expression also promoted angiogenesis of GC cells, accelerating growth of subcutaneous xenograft of the cancer cells *in vivo*.

Conclusions: Over-expression of apelin promotes proliferation and metastasis of GC cells via the ERK/Cyclin D1/MMP-9 signaling pathway and is associated with adverse events of the cancer. Consequently, apelin is a potential therapeutic target for human GC.

Background

Gastric cancer (GC) is the fifth most prevalent and aggressive malignancy and the third most fatal cancer globally [1, 2]. Despite the recent advances such as radical surgical resection, adjuvant chemoradiation and targeted therapy, the prognosis for GC patients remains poor, with the overall 5-year survival rate only between 20–40% [3–5]. Although several studies have identified numerous proteins associated with the clinical outcomes of GC patients, none of them has proposed early and accurate diagnostic as well as prognostic molecular marker for GC [6, 7].

Apelin is a bioactive peptide recently identified as an endogenous ligand that binds APJ, a human G protein-coupled receptor [8]. Apelin, a member of the adipokine family secreted by adipose tissue, is expressed in numerous cell types [9]. The Apelin gene encodes a prepropeptide consisting of 77 amino acids that promotes proteolytic maturation and subsequently generate different bioactive peptide fragments: Apelin-17, Apelin-36 and Apelin-13, all predominant in human plasma [8, 9]. Apelin/APJ signaling pathway, mostly associated with the cardiovascular system, participates in numerous

physiological and pathological processes such as angiogenesis, heart failure, energy metabolism and cancer progression [9, 10]. Dysfunctional and abnormal vasculature plays a critical role in the development and growth of tumors [10–12]. Accordingly, abnormal expression of VEGF and its receptor, associated in angiogenesis, have been therapeutic cancer targets for over 30 years [11, 12]. Apelin overexpression stimulates proliferation of endothelial cells, enhances tumor vascularization and accelerating forming capillary tubes demonstrated both *in vivo* and *in vitro* [13, 14]. In addition, over expression of Apelin is associated with poor clinical outcomes in patients with certain cancers [15, 16].

However, the role and molecular mechanisms of Apelin in GC is not well understood. Therefore, we investigated the correlation between Apelin expression and clinical outcomes of human GC and as well the mechanisms underlying Apelin expression and the occurrence of adverse events in GC cells.

Patients And Methods

Patients

We analyzed histopathologically confirmed GC cancer tissues of 178 patients attending the Binzhou Medical College Affiliated Hospital between January 2009 and December 2011. The patients underwent radical D2 lymphadenectomy followed by chemotherapy alone or in combination with radiotherapy. All patients underwent computed tomography (CT) of the neck, chest and abdomen. The anti-tumor therapies were only administered after surgery. Postoperative chemotherapy with fluoropyrimidine or capecitabine in combination with oxaliplatin or paclitaxel, repeated for at least 4 cycles, was introduced one month after the surgery. Eligible patients underwent intensive modulation radiotherapy (IMRT) of 45-50.4 Gy, given in 1.8-2.0 Gy fractions. The progression-free survival (PFS) time was defined as the period between the operation and the re-occurrence of the cancer, death of the patient or the end of the study. Overall survival (OS) was assessed between time of surgery to death or December 31, 2016.

This study was approved by the Ethics Committee of Binzhou Medical College Affiliated Hospital. All procedures involving human participants were performed in accordance with ethical standards of the board and the 1964 Helsinki declaration and its later amendments. The written informed consent was obtained from all patients in this study.

Immunohistochemical analysis

Immunohistochemical analyses were performed on tumor tissues previously fixed in formalin and embedded in paraffin. The tissue sections (4 μ m) were deparaffinized with xylene and rehydrated in several dilutions of alcohol. Heat-induced antigen retrieval was performed in 0.01 M citrate buffer (PH = 6) for 5 minutes, before heating the slides for 15 minutes at 99°C. The tissue sections were incubated in 3% H₂O₂-methanol solution at 37.0°C for 20 minutes to block endogenous peroxidase. Thereafter, the tissues were blocked for 10 minutes in albumen, before overnight incubation at 4°C with anti-Apelin, anti-VEGF or anti-CD34 antibodies at dilution ratios of 1:200, 1:100 or 1:200, respectively (Abcam, the USA). The tissues were stained and counter stained with diaminobenzidine and Mayer's modified hematoxylin

respectively, and viewed using the EnVision-HRP detection system (Dako, Carpinteria, CA, the USA). Tissues were then incubated at 37°C for 30 minutes with 80 µL horseradish peroxidase conjugated goat anti-rabbit or rabbit anti-mouse immunoglobulins G (IgG) (1:250) (Dako, Carpinteria, CA, the USA). The tissues were evaluated independently by two investigators, oblivious to the clinical data of the patients. Disagreements were resolved through discussion. Apelin or VEGF immunostaining score was calculated by multiplying the positive cell area score and the staining intensity score. An immunoexpression score > 3 was considered positive, with those ≤ 3 was considered negative. The staining intensity was classified into four levels: no staining (0), light staining (1), moderate staining (2) and deep staining (3). The positive cell area score was based on the percentage of positive cells, classified as follows: Negative (0), less than 10% positivity (1), 11–50% positivity (2), 51–75% positivity (3) and greater than 76% positivity (4) [15,16]. Microvessel densities (MVD) were established after labeling the capillaries (0.02–0.10 mm) with CD34 (Dako, Carpinteria, CA, the USA). Morphometric analysis of three sections per slide was performed using computer-aided CUE-2 software (Olympus Vanox, Tokyo, Japan) as previously described [17].

Cell culture

HGC-27, MGC-803 and SGC-7901 human GC cell lines were purchased from Anhui Medical University (Anhui, China). They were all cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, CA, the USA) supplemented with 10% FBS (Gibco, CA, the USA) at 37°C under 5% CO₂.

Construction of stable transfected cell lines

Apelin complementary DNA was purchased from Thermo Fisher Scientific (Shanghai, China). DNA encoding areas were amplified using the following primers: 5'-CGCGAATTCGGCATGAATCTGCGGCTCTG-3' and 5'-GCGCTCGAGTCAGAAAGGCATGGGTCC-3'. The amplified PCR products were ligated to Apelin cDNA and cloned into the pcDNA vector containing *EcoRI* and *XhoI* restriction sites (Invitrogen, Carlsbad, CA, USA). The expression of the hybrid DNA in the vectors was validated by sequencing. The MGC-803 cell were transfected with the pcDNA 3.1 or empty vectors using the Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Shanghai, China), according to the manufacturer's protocol.

RNA isolation and quantitative real-time reverse transcription-polymerase chain reaction

Total RNA from each human GC cell line was extracted using TRIzol (Invitrogen, Carlsbad, CA, the USA), following the manufacturer's protocol. Corresponding cDNA was synthesized through reverse transcription of the RNA, using the murine leukemia virus. The resultant DNA was amplified under the following cyclic conditions through 42 cycles: initial denaturation 95°C for 20 seconds, subsequent denaturations at 95°C for 5 seconds, annealing elongation at 60°C for 30 seconds. PCR products were stained with ethidium bromide, separated through gel (2%) electrophoresis and visualized under UV. The primer sequences for PCR were as follows: Apelin: 5'-GATGCCGCTTCCCGATG-3' (forward) and 5'-ATTCCTTGACCCTCTGGGCT-3' (reverse), β-actin; 5'-TGCTGTCCCTGTATGCCTCT-3' (forward) and 5'-AGGTCTTTACGGATGTCAACG-3' (reverse). The expression levels of mRNA for the above proteins were

calculated based on the $2^{-\Delta\Delta Ct}$ method [18]. Gene expression level of Apelin mRNA in MGC-803 cell line was determined using the same method, 48 hours after transfection with control or Apelin-encoding pcDNA 3.1 vector.

Protein Extraction and Western Blot analysis

Cultured cells at logarithmic growth phase were resuspended in lysis buffer containing 5 μ L Protease Inhibitor Cocktail (cOmplete, Sigma, Germany) before lysis as previously [19]. The concentration of xyz proteins in the supernatants was measured using the Bradford protein assay kit (Bio-Rad, Hercules, CA, the USA). Equal amounts of protein samples (80 μ g/lane) were fractionated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto polyvinylidene fluoride (PVDF) membranes at 0.8mA/cm² for 20–30 minutes. The membranes were blocked for 2 hours at 37°C with Tris-buffered saline (TBS) containing 10% nonfat milk before overnight incubation at 4°C with primary antibodies against Apelin, β -actin, pERK, pAkt, Cyclin D1 and MMP-9. Membranes were washed four times with TBST and thereafter incubated for 2 hours at 37.0°C with horseradish peroxidase (HRP)-conjugated secondary antibodies. The proteins were visualized after staining with the ECL reagent and thereafter quantified using the Quantity One software. The dilutions for the primary antibodies were as follows; 1:200 for anti-Apelin (Phoenix Pharmaceuticals, USA), 1:2,000 for anti- β -actin (Maxim, China), 1:200 for anti-pERK (Cell Signaling Technology, France), 1:1000 for anti-pAkt (Santa Cruz, USA), 1:200 for anti-Cyclin D1 (Santa Cruz, USA) and 1:200 for anti-MMP-9 (Santa Cruz, USA). Proteins were extracted from MGC-803 cells for western blotting after 48 hours of transfection with pcDNA3.1-Apelin or control vectors following activation with Apelin-13 (Phoenix Pharmaceuticals, Inc, the USA). Analysis of pERK, pAkt, Cyclin D1 and MMP-9 expression in MGC-803 cells was performed as described in the preceding sections.

ELISA

The concentrations of Apelin in MGC-803 cell culture supernatants (500 μ L) were measured using human Apelin ELISA kit (Phoenix Pharmaceuticals, USA) following the manufacturer's instructions. Similarly, the concentration of Apelin after transfection with pcDNA3.1-Apelin or control vector was performed in similar manner. The experiments were performed in duplicate

Cell Proliferation assays

To assess cell proliferation, cells (1×10^4 / well) were seeded in flat-bottomed 96-well microplates and cultured for 24 hours. Then the cells were washed twice using PBS and cultured in DMEM supplemented 0.5% FBS for 12 hours. The cells were treated with MTT (5mg/mL) for 4 hours at 37.0°C after 24 h incubation with different concentrations of Apelin-13 (0.02 μ mol/L, 0.1 μ mol/L, 0.5 μ mol/L, 2.5 μ mol/L, 12.5 μ mol/L, 62.5 μ mol/L), the cells were treated with. Thereafter, 150 μ L of dimethyl sulfoxide (DMSO) was added to each well to remove cells that were not reduced by MTT reagent. The absorbance of cell supernatants was measured at 570 nm using a microplate reader (MA, USA). The absorbance was also measure at 570 nm using the optimal concentration obtained from the previous experiment at different time points (6h, 12h, 24h, 36h and 48h). The absorbance was also measured at 570 nm at the

aforementioned time points to validate change in cell proliferation after transfection with pcDNA 3.1 or pcDNA 3.1-Apelin vector.

Cell Migration and Invasion Analysis

The migration and invasion of cells were evaluated using the Transwell chambers (BD, the USA). Briefly, MGC-803 cells were divided into 4 groups: Non-treatment group, Apelin-13 treatment group, pcDNA 3.1-transfection group and pcDNA 3.1-Apelin – transfection group. The cells (5×10^4 cells/ well) were seeded in the upper chamber pre-coated with serum free Matrigel (BD, the USA). The lower chamber contained 600 μ L of DMEM. After 24 h of incubation, cells that had not penetrated the membrane were removed. Cells that migrated across the membrane were counted using an inverted microscope at 200 $\times$ magnification (Olympus, Japan), after 15 minutes staining with 4% polyoxymethylene and 0.2% crystal violet.

Apoptosis of MGC-803 cell

Apoptosis of MG-803 cells was induced using Suberoylanilide hydroxamic acid (SAHA) [20], a member of histone deacetylase inhibitor (HDACI) family. Here, MGC-803 cells were divided into 5 subgroups: Controls, SAHA- treatment group (2 μ mol/L for 24 hours), SAHA + Apelin-13 treatment group (2.5 μ mol/L for 24hours), SAHA + pcDNA3.1 treatment group (24hours after transfection) and SAHA + pcDNA3.1-Apelin treatment group (24hours after transfection). After re-suspension in 300 μ L of binding buffer (BD, USA), the cells were mixed with 5 μ L of Annexin V-FITC for 15 minutes and thereafter in 5 μ L of propidium iodide (PI) for 5 minutes at 37.0°C, both in the dark. Finally, cells were mixed with 200 μ L of binding buffer and examined using a flow cytometer (BD, USA). U1 subset (Annexin V-FITC (-)/ PI (+)) represented the degree of necrosis, U2 subset (Annexin V-FITC (+)/PI (+)) represented late-staged apoptosis, U3 subset (meaning Annexin V-FITC (-)/PI (-)) represented the control whereas U4 subset (Annexin V-FITC (+)/PI (-)) represented early-staged apoptosis. The rate of apoptosis was calculated as: $(U2 + U4) / (U1 + U2 + U3 + U4) \times 100$.

Construction of MGC-803 cell stably expressing Apelin

To determine the optimal concentration of puromycin (Beijing, China), cells (2×10^4 cells/well) at the logarithmic phase were cultured overnight in 24-well microplates under different puromycin concentrations (0, 1, 2.5, 5, 7.5, 10, 15 μ g/mL). We found 7.5 μ g/mL of puromycin (the lowest concentration) killed all MGC-803 cells after 72 hours of treatment, thus was selected for subsequent experiments. MGC-803 cells were cultured in 24-well plates containing FBS or antibiotic free DMEM. The cells were cultured to 80% confluent. The cells were further incubated at 37°C for 4 hours after addition of a mixture of 2mL polybrene (Beijing. China) (final concentration 6 μ g/mL) and viral supernatant (0.5mL). Thereafter, fresh DMEM was added in each well followed by another 24 h incubation at 37°C. Puromycin (7.5 μ g/mL) was added to each well after 3 days, followed by x h incubation at 37°C. The medium was changed every 2–3 days until colonies were formed. Negative colonies were removed, whereas the puromycin-resistant colonies were transferred to a 24-well microplate divide to more than half of the dish.

mRNA and protein expression of target genes were determined using RT-PCR and Western blot assays, respectively.

Subcutaneous xenografts in vivo

The animal studies were implemented in accordance with the rules of Laboratory Animal Welfare Ethics Committee at Binzhou Medical College Affiliated Hospital (Certificate of Approval NO. SYXK 20180022). Additionally, the use of animal in study was carried out in compliance with the ARRIVE guidelines. All institutional and national guidelines for the care and use of laboratory animals were followed. The mice required euthanasia for humane reasons in accordance with the guideline of animal ethics approval.

Fourteen nude BALB/c nu/nu, female, 5–6 weeks old mice were purchased from the Laboratory Animal Resources of Chinese Academy of Sciences (Shanghai, China). The mice were raised in pathogen-free conditions. The xenograft tumors were established by subcutaneous injection of 0.2 mL MGC-803 cells (1×10^7 /mL) stably expressing Apelin or control into the mice. The long diameter (a), short diameter (b) and weight of the tumors were measured twice a week by the same investigator. The tumor volumes (mm^3) was calculated based on $V = ab^2/2$. The resultant volumes were used to plot a tumor growth curve. Mice were euthanized after 35 days of xenograft for further analyses. The tumor proliferation rate was calculated as; (the averaged weight of xenograft tumors in the experiment - the averaged weight of control group) / the averaged weight of xenograft tumors $\times 100\%$.

Immunohistochemistry of MVD in Vivo

Immunohistochemical staining was performed for MVD in murine tumors as described in the previous section.

Statistical Analysis

Data was analyzed using SPSS software, version 16.0 (NY, USA). The association between clinicopathologic factors and Apelin as well as VEGF expression was analyzed using chi-square test. Continuous variables such as MVD were expressed as mean $\pm$ S.E.M. Differences between groups were analyzed using Student *t*-test or nonparametric tests. The association between expression of Apelin and VEGF was analyzed using Spearman's rank correlation test. The association between Apelin and prognosis of GC was assessed using Kaplan-Meier, Cox proportional hazards survival analyses and log-rank test. $P < 0.05$ was considered statistically significant.

Results

Among the 178 participants, 141 (79.21%) were males whereas 37 (20.79%) were females, with a combined median age of 62 years (range 34–38 years). In addition, 61, 14, 49 and 54 patients presented with primary lesions in the cardiac region, gastric fundus, gastric body and gastric antrum, respectively. The tumors were divided into well or moderately differentiated (82 cases), poorly differentiated (75 cases), mucinous adenocarcinoma (17 cases) and signet ring cell carcinomas (2 cases) based on the

degree of differentiation, based on the AJCC/UICC TNM classification method, revised in 2010. In addition, 5, 98, 75 patients presented with I, II and staged III, respectively, of GC.

Immunohistochemical analyses revealed Apelin was expressed in 53 of 178 normal gastric tissues adjacent to tumor tissues and 88 of 178 tumor tissues (29.78% vs 49.44%, $P < 0.001$, Table 1.1). Additionally, immunostaining revealed that in tumor tissues, apelin was mostly distributed in the cytoplasm (Fig. 1.1). On the other, VEGF was expressed in 55.62% of tumor cells, compared to 39.89% in adjacent normal tissues ($P = 0.003$, Table 1.1). Similarly, just like apelin, VEGF proteins displayed cytoplasmic distribution (Fig. 1.2).

Table 1.1
Expression of Apelin and VEGF in gastric cancer tissues and adjacent tissues

			cancer tissues	adjacent tissues	χ^2 value	<i>P</i> value
Apelin	positive	88		53	14.386	<0.001
	negative	90		125		
VEGF	positive	99		71	8.827	0.003
	negative	79		107		

Chi-square test revealed that apelin expression was strongly and positively correlated with vessel invasion and lymph node metastasis ($P < 0.01$), ($P < 0.01$) (Table 1.2). In addition, Apelin expression was associated with late-staged tumor (T), pathological type and nerve invasion ($P < 0.05$). Contrarily, we found no significant associations between Apelin expression and gender, age and the site of primary lesion ($P > 0.05$). Meanwhile, VEGF expression was significantly high during late T stage ($P < 0.01$), vessel invasion and N stage ($P < 0.05$). However, there was no significance difference in VEGF expression between gender, across age groups, site of primary lesion, pathological type and nerve invasion ($P > 0.05$).

Table 1.2
The relationship between Apelin and VEGF and clinicopathologic factors

Characteristics	Apelin		χ^2 value	<i>P</i> value	VEGF		χ^2 value	<i>P</i> value
	+	-			+	-		
	(n)	(n)			(n)	(n)		
Gender			1.001	0.317			0.810	0.368
Male	67	74			76	65		
Female	21	16			23	14		
Age(years)			0.840	0.772			1.585	0.208
≤ 62	46	49			57	38		
>62	42	41			42	41		
site of primary lesion			3.240	0.356			6.420	0.093
Cardia	28	33			26	35		
Fundus	7	7			9	5		
Body	21	28			31	18		
Antrum	32	22			33	21		
Pathological differentiation			11.240	0.024			5.103	0.277
Well or moderately differentiated	33	49			43	39		
Poorly differentiated	42	33			42	33		
Mucinous adenocarcinoma	11	6			10	7		
Signet ring cell carcinoma	0	2			2	0		
Other	2	0			2	0		
Nerve invasion			5.731	0.017			3.116	0.078
Yes	49	34			52	31		
No	39	56			47	48		
Vessel invasion			7.910	0.005			5.807	0.016
Yes	38	21			40	19		
No	50	69			59	60		
T stage			9.845	0.020			11.597	0.009
T1	1	1			1	1		

Characteristics	Apelin		χ^2 value	<i>P</i> value	VEGF		χ^2 value	<i>P</i> value
	+	-			+	-		
	(n)	(n)			(n)	(n)		
T2	2	11			3	10		
T3	58	63			64	57		
T4	27	15			31	11		
N stage			14.433	0.002			8.653	0.034
N0	18	23			21	20		
N1	13	26			16	23		
N2	22	27			35	14		
N3	35	14			27	22		

Meanwhile, the MVD was greater in Apelin positive (33.086 ± 7.862 ; $P < 0.05$) than Apelin-negative (21.071 ± 6.320) tumor tissues. Similarly, a statistically significant difference was observed between the VEGF-positive subgroup with high MVD expression (29.075 ± 8.193) and VEGF-negative subgroup with low MVD expression (19.638 ± 5.614 ; $P < 0.05$) (Table 1.3 and Fig. 1.3).

Table 1.3
The relationship between the expression of Apelin, VEGF and MVD

Characteristics		MVD	<i>t</i>	<i>P</i>
Apelin	positive	33.086 ± 7.862	2.917	0.013
	negative	21.071 ± 6.320		
VEGF	positive	29.075 ± 8.193	2.514	0.025
	negative	19.638 ± 5.614		

Immunohistochemical analyses further revealed that VEGF was expressed in more 72.7% (64/88) Apelin-positive tumor cells than Apelin-negative tumor cells 38.9% (35/90). In the reverse, Apelin was expressed in 64 (64.6%) of 99 VEGF-positive tissues, compared to 24 (30.3%) of 79 of VEGF-negative tissues. Overall, expression of Apelin proteins strongly and positively correlated with that of VEGF ($R = 0.856$; $P < 0.01$, Table 1.4).

Table 1.4
The association between Apelin and VEGF expression

Characteristics		Apelin		r_s	P
		positive	negative	0.856	0.004
VEGF	positive	64	35		
	negative	24	55		

The 1 year, 3 years and 5 years OS rates of patients expressing Apelin were 97.73%, 62.50% and 28.41%, respectively (Fig. 1.4). That of patients not expressing Apelin were 97.78%, 76.67% and 53.33% for respective 1 year, 3 year and 5 year OS. The median OS of patients with Apelin positive tissues was 40.7 months, compared to 60.2 months for Apelin negative patients ($\chi^2 = 12.549$, $P < 0.001$). The 1 year PFS rates of patients not expressing Apelin was 94.44%, compared with 96.60% of those not expression apelin. For 3 year PFS, that of apelin positive patients was 65.56%, compared to 44.32% of apelin negative patients. On the other hand, the 5 year PFS of patients not expression apelin was 40.00%, compared to 19.32% for apelin negative patients (Fig. 1.5). Furthermore, there was a substantial difference in median PFS between apelin negative and apelin positive groups (49.8 months vs 30.5 months, $\chi^2 = 9.537$, $P = 0.002$). Overall, Apelin expression was associated with poor OS and shorter PFS.

Univariate Cox proportional hazards analysis revealed that except for gender, age and the site of primary tumor or nerve invasion, Apelin and VEGF expression, T stage, lymph node (N) stage, vessel invasion and histological type (all $P < 0.05$) were all associated with poor prognosis of GC (Table 1.5). Further multivariate regression analysis revealed that lymph node metastasis ($P < 0.001$), late-staged status ($P = 0.008$), poor differentiation ($P = 0.027$) and histological type (including mucinous adenocarcinoma, signet ring cell carcinoma and small cell carcinoma) were independent factors associated with poor prognostic and OS of GC ($P = 0.012$) (Table 1.6).

Table 1.5

COX univariate analysis of factors associated with survival in 178 patients with gastric cancer

Characteristics	5 years overall survival(OS)		
	HR	95%CI	Pvalue
Age(years)			0.451
≤ 62	1.000	Ref.	
> 62	1.284	0.786–1.852	
Gender			0.423
Male	1.000	Ref.	
Female	0.936	0.605–1.633	
Position			
Cardia	1.000	Ref.	—
Fundus	0.972	0.112–6.668	0.503
Corpora	0.774	0.276–1.942	0.137
Sinuses	0.865	0.413–1.826	0.257
Pathological differentiation			
Well/moderately differentiated	1.000	Ref.	—
Poorly differentiated	2.336	1.324–3.735	0.030
Other ^a	4.413	1.041–12.546	<0.001
Nerve invasion			0.068
No	1.000	Ref.	
Yes	1.258	0.646–2.425	
Vessel invasion			0.025
No	1.000	Ref.	
Yes	2.369	0.983–4.337	
T stages			0.016
T1 + T2	1.000	Ref.	
T3 + T4	3.007	1.353–5.724	

a: Mucinous adenocarcinoma, signet ring cell carcinoma and small cell carcinoma. b: N₁□N₂□N₃

Characteristics	5 years overall survival(OS)		
	HR	95%CI	<i>P</i> value
N stages			<0.001
N0	1.000	Ref.	
N + ^b	4.591	2.081–8.852	
Apelin			0.001
Negative	1.000	Ref.	
Positive	3.984	1.276–6.824	
VEGF			0.027
Negative	1.000	Ref.	
Positive	2.322	1.020–5.316	
a: Mucinous adenocarcinoma, signet ring cell carcinoma and small cell carcinoma. b: N ₁ –N ₂ –N ₃			

Table 1.6

Multivariable analysis of the factors associated with survival in 178 patients with gastric cancer

Characteristics	5 years overall survival(OS)		
	HR	95%CI	P value
Apelin			0.095
Negative	1.000	Ref.	
Positive	1.226	0.559–2.673	
VEGF			0.139
Negative	1.000	Ref.	
Positive	1.178	0.420–2.569	
Pathological differentiation			
Well/ moderately differentiated	1.000	Ref.	—
Poorly differentiated	2.605	1.228–4.947	0.027
Other ^a	3.239	1.153–10.345	0.012
Vessel invasion			0.069
No	1.000	Ref.	
Yes	1.427	0.698–3.566	
T stages			0.008
T1 + T2	1.000	Ref.	
T3 + T4	3.225	1.547–6.035	
N stages			<0.001
N0	1.000	Ref.	
N + ^b	3.987	1.836–8.148	
a: Mucinous adenocarcinoma, signet ring cell carcinoma and small cell carcinoma. b: N ₁ □N ₂ □N ₃			

Cell culture tests further validated the over-expression of apelin in MGC-803, SGC-7901 and HGC-27 GC cell lines. RT-PCR further revealed that the expression of apelin mRNA was significantly higher in MGC-803, SGC-7901 and HGC-27 GC cell lines than normal adjacent cells ($P < 0.05$, Fig. 2.1). Apelin was expressed lowest in MGC-803 cells ($Ct = 1.000 \pm 0.344$), a 30-fold difference when compared with HGC-27 cells ($Ct = 29.755 \pm 5.067$).

Western blot revealed that Apelin proteins were expressed lowest in MGC-803 cells, consistent with qRT-PCR analyses ($P < 0.05$, Fig. 2.2).

ELISA test also revealed that Apelin protein and its mRNA were expressed lowest in MGC-803 cells (132.00 ± 31.97 pg/mL) ($P < 0.05$, Fig. 2.3).

The pcDNA3.1-Apelin vector transfected MGC-803 cells were selected for subsequent analyses, down to the lowest Apelin expression. We found Analyses Apelin mRNA and Apelin proteins were over-expressed in MGC-803 cells, relative to controls. Moreover, Apelin expression improved significantly, after transfection with pcDNA3.1-Apelin (Fig. 2.4, 2.5 and 2.6).

MTT assay revealed that inhibition of Apelin expression modulated the proliferation of GC cells in a concentration and time dependent manner, with 2.5 μ mol/L of Apelin-13 being the optimal concentration (Fig. 2.7 and Fig. 2.8).

The proliferation rate of pcDNA3.1-Apelin vector trasnsfected MGC-803 cells was greater than that of control within 24–48 hours of incubation (Fig. 2.9)

The migration and invasion assay of MGC-803 cells revealed that compared to controls, Apelin-13 positive cells exhibited significant improvement greater membrane infiltration capacities ($t = -9.577$, $P = 0.001$) (Fig. 2.10). A similar observation was also made for pcDNA3.1-Apelin trasnfected cells and controls ($t = -5.142$, $P = 0.007$).

Meanwhile, SAHA treated cells exhibited grater apoptosis relative to the controls ((15.100 ± 3.997) % vs (7.300 ± 1.745) %, $P < 0.05$) (Fig. 2.11). Strikingly, there was difference in the proportion of apoptotic cells after SAHA and Apelin-13 treatment ((14.000 ± 3.899) %) or SAHA and pcNDA3.1-Apelin treatment ((14.425 ± 4.393) %). There was no significant difference in apoptosis relative among the SAHA and SAHA + Apelin-13 and SAHA and SAHA + pcNDA3.1-Apelin treatment groups ($t = 0.342$, $P = 0.774$; $t = 0.816$, $P = 0.446$).

Western blot assays revealed that pERK1/2, pAkt, cyclin D1 and MMP-9 cytokines as well as Apelin overexpression were associated with characteristic biological behavior of MGC-803 cells. As previously mentioned, there were four subgroups, untreated cells, Apelin-13 induced cells (incubated with a concentration of 2.5 μ mol/L Apelin for 24 hours), mock-vector transfected cells (incubated for 24 hours after transfection with pcDNA3.1 vector) and pcDNA3.1-Apelin transfected cells (incubated for 24 hours after transfection with pcDNA3.1-Apelin vector). Overall, there was no significant difference in the expression of pAkt proteins between the experimental groups and controls ($P > 0.05$). However, pERK1/2, cyclin D1 and MMP-9 proteins were over-expressed in MGC-803 cells after Apelin-13 treatment and pcDNA3.1-Apelin transfection ($P < 0.05$, Fig. 2.12).

Real time RT-PCR analyses further revealed that expression of Apelin mRNA was significantly higher in pLV-puro-Apelin transfectedMGC-803 cells than the controls ($t = -4.236$, $P = 0.013$, Fig. 3.1). Western blot

analysis revealed that indeed the expression of Apelin proteins was substantially higher in pLV-puro-Apelin transfected MGC-803 cells, relative to controls ($t=-3.833$, $P=0.019$) (Fig. 3.2).

We established 14 gastric cancer xenografts in the nude mice through subcutaneous injection with MGC-803 cells transfected with pLV-puro-Apelin or pLV-puro. One mouse died three days after injection with pLV-puro-Apelin. Control mice did not develop tumors. Mice over-expressing apelin gradually became dull, had poor appetite till 24th day after injection. Furthermore, pLV-puro-Apelin mice displayed greater growth of the tumors from day 14 of injection with tumor cells (Fig. 3.3 and Table 3.1). A maximum body weight of the mice and the mean tumor size of 2.96 ± 0.61 g was observed in the mice over-expressing -Apelin, whereas the minimum body weight of the mice and the mean tumor size (1.70 ± 0.43 g) was observed in the control group mice (Fig. 3.4). Overall, these findings suggest that Apelin promotes proliferation of gastric cancer cells both *in vitro* and *in vivo*.

Table3.1 The volume of gastric cancer xenografts in nude mice and statistical analysis

Time(day)	Control($\bar{x} \pm s$)	Overexpression of Apelin($\bar{x} \pm s$)	t value	P value
7	22.656±8.702	26.775±11.745	-0.630	0.543
10	61.517±23.151	80.836±26.352	-1.232	0.246
14	106.115±36.515	196.010±66.854	-2.639	0.025
17	306.789±105.315	567.346±198.854	-2.598	0.027
21	748.919±214.059	1399.063±499.872	-2.673	0.023
24	1367.348±444.701	2557.645±684.409	-3.261	0.009

MVD assay by CD34 immunolabeling assays revealed that was performed to explore the mechanism underlying the differences in neoplastic growth velocity between over-expression of Apelin promotes angiogenesis of cancer cells. Xenograft tumors over-expressing apelin displayed greater MVD (168.833 ± 35.078) relative to controls (112.333 ± 29.859 ; $t=-2.734$, $P=0.021$) (Fig. 3.5).

Discussion

Local recurrence and metastasis, the principal factors associated with poor prognosis, are regulated by numerous genes and cytokines [21, 22]. The novel relationship between angiogenesis and proliferation, metastasis as well as recurrence of tumors has prompted greater research in various factors associated with angiogenesis. In particular, VEGF signaling pathway is thought to play key roles in tumor angiogenesis. Consequently, VEGF-targeted therapy has resulted in significant benefits into the treatment of several tumors cancers such as lung and colon cancers [23, 24]. Previous works have demonstrated that VEGF signaling pathway participates in tumorigenesis and development of GC and over expression of VEGF is associated with poor prognosis of GC [23–25]. However, the clinical benefits of anti-VEGF drug

in human GC have been sub-optimal [25]. Therefore, it is necessary to identify novel biomarker and new vascular therapeutic targets for GC.

Apelin is an endogenous ligand recently thought to regulate angiogenesis [9, 13, 14, 26]. Previous studies have demonstrated dysregulated expression of Apelin in several human solid tumors [13, 26]. However, only few studies have evaluated the role of Apelin in tumorigenesis and progression of GC. In our study, we found that VEGF was not only over-expressed in tumor tissues, but also its expression exhibited a strong positive correlation with Apelin. We also found that over expression of Apelin or VEGF predicated poor prognosis of GC. Nevertheless, further studies are needed to evaluate any synergistic relationship between Apelin and VEGF expression in promoting angiogenesis. Feng and colleagues demonstrated that expression of Apelin in tumor tissues, rather than serum, was associated with worse clinicopathological features of tumors. In addition, expression of apelin in tissues is an independent poor prognostic factor for GC [27]. Although we found Apelin was not an independent prognostic factor, multivariate Cox regression analysis revealed that over expression of Apelin was associated with poor prognosis. In addition, the strong correlation between Apelin over-expression and late - T and N stages, as well as vessel invasion suggests that Apelin promotes local recurrence and lymph node metastasis of tumors.

RT-PCR, western blot and ELISA further revealed that both Apelin proteins and its mRNA were under expressed in MGC-803 cells. However, previous works have demonstrated that Apelin proteins and its mRNA are expressed differently in colon adenocarcinomas [28]. A similar observation was made for human non-small lung cancer [15]. MGC-803 cell line is poorly differentiated, HGC-27 cell line is undifferentiated whereas SGC-7901 cell line exhibits metastatic properties. These distinct features have been linked to the differential expression of Apelin in the cells.

In the present study, we used the MGC-803 cell line. Overall, RT-PCR, western blot and ELISA analyses revealed that Apelin was over-expressed in MGC-803 cells transfected with pcDNA3.1-Apelin. The findings suggested that Apelin expressed in cancer cells function in paracrine and autocrine manner. Furthermore, we found that both over-expressed exogenous and endogenous Apelin substantially modulated the proliferation and migration of MGC-803 cells. Previous studies have demonstrated that exogenous Apelin can enhance proliferation and migration of cancer cells such as lung adenocarcinomas cell line A549 [29] and human prostate cancer cell line LNCaP [30]. Moreover, hepatocellular carcinoma cell (HepG2) [16] and vascular smooth muscle cells (VSMCs) [31] over expressing apelin were resistant to apoptosis. Even though the effects of Apelin overexpression on the proliferation of colorectal cancer cells have not been reported, over-expression of apelin promotes proliferation of xenograft tumors in mice [28].

SAHA, a member of histone deacetylase inhibitor, induces apoptosis in MGC-803 cells [20]. To our knowledge, the relationship between overexpression of Apelin and malignant biological behavior of human GC cells both *in vitro* and *in vivo* has not been reported. In this study, we found SAHA had no effect on MGC-803 cells transfected with pcDNA3.1-Apelin or treated with exogenous Apelin.

Studies shows that both mitogen-activated protein kinase kinase/(MAPK)/ERK and phosphoinositide 3-kinase (PI3K)/Akt signaling pathways regulate the proliferation, migration, angiogenesis and inhibition of

apoptosis of tumor cells, including the human GC cells [32, 33]. Tumor cells promote ERK phosphorylation, inducing transcription of cyclin D1 that mainly promote growth of tumor cells via the ERK signaling pathway. Cyclin D1 promotes transition of cells from G1 to S phase and proliferation of cells [34]. Both MAPK/ERK and PI3K/Akt signaling pathways regulate invasion and metastasis of cancer cells primarily by up-regulating MMP-9 transcription [35, 36]. The apoptosis is inhibited by the expression of Bcl-2 family regulated via the MAPK/ERK and PI3K/Akt signaling pathway or inhibition of the caspase signaling pathway.

Our findings demonstrated that the expression of phosphorylated ERK (pERK), cyclin D1 associated with cell proliferation and MMP-9 linked to invasion of cancer cells were all over-expressed following Apelin treatment. Strikingly, there was no significant difference in the expression of pAkt between treatment and control groups. Apelin/APJ signaling pathway regulates apoptosis of cells through phosphorylation of Apelin [28]. MG132 is a proteasome inhibitor, which participates in inducing apoptosis of cells by downregulating the expression of pAkt in multiple tumor cells [37]. Apelin/APJ modulates apoptosis of colorectal cancer cells by inducing phosphorylation of Akt [28]. Remarkably, exogenous Apelin neither promoted nor modulated the expression of pERK in LoVo cells [28]. Yang *et al.* reported that Apelin-13 induces cell proliferation and promotes autophagy through phosphorylation of ERK. Phosphorylated ERK intern activates downstream transduction cascades in human lung cancer cell line A549 [29]. Moreover, exogenous Apelin promotes migration and inhibits apoptosis of cancer cells but not proliferation of cancer cells by inducing phosphorylation of ERK and Akt in human lymphatic endothelial cells [31].

On the other hand, Apelin had no effect on apoptosis *in vitro* induced by SAHA and phosphorylated Akt. There relationship between SAHA and the MAPK/ERK and PI3K/Akt signaling pathways is not straightforward. Apelin regulated apoptosis of GC cells via the MAPK/ERK signaling pathway. Therefore, further studies should be conducted to unravel the relationship between Apelin and SAHA expression. Notably, we observed a positive feedback between Apelin expression and ERK activation. Nevertheless, we did not down-regulate Apelin expression in the HGC-27 GC cell lines for lack of an effective SiRNA.

To the best of our knowledge, this is the first study to establish human GC cell xenograft models over-expressing Apelin. Overall our findings suggest that Apelin overexpression promotes growth and angiogenesis of human GC cells *in vivo*. Apelin is an angiogenic factor that stimulates proliferation and migration of vascular endothelial cells through extrinsic and intrinsic pathways. In addition, Apelin or APJ knockdown efficiently blocks angiogenesis [38]. Sorli *et al.* revealed that Apelin overexpression increased the formation of tumorous vessels and accelerated tumor growth and tumor neoangiogenesis in paracrine manner [39]. Kidoya *et al.* demonstrated that the Apelin/APJ signaling pathway regulates maturation and function of tumor vascular system [40]. MVD is the gold standard for estimating tumor angiogenesis, closely associated with tumor invasion and metastasis and is an independent prognosis factor for GC [39]. Berta *et al.* observed that Apelin overexpression stimulates tumor growth and increase MVD and capillary diameter [15]. Apelin-13 antagonist blocks tumor proliferation or angiogenic activity of hepatocellular carcinoma xenograft model, validating the role of Apelin overexpression in tumourigenesis

through angiogenesis [41]. Overall, overexpression of Apelin promotes proliferation of tumor cells, while simultaneously enhancing the MVD.

Conclusion

In general, compared to normal adjacent cells, human gastric cancer tissues over-express apelin. The over-expression of Apelin is associated with vessel invasion, N and T tumor stage, expression of VEGF and high MVD. These factors are intern associated with poor prognosis of GC. Overall, over-expression of Apelin promotes proliferation and invasion of cancer cells via the ERK/Cyclin D1/MMP-9 signaling pathway. However, it does not inhibit SAHA-induced apoptosi of cancer cells (MGC-803 cell line). Moreover, high Apelin levels enhance angiogenesis of subcutaneous xenograft. Given that occurrence and development of cancers are closely linked to angiogenesis, Apelin antagonist can be a novel therapeutic target for human GC.

Declarations

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

LJT:Conceptualization, Methodology, Formal analysis and Writing - Original Draft; HZL: Software, Dataanalysis and Data Curation; QZ: Investigation and Resources. DZG: Validation.

JY: Visualization.

HTG: Dataanalysis.

YJZ:Investigation.

YQH: Project administration.

YZH:Writing - Review & Editing and Supervision.

All authors are accountable for all aspects of the work.

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Figures

Figure 1

Immunohistochemical staining for Apelin in gastric cancer tissues (A, B) and adjacent normal tissues (C, D) (Magnification, ×200 (A, C) and ×400 (B, D)).

Figure 2

Immunohistochemical staining for VEGF in gastric cancer tissues (A, B) and adjacent normal tissues (C, D) (Magnification, ×200 (A, C) and ×400 (B, D)).

Figure 3

Immunohistochemical staining for MVD in gastric cancer tissues. A.MVD in Apelin-positive cells, B: MVD in Apelin-negative GC cells C MVD in VEGF-positive GC cells; D:MVD in VEGF-negative GC cells (Magnification×200)

Figure 4

Kaplan-Meier curves for the relationship between expression of Apelin in GC cells and OS of patients. Over-expression of Apelin-negative was associated with poor OS (60.2 months for the experimental vs 40.7 months for the control group).

Figure 5

Kaplan-Meier curves for the PFS the relationship between expression of Apelin and OS. Over-expression of Apelin was associated with shorter subgroup PFS. Figure 2.1 RT-PCR for Apelin mRNA expression in different GC cell lines (* $P \leq 0.05$ ** $P \leq 0.01$)

Figure 6

RT-PCR for Apelin mRNA expression in different GC cell lines (* $P \leq 0.05$ ** $P \leq 0.01$)

Figure 2.2 Expression of Apelin protein in different GC cell lines by Western blot

Figure 7

Western blot analysis for the expression of Apelin protein in different GC cell lines (* $P \leq 0.05$ ** $P \leq 0.01$)

Figure 8

ELISA for the expression of Apelin protein in different GC cell lines (** $P \leq 0.01$ *** $P \leq 0.001$)

Figure 9

RT-PCR analysis for the relative expression of Apelin mRNA before and after transfection. Transfection of MGC-803 cells with pcDNA3.1-Apelin increased the relative expression of Apelin in GC cell lines (** $P \leq 0.01$)

Figure 10

Western blot analysis for the expression of Apelin in MGC-803 cells after transfection with pcDNA3.1-Apelin (** $P < 0.01$)

Figure 11

ELISA for the expression of Apelin protein before and after transfection with with pcDNA3.1-Apelin (**P<0.01)

Figure 12

MTT assay for the proliferation of MGC-803 cells under different concentrations of Apelin-13. (*P<0.05, ** P<0.01)

Figure 13

The MTT assay for the proliferation of MGC-803 cells at different time points following Apelin-13(2.5 μ mol/L) treatment (* $P \leq 0.05$, ** $P \leq 0.01$).

Figure 14

MTT assay for the proliferation of MGC-803 cells after transfection with pcDNA3.1-Apelin or pcDNA3.1 at different time point. (* $P < 0.05$, ** $P < 0.01$).

Figure 15

Transwell assay for the effect of Apelin-13 on the migration and invasion of MGC-803 cells by. A: Untreated group; B: Apelin-13 treatment group; C: pcDNA3.1 transfected group; D: pcDNA3.1-Apelin transfected group. (** $P \leq 0.01$).

Figure 16

Transwell assay for the effect of Apelin-13 on apoptosis of MGC-803 cells. A Control group; B SAHA treatment group; C SAHA + Apelin-13 treatment group; D SAHA + pcDNA3.1 transfection group; E SAHA + pcDNA3.1-Apelin transfection group. * A group $P \leq 0.05$ ** $P \leq 0.01$ # $P \leq 0.05$

Figure 17

Western blot analysis for the expression of xyz in MGC-803 cells in controls, apeln-13 treatment group (incubated with a concentration of 2.5 μ mol/L for 24 hours), mock-vector transfected group (24 hours after transfection with pcDNA3.1 vector) and pcDNA3.1-Apelin transfected group (24 hours after transfection with pcDNA3.1-Apelin vector)

Figure 18

RT-PCR for the relative expression of Apelin mRNA in MGC-803 cells over-expressing Apelin and controls
 $*P \leq 0.05$

Figure 19

Western blot analysis for the relative expression of Apelin proteins - in vivo. $*P \leq 0.05$

Figure 20

The effect of Apelin on subcutaneous tumor graft into the nude mice within 24 days (* $P < 0.05$ ** $P < 0.01$)

Figure 21

The growth of gastric cancer xenografts in nude mice. (A) The GC xenografts extracted from the mice. (B) Mean weight of tumors over-expressing Apelin, relative to controls (** $P < 0.01$)

Figure 22

The MVD in gastric cancer xenografts in nude mice (CD34 marked) (magnification $\times 200$). A: Xenografts tumors over-expressing Apelin; B: Control Xenografts tumors (under-expressing apelin) ($*P < 0.05$).