Galanin expression is down-regulated in patients with gastric cancer

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Abstract

Objective: To investigate whether galanin and its three receptors (Gal-R1, Gal-R2, Gal-R3) contribute to development of gastric cancer.

Methods: Preoperative and postoperative fasting venous blood samples were collected from 34 patients with gastric cancer and 13 healthy individuals. Plasma galanin contents, as well as expression levels of galanin and its receptors, were quantitatively examined in a cohort of human gastric cancer tissues and corresponding adjacent tissues.

Results: Statistically significantly lower galanin levels were found in the preoperative samples from patients with gastric cancer, compared with postoperative samples from these same patients, as well as with samples from healthy donors. Furthermore, galanin and Gal-R1 expression levels were dramatically reduced in gastric cancer tissues, compared with corresponding adjacent tissues, whereas Gal-R2 and Gal-R3 levels remained unchanged. Furthermore, galanin mRNA and protein expression levels in the preoperative samples from patients with gastric cancer were significantly correlated with lymph node metastasis, tumor node metastasis stage, and size of the gastric cancer.
Conclusions: Overall levels of galanin and Gal-R1 expression were down-regulated in patients with gastric cancer; local levels were also specifically downregulated in gastric cancer tissues. Galanin and its receptor, Gal-R1, may contribute to development of gastric cancer.

Keywords
Galanin, Gal-R1, gastric cancer, diagnostic screening, carcinogenesis, clinical prognosis

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Introduction
Gastric cancer is the fourth most common cancer worldwide, and comprises one of the most important causes of cancer-related death.1,2 Most gastric cancers are thought to develop from areas of flat dysplasia via preneoplastic lesions, including atrophic gastritis and intestinal metaplasia. Current diagnostic screening methods are suboptimal; therefore, novel diagnostic approaches are needed.

Galanin, a 29/30-amino-acid peptide, was first isolated in 1983 from porcine intestine by Tatemoto and collaborators.3 It is synthesized in the brain and gut; the primary functions are to modulate food intake, energy metabolism, gut smooth muscle contraction, and gastric acid secretion.4 Galanin exerts its biological functions primarily through the activation of galanin receptor subtypes (Gal-R1, Gal-R2 and Gal-R3).5 Prior studies have shown that galanin is expressed in endocrine-related cancers6 and in non-endocrine gastric cancer.7–11 Galanin-immunoreactive nerve fibers are distributed in the submucosa, smooth muscle layers, and intramural ganglia in the rat gastrointestinal tract.12 The enteric nervous system in the human gastrointestinal tract is strongly represented in intramural nerve fibers, as well as in neurons distributed in numerous ganglia.13 Notably, these neurons contain numerous neuronal factors, such as galanin,14 and treatment with galanin has been shown to inhibit development of gastrointestinal cancer in mice and rats.9,10 In skin xeno-grafts of human gastric cancer cells in nude mice, galanin treatment reduced tumor volume and weight, although it did not alter the rate of apoptosis.9 In addition, plasma galanin levels and galanin mRNA expression in colon carcinoma tissue were strongly increased in patients with advanced colon cancer, compared with controls.7,8,15 High galanin expression is correlated with poor disease-free survival among patients with early-stage colorectal tumors, as well as poor prognosis of patients with stage II colorectal tumors.7,11 Galanin expression is reportedly down-regulated in human gastric cancer cell lines, although galanin receptor expression (Gal-R1-3) is not.16 Because there is minimal literature regarding the alteration of galanin levels in gastric cancer patients, the present study aimed to evaluate the expression of galanin and its receptors (Gal-R1, Gal-R2, Gal-R3) in patients with gastric cancer.

Materials and methods
Study subjects
Gastric cancer tissues and corresponding adjacent tissues were obtained from 34 patients with gastric cancer during gastric
ablative surgery in the Clinical Medical College, Yangzhou University. All cancer tissues were obtained from the stomach body of each patient, and the adjacent tissues were taken from macroscopically-unchanged stomach wall, approximately 5 cm from the tumor. The detailed clinical characteristics, including medical history, medications and anthropometric data, were recorded for each subject. None of the included patients had malignant diagnoses, concurrent malignancies, or chemotherapy prior to surgery. In addition, 13 age- and sex-matched healthy individuals were recruited as controls. Written informed consent was obtained from all participants, or from their relatives; the protocol of the study was approved by the Ethics Committee of Clinical Medical College, Yangzhou University.

**Blood sample assay**

To determine levels of galanin, fasting venous blood samples were collected from the gastric cancer patients at 2 days prior to the surgery and at 7 days after the surgery; fasting venous blood samples were collected from the healthy volunteers on the day that each volunteer was screened for inclusion in the study. The blood samples (2 ml) were collected in prechilled EDTA tubes, containing 100 µl aprotinin (1 µg/ml); within 30 min after collection, the tubes were centrifuged for 15 min at 1000 × g at 4°C. Plasma was separated into vials and stored at −80°C until measurement. One hundred microliters of plasma from each assay point was used to analyze galanin content using an enzyme-linked immunosorbent assay (CUSABIO, Inc., Wuhan, China). All measurements were performed in duplicate, and the mean of the two measurements was used for statistical analyses. According to the manufacturer’s specifications, the range of the assay was 4.7–300 pg/ml, and the average sensitivity of the assay was 1.17 pg/ml. Intra-assay precision coefficient of variation was <8% and inter-assay precision coefficient of variation was <10%.

**Real-time quantitative PCR**

Human gastric cancer and corresponding adjacent tissues were used to analyze the mRNA levels of galanin and its receptors, using the real-time polymerase chain reaction (PCR) method. Briefly, collected biopsy specimens were placed in ice-cold saline and transported to the laboratory within 15 minutes. Samples were cleaned by removing visible blood clots, and stored at −80°C. Total RNA was isolated from 100 mg of frozen gastric cancer and corresponding adjacent tissues by using Trizol (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer’s instructions. The concentration and integrity of the RNA were assessed by spectrophotometric analysis at 260/280 nm. cDNA was synthesized from 1 µg RNA using the MMLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). mRNA expression levels were determined using real-time fluorescent detection in an Applied Biosystems 7500 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). The primers used for gene amplification were as follows:

- Gal forward 5’-CCGGCCAAGGAAAAACGAG-3’;
- reverse 5’-GAGGCCATTCTTGTGCTG-3’;
- Gal-R1 forward 5’-TCGGGACAGCAACAAAC-3’;
- reverse 5’-TGCAGATGATTGAGATTCTTGG-3’;
- Gal-R2 forward 5’-GCCGCCATCGGCTCATCT-3’;

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reverse 5’-GTCGAGGTGCGCTCCATGCT-3’;

*Gal-R3* forward 5’-ACAGATCTCTTCATCCTCAACTT-3’;

reverse 5’-GTGAGGTAGATGAGCAGATGTAC-3’;

*GAPDH* forward 5’-CCGGCCAAGGAAAACGAG-3’;

reverse 5’-GAGGCCATTCTTGTCGCTGA-3’.

Reaction conditions were: 95°C for 15 min, one cycle; 94°C for 10 s, 60°C for 1 min, 40 cycles. Fold differences in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method, with *GAPDH* as the endogenous control gene.

**Western blot analysis**

Western blot analyses were used to determine galanin levels in gastric cancer tissue and corresponding adjacent tissues. Briefly, 100 mg frozen tissue was homogenized on ice in 1 ml RIPA buffer. Homogenates were centrifuged (14,000 × g for 10 min at 4°C) and supernatants were harvested. Protein concentrations of cell supernatants were determined by the bicinchoninic acid method, using bovine serum albumin to generate a standard curve. Protein lysates (25 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked in Tris-buffered saline (pH 7.5) containing 0.05% Tween-20 (1×TBST) and 5% skim milk for 2 h at 37°C, then probed overnight at 4°C with anti-galanin antibody (1:2000; No. sc-25446, Santa Cruz Biotechnology Inc., Dallas, TX, USA) and anti-GAPDH antibody (1:1000; No. PB0141, Boster Biological Technology Inc., Wuhan, China), respectively. Membranes were washed three times with 1×TBST for 10 min and then incubated for 2 h at 37°C with peroxidase-conjugated goat anti-rabbit secondary antibody (1:2000; No. BA1054, Boster Biological Technology Inc.). Finally, immunoreactive bands were visualized by enhanced chemiluminescence and quantified by densitometry, using a Bio-Rad Image Analysis System (Bio-Rad, Hercules, CA, USA), as galanin/GAPDH.

**Statistical analysis**

Data were expressed as mean ± SD or the number and percentage, respectively. Differences between preoperative and postoperative fasting plasma galanin levels were analyzed with paired t-tests. Differences in galanin protein and mRNA levels between gastric cancer tissues and corresponding adjacent tissues were analyzed with independent t-tests. Possible correlations between parameters were evaluated by Spearman correlation coefficient analyses. Differences were considered to be statistically significant when $P < 0.05$. All analyses were performed using SPSS software for Windows (version 17.0; SPSS, Inc., Chicago, IL, USA).

**Results**

The primary characteristics of patients with gastric cancer in this study are listed in Table 1. Interestingly, a significantly lower galanin level was found in preoperative samples from patients with gastric cancer, compared with postoperative samples from the same patients ($P < 0.01$) or compared with samples from healthy donors ($P < 0.01$; Figure 1). Furthermore, levels of *Gal* and *Gal-R1* mRNA expression were dramatically lower in gastric cancer tissues than in corresponding adjacent tissues ($P < 0.05$), while *Gal-R2* and *Gal-R3* levels remained unchanged (Figure 2). Galanin protein levels were significantly lower in gastric cancer tissues than in corresponding
Table 1. Tumor clinicopathological and biochemical parameters of patients with gastric cancer.

| Characteristics                          | Variables | Relative mRNA expression of galanin |
|------------------------------------------|-----------|-------------------------------------|
| Age (years)                              |           |                                     |
| Healthy donors                           | 62.15 ± 6.18 |                                     |
| Gastric cancer patients                  | 69.36 ± 7.53 |                                     |
| ≥60                                      | 18        | 20.46 ± 2.76                        |
| <60                                      | 16        | 20.63 ± 2.31                        |
| Sex                                      |           |                                     |
| Healthy donors                           |           |                                     |
| Male                                     | 8         | 35.62 ± 5.81                        |
| Female                                   | 5         | 35.74 ± 5.22                        |
| Gastric cancer patients                  |           |                                     |
| Male                                     | 23        | 20.38 ± 2.77                        |
| Female                                   | 11        | 20.89 ± 1.99                        |
| Tumor size                               |           |                                     |
| <5 cm                                    | 17        | 22.22 ± 1.98                        |
| ≥5 cm                                    | 17        | 18.87 ± 1.81                        |
| Invasion depth                           |           |                                     |
| T1 + T2                                  | 14        | 20.96 ± 2.34                        |
| T3 + T4                                  | 20        | 20.25 ± 2.66                        |
| Lymph node metastasis                    |           |                                     |
| No                                       | 12        | 22.36 ± 2.79                        |
| Yes                                      | 22        | 19.55 ± 1.74                        |
| Differentiation                          |           |                                     |
| High                                     | 14        | 20.71 ± 2.09                        |
| Middle                                   | 12        | 20.59 ± 2.97                        |
| Low                                      | 8         | 20.19 ± 2.81                        |
| Tumor node metastasis stage              |           |                                     |
| I                                        | 8         | 23.54 ± 2.29                        |
| II                                       | 8         | 20.37 ± 1.54                        |
| III                                      | 18        | 19.29 ± 1.82                        |
| Plasma galanin concentration (pg/ml)     |           |                                     |
| Preoperative patients                    | 20.73 ± 2.52 |                                     |
| Postoperative patients                   | 32.74 ± 4.15 |                                     |
| Healthy donors                           | 36.27 ± 5.94 |                                     |
| Levels of galanin mRNA \(2^{-\Delta C_t}\) |           |                                     |
| Cancer tissues                           | 104.64 ± 22.52 |                                   |
| Adjacent tissues                         | 241.97 ± 66.68 |                                   |
| Levels of Gal-R1 mRNA \(2^{-\Delta C_t}\) |           |                                     |
| Cancer tissues                           | 30.74 ± 10.76 |                                   |
| Adjacent tissues                         | 91.30 ± 21.57 |                                   |
| Levels of Gal-R2 mRNA \(2^{-\Delta C_t}\) |           |                                     |
| Cancer tissues                           | 31.44 ± 10.36 |                                   |
| Adjacent tissues                         | 40.44 ± 14.58 |                                   |
| Levels of Gal-R3 mRNA \(2^{-\Delta C_t}\) |           |                                     |
| Cancer tissues                           | 49.21 ± 21.44 |                                   |
| Adjacent tissues                         | 73.78 ± 33.56 |                                   |
| Levels of galanin protein                |           |                                     |
| Cancer tissues                           | 0.63 ± 0.15  |                                   |
| Adjacent tissues                         | 1.36 ± 0.66  |                                   |

Variables are presented as counts or mean ± standard deviation.
adjacent tissues ($P < 0.05$) (Figure 3). There was no significant difference in plasma galanin levels between postoperative samples from gastric cancer patients and samples from healthy donors (Figure 1).

In addition, we examined correlations between galanin protein and mRNA levels and various tumor parameters in patients with gastric cancer. Preoperative plasma galanin levels were significantly higher than those in healthy controls (Figure 1). Figure 2 and Figure 3 show the expression levels of galanin and its receptors (Gal-R1, Gal-R2, and Gal-R3) in gastric cancer tissues compared to adjacent tissues. The data indicate a significant increase in galanin mRNA levels in cancer tissues, with no significant changes in the mRNA levels of galanin receptors.

**Figure 1.** Levels of plasma galanin in healthy controls (HC, $n = 13$), preoperative gastric cancer patients (Pre-op, $n = 34$), and postoperative gastric cancer patients (Post-op, $n = 34$). Data are expressed as mean ± SD. $^{++}P < 0.01$ vs. HC, $^{##}P < 0.01$ vs. Pre-op.

**Figure 2.** Galanin and galanin receptor (Gal-R1, Gal-R2, and Gal-R3) mRNA expression in gastric cancer tissues ($n = 34$) compared with the corresponding adjacent tissues ($n = 34$). Data are expressed as mean ± SD. $^{#}P < 0.05$ vs. control tissues. 1,3,5 Control tissues; 2,4,6 Cancer tissues.

**Figure 3.** Galanin protein levels in gastric cancer tissues ($n = 34$) compared with the corresponding adjacent tissues ($n = 34$). Data are expressed as mean ± SD. $^{#}P < 0.05$ vs. control tissues. 1,3,5 Control tissues; 2,4,6 Cancer tissues.

In summary, the study highlights the potential role of galanin in gastric cancer, with increased plasma levels and altered mRNA expression patterns.
galanin levels were significantly associated with lymph node metastasis ($r = -0.309, P < 0.05$), size of gastric cancer ($r = -0.425, P < 0.05$), and tumor node metastasis stage ($r = -0.368, P < 0.05$). Conversely, preoperative galanin levels were not correlated with patient age and sex, or with invasion depth and differentiation of gastric cancer in this study. Moreover, galanin mRNA expression was significantly associated with the size of gastric cancer ($r = -0.380, P < 0.05$) and preoperative plasma galanin levels ($r = 0.465, P < 0.01$), but not with patient age and sex, lymph node metastasis, tumor node metastasis stage, invasion depth, or gastric cancer differentiation.

**Discussion**

Thus far, compelling evidence has demonstrated a relationship between galanin expression in endocrine-related tumor tissues and the clinical prognosis of cancers.⁴,⁶,¹²,¹⁷,¹⁸ Galanin has also been detected in a variety of non-neuroendocrine human tumors, including head and neck squamous cell carcinoma¹²,¹⁷,¹⁸ and colon cancer.⁷,⁸,¹⁰,¹¹,¹⁵ An increasing number of experimental results have shown that colorectal cancer tissues exhibit significantly greater galanin levels than corresponding noncancerous tissues.⁷,⁸,¹¹,¹⁵ Galanin mRNA levels have been directly correlated with adenocarcinoma size and stage.⁸ Nevertheless, the present study showed that there was a lower level of plasma galanin in preoperative samples from patients with gastric cancer, compared with postoperative samples from those same patient, or compared with samples from healthy donors. Furthermore, galanin and Gal-R1 expression levels were significantly lower in gastric cancer tissues than in corresponding adjacent tissues. A probable explanation for this inconsistency is that galanin expression in patients with cancer, as well as in cancer tissues, may change according to the type of cancer and the involved tissues.

In addition, our study showed that the plasma galanin levels of patients with gastric cancer were significantly associated with lymph node metastasis, size of gastric cancer, and tumor node metastasis stage. Because circulating galanin levels may be affected by altered expression of galanin in cancer tissues, these levels are likely to be influenced by cancer growth and lymph node metastasis. Galanin mRNA expression was significantly associated with the size of gastric cancer, as well as preoperative plasma galanin levels. Thus, elevation of galanin mRNA expression levels was consistent with increased galanin synthesis and release.¹⁹

Notably, galanin treatment may have a negative effect on volume and weight of tumors, although it has not shown an effect on apoptotic rate in murine models of gastric cancer.⁹ Carcinogenesis was considerably suppressed by parallel injection of galanin in gastric or colon cancer in rat models of disease.⁹,¹⁰ The number of blood vessels in tumor tissues was significantly reduced in mice who received continuous intraperitoneal infusion of galanin, compared with control mice with tumors who did not receive infusion of galanin.¹⁰ In vitro exposure of tumors to galanin reduced the number of viable cells and proliferation index.²⁰ Because galanin constitutes an inhibitory factor with regard to regulating cell proliferation, low galanin expression may be beneficial for lymph node metastasis and cancer growth. The mechanism of myenteric plexus decomposition in the stomach wall of cancer patients was elevated in the presence of high expression of CASP3 or CASP8, and was accompanied by reduced expression of Gal.¹⁴ Thus, low galanin levels may constitute a biomarker of gastric carcinogenesis.
A limitation of the current investigation was the size of the control cohort. It is a well-accepted practice to collect samples of cancer tissue and corresponding adjacent tissues from patients with gastric cancer during tumor resection; however, this is generally not appropriate for volunteers or other patient groups. Another limitation is that it is not optimal to use adjacent tissues near the tumor area as controls, because cancer-induced changes may have also occurred in these tissues.

The exact cause that results in down-regulated galanin expression in patients with gastric cancer remains poorly understood. The sole clue regarding the relationship between low galanin expression and gastric cancer is that a higher plasma galanin level has been observed in obese men, compared with normal-weight men;\(^ {21,22}\) moreover, most patients with gastric cancer exhibit dyspepsia and nutrient deficiency, accompanied by weight loss and thinning, which may result in low circulating galanin levels. Thus, the underlying mechanism should be further clarified in the future.

In conclusion, our investigation showed that galanin was down-regulated in preoperative samples of plasma and cancer tissues from patients with gastric cancer. Galanin protein and mRNA expression levels were significantly correlated with the severity and progression of the disease. Careful prospective clinical studies with large study populations and long follow-up times are needed to further clarify the impact of down-regulated galanin expression on the pathogenesis of gastric cancer. Notably, the circulating galanin level may serve as a biomarker for the early detection and prognostic assessment of gastric cancer.

Declaration of conflicting interest
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