Nesfatin-1 is a potential diagnostic biomarker for gastric cancer

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Abstract. The lack of reliable plasma biomarkers limits their use in the diagnosis of gastric cancer (GC). The current study aimed to determine whether plasma nesfatin-1 can be used as a novel non-invasive biomarker for the diagnosis of GC. The levels of nesfatin-1 in 40 patients with GC and 40 healthy individuals, who were selected from the Chaohu Hospital Affiliated to Anhui Medical University, were assessed. ELISA was used for the measurement of plasma nesfatin-1 levels, while immunohistochemistry was applied to determine Ki67 protein expression in GC and normal gastric tissues. The diagnostic value of plasma nesfatin-1 for GC was further assessed using receiver operating characteristic (ROC) curve analysis. The results revealed that, compared with the controls, the mean nesfatin-1 levels in patients with GC were significantly increased. Furthermore, the protein expression of Ki67 in GC tissue was significantly upregulated compared with that in normal gastric tissue. Plasma nesfatin-1 levels were also demonstrated to be correlated with Ki67 protein expression in GC tissues. Additionally, ROC curve analysis indicated the potential diagnostic value of nesfatin-1, and the area under the ROC curve (AUC) for nesfatin-1 was 0.857 (95% confidence interval, 0.769-0.946). At a threshold nesfatin-1 level of 1.075 ng/ml, the optimal sensitivity and specificity were 70.0 and 95.0%, respectively, in discriminating patients with GC from healthy controls. These results indicated that plasma nesfatin-1 may serve as a novel biomarker for the diagnosis of GC and determination of GC cell proliferation.

Introduction

Gastric cancer (GC) is the third most common cause of cancer-associated mortality worldwide and represents a major global health issue (1). According to the results of the 2015 China Cancer Statistics, released by the National Central Cancer Registry of China, the estimated incidence of GC in China is ~679,000 cases in 2015, second only to lung cancer (2). Epidemiological data has revealed that the 5-year survival rate is >90% for early-stage GC, with a poor prognosis observed for advanced GC cases (3). Therefore, improving the diagnostic rate of GC and its precancerous form is of great significance in the treatment and prognosis of GC.

Endoscopy and pathological examination are the gold standards for the clinical diagnosis of GC (4). However, their clinical application is prevented due to the patient discomfort, invasive nature and high cost of these examinations. Therefore, in order to reduce the burden of GC, there is an urgent requirement for simple, less invasive, cost-effective, sensitive and specific screening tools, which can be achieved by developing plasma protein biomarkers (5). However, there are a limited number of clinically available plasma biomarkers for GC, and the optimal serum biomarker for the detection of GC is currently being studied (6,7).

Nesfatin-1 is a novel anorexigenic factor that is cleaved from its precursor nucleobindin-2 (NUCB2). Previous studies have demonstrated that chronic intracerebroventricular injection of nesfatin-1 reduced the body weight of rats, whereas the animals gained body weight following the chronic intracerebroventricular injection of antisense morpholino oligonucleotide against the gene encoding NUCB2 (8). Further studies have indicated that nesfatin-1 can cross the blood-brain barrier (9), and can be expressed in a variety of peripheral tissues, indicating that nesfatin-1 exhibits a wide range of physiological activities (10). Recently, it has been revealed that NUCB2/nesfatin-1 is highly expressed in the gastric mucosa compared with other viscera and the brain (11). Furthermore, the expression of NUCB2/nesfatin-1 mRNA in gastric endocrine cells was significantly downregulated following a 24-h period of fasting in rats, indicating a regulatory anorexigenic role of peripheral NUCB2/nesfatin-1 in energy homeostasis (11). Preclinical studies have further demonstrated that nesfatin-1 may be associated with the pathogenesis of GC stress-related depression (12).

The imbalance of cell proliferation is a characteristic of a variety of cancer types (13). Nesfatin-1 has been reported to be linked to the mammalian target of rapamycin (mTOR) pathway (14), an important signaling cascade that is associated with the dysregulation of cell proliferation (15), indicating that nesfatin-1 may serve a pivotal role in the proliferation of GC. Furthermore, the antigen Ki67, which is closely associated with the cell cycle, is known to be expressed during the proliferation and synthesis phases of the cell cycle, but not in the resting phase (16). A negative correlation has been revealed

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between the overexpression of Ki67 and carcinoma differentiation (17). It has also been reported that routine assessment of Ki67 levels may be a useful tool for identifying patients with more aggressive diseases and can be used to improve treatment strategies (18). In addition, the Ki67 proliferating index increases in GC, and is a good indicator of the proliferative and differentiation ability of GC cells (19).

The aim of the present study was to investigate whether plasma nesfatin-1 can be used as a novel non-invasive biomarker for GC. Furthermore, the association between plasma nesfatin-1 levels and Ki67 protein immunoexpression was investigated in the present study.

Materials and methods

Subjects. A total of 40 patients with GC, who were admitted to Chaohu Hospital Affiliated to Anhui Medical University (Hefei, China) between June 2017 and June 2018, were enrolled into the present study. All patients exhibited upper abdominal discomfort and were diagnosed with GC by pathological examination (20). Healthy subjects were also selected as the controls during the same time period. All the subjects in the control group were healthy individuals who volunteered to participate in a free health examination to detect any organic lesions in the stomach. Clinical information was obtained from the clinical records of the subjects. The exclusion criteria were as follows: i) Patients receiving radiotherapy or chemotherapy; ii) patients with other types of cancer or major organ diseases, including in the heart, liver, kidney or lungs; iii) patients with severe active infectious diseases; and iv) patients with severe blood diseases, bone marrow transplantation, severe trauma or immune diseases. According to the AJCC/UICC TNM staging system (7th edition) (21), patients with GC were divided into four subgroups (stage I to IV disease). The present study was approved by the Ethics Committee of Chaohu Hospital Affiliated to Anhui Medical University. Informed consent was obtained from all individual participants included in the study.

Plasma collection and measurements. Body weight and height measurements, and body mass index (BMI) calculations were performed on all subjects. Blood samples were collected from the forearm vein at ~8 a.m while the subjects were in a fasting state. Furthermore, blood samples were drawn prior to drug treatment. Tubes with a 5 ml capacity containing EDTA were used for blood collection. Plasma was obtained by centrifugation at 1,200 x g for 5 min at 4˚C, and the separated plasma was stored at -80˚C until the assays were performed. The concentrations of nesfatin-1 were measured using commercially available ELISA kits (Jianglai Bio, Shanghai, China), according to the manufacturer's protocol.

Immunohistochemistry. For immunohistochemistry studies, a labeled-streptavidin-biotin (LAB-SA) method was performed with the Histostain®-Plus Bulk Kit Zymed® 2nd generation LAB-SA detection system (cat. no. 85-9043, Zymed; Thermo Fisher Scientific, Inc.) (22,23). Gastric tissue specimens were obtained during surgery and fixed in 10% neutral buffered formalin for 24 h at room temperature, and were subsequently, conventionally dehydrated, embedded in paraffin and cut into 4-μm sections. The sections were deparaffinized in xylene and dehydrated in a descending dilution of ethanol. For antigen retrieval, all slides were microwaved in 10 mmol/l sodium citrate buffer (pH 6.0) at 10-min intervals for a total of 20 min. Next, the endogenous peroxidase activity was blocked with 3% H_2O_2 (reagent A) for 10 min at room temperature. Subsequent to washing with PBS, the sections were incubated with antibodies targeting Ki67 (1:100; cat. no. ab16667; Abcam) overnight at 04˚C. The sections were then washed with PBS and incubated with polymerase auxiliaries (reagent B) for 20 min. After washing with PBS, the sections were incubated with biotinylated secondary antibody (reagent C) for 30 min at room temperature. DAB was then added for visualization, and tissues were counterstained with hematoxylin. A negative control was designed using PBS instead of the primary antibody. Subsequently, sections were then scored using light microscopy. Ki67-positive tissue sections were examined to determine the presence of brown-stained nuclei. By scanning the sections at a magnification of x100, the most heavily Ki67-labeled areas were identified. Cell counts were performed in five randomly selected areas with a magnification of x400 and using a compound light microscope. The number of positively stained nuclei was expressed as a percentage of the total number of complete epithelial cells. The Ki67 labeling index was calculated as the number of immunohistochemical positive cells x100 over the total number of observed cells (24,25). Scoring of immunostaining was categorized as follows: Score of 0, <10% of cells stained; score of 1, 10-49% of cells stained; and score of 2, >50% of cells stained (26).

Statistical analysis. All statistical analyses were performed using SPSS software, version 12.0.1 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant result. One-sample Kolmogorov-Smirnov test showed a normal distribution of continuous variables (age, BMI and concentration of nesfatin-1) in the patient and control groups. Student's t-test was used to evaluate the differences between groups (age, BMI and concentration of nesfatin-1). A statistical analysis of the plasma nesfatin-1 concentrations between the control group and the four subgroups of patients with GC was performed using one-way analysis of variance (ANOVA), followed by a least significant difference post-hoc test. To analyze the sex difference between groups, the χ^2 test was used. Receiver operating characteristic (ROC) curve analysis was performed to determine the cut-off values of plasma nesfatin-1. Correlational analyses were also performed using Pearson and Spearman correlation tests.

Results

Subject demographics. No significant differences were observed in the demographic characteristics between patients with GC and control individuals. As presented in Table I, the age, BMI or sex were not significantly different between the two groups.

Plasma nesfatin-1 levels. The plasma concentration of nesfatin-1 in the control group ranged between 0.69 and 1.21 ng/ml, with a mean value of 0.89 ng/ml, while the plasma concentration of nesfatin-1 in the GC group ranged between 0.64 and 1.67 ng/ml

Table I. Comparison of mean values (or ratio) of age, sex and BMI between the GC and control groups (mean ± standard deviation).

Variable	Control group	GC group	Statistics (t or χ^2)	P-value
Age, years	63.60±7.38	67.23±11.93	-1.634	0.106
Sex (female/male)	12/28	10/30	0.251[a]	0.617
BMI, kg/m^2	22.80±2.15	23.48±1.57	-1.629	0.107

[a]Represents the χ^2 score. GC, gastric cancer.

with a mean value of 1.19 ng/ml. As presented in Fig. 1, the plasma concentrations of nesfatin-1 (t=-6.876; P<0.001) were significantly higher in patients with GC as compared with those in the control group. Furthermore, the tumor stage was classified according to the AJCC/UICC TNM staging system (7th edition) (21), and the number of patients with stage I to IV disease was 5 (12.5%), 16 (40.0%), 13 (32.5%) and 6 (15.0%), respectively. According to the results of one-way ANOVA, compared with the control group, the plasma concentrations of nesfatin-1 in the four subgroups of GC patients were all significantly increased, indicating that nesfatin-1 may be used as a biomarker for the diagnosis of GC (Table II). According to Spearman correlation analysis, there was no significant correlation between the plasma concentration of nesfatin-1 and the tumor stage in the GC group (r,-0.191; P=0.237; Fig. 2).

Ki67 protein expression in gastric tissues. The results of immunohistochemical analysis of GC and normal tissues are presented in Fig. 3. The Ki67 protein staining was found to be localized in the nucleus of the GC and normal gastric tissues (Fig. 3A). In addition, the expression of Ki67 protein in GC tissues was significantly higher compared with that in normal gastric tissues (t=-3.515; P=0.001; Fig. 3B). The results of the Pearson correlation analysis further demonstrated that the plasma nesfatin-1 concentrations were positively correlated with the protein expression of Ki67 in GC tissues (r=0.706; P<0.001; Fig. 4).

ROC curve analysis. The results of ROC curve analysis indicated the potential diagnostic values of plasma nesfatin-1 (Fig. 5). The area under the ROC curve (AUC) for nesfatin-1 was 0.857 (95% confidence interval, 0.769-0.946). Furthermore, at a cut-off nesfatin-1 value of 1.075 ng/ml, the sensitivity and specificity for discriminating patients with GC from the healthy controls were 70.0 and 95.0%, respectively. According to the cut-off nesfatin-1 value of 1.075 ng/ml, the positive and negative cases in the control group were 2 and 38, respectively, while the positive and negative cases in the GC group were 28 and 12, respectively. In comparison to the actual results, the ROC-determined cut-off value for nesfatin-1correctly diagnosed 95% (38/40) of cases in the control group, whereas 70% (28/40) in the GC group.

Discussion

The current study is, to the best of our knowledge, the first to examine the plasma nesfatin-1 levels in patients with GC.

Table II. Comparisons of mean plasma nesfatin-1 levels between the GC subgroups and the control group (mean ± standard deviation).

Group	Nesfatin-1 (ng/ml)	P-value[a]
Control	0.89±0.12	-
GC		
Stage I	1.27±0.15	<0.01
Stage II	1.22±0.27	<0.01
Stage III	1.15±0.18	<0.01
Stage IV	1.12±0.40	<0.01

[a]P-value vs. control group. GC, gastric cancer.

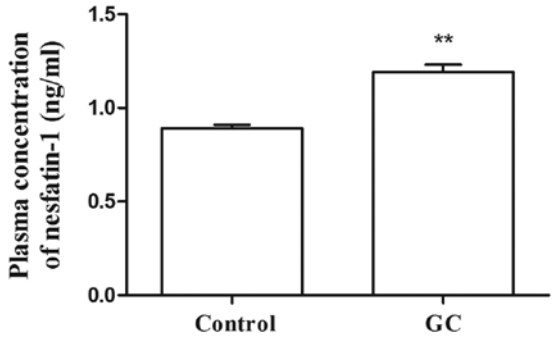

Figure 1. Comparison of mean values of plasma nesfatin-1 in the GC and control groups. The data are presented as the mean ± standard deviation, with n=40 in each group. **P<0.01 vs. control group. GC, gastric cancer.

The results demonstrated that the plasma nesfatin-1 concentrations were significantly increased in patients with GC when compared with those in healthy controls. In addition, the results of immunohistochemical analysis indicated that the protein expression of Ki67 in the tissues of patients with GC was higher compared with that detected in the normal gastric tissues of healthy controls. A positive correlation was revealed between plasma nesfatin-1 concentration and Ki67 protein expression in GC tissues. Furthermore, the results of the ROC analysis revealed an AUC value of 0.857, with 70.0% sensitivity and 95.0% specificity of nesfatin-1 in discriminating patients with GC from healthy controls.

Nesfatin-1, a newly discovered feeding regulator, has been suggested to serve an important physiological role in the central

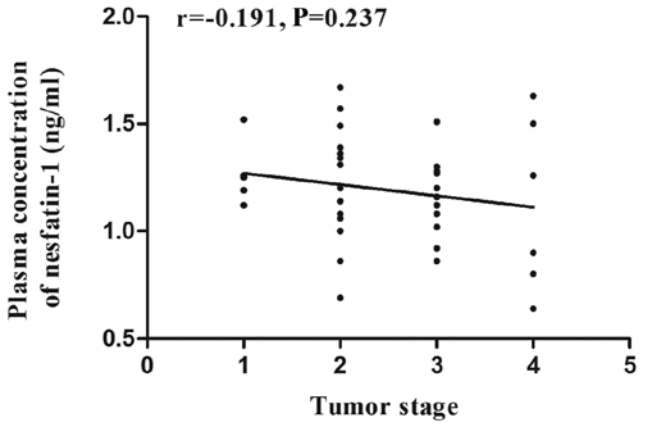

Figure 2. Correlation between the plasma concentration of nesfatin-1 and the tumor stage in the GC group. No significant correlation between the plasma concentration of nesfatin-1 and the tumor stage in the GC group was found by Spearman correlation analysis. GC, gastric cancer.

nervous system and peripheral tissues (27). A link between nesfatin-1 level and a variety of cancer types has previously been demonstrated (28). It has also been reported that a high level of nesfatin-1/NUCB-2 is associated with poor prognosis and promotes cell migration in breast cancer (29). By contrast, decreased serum expression of nesfatin-1 was demonstrated in patients with lung cancer and weight loss (30). Furthermore, an *in vitro* study suggested that nesfatin-1 enhanced the migration, invasion and epithelial-mesenchymal transition (EMT) in colon cancer cells through the LKB1/AMPK/TORC1/ZEB1 pathways (31). The current study investigated the changes in nesfatin-1 expression in patients with GC, and revealed significantly higher plasma levels of nesfatin-1 in these patients as compared with those in normal subjects. GC is often accompanied by the clinical symptom of appetite loss that is often caused by the invasion of normal tissues by cancerous tissues, which may lead to impaired gastric function (32). This symptom may also be associated with elevated nesfatin-1 levels. It has been reported that the central and peripheral administration of nesfatin-1 reduced food intake in rats and led to the loss of body weight (33,34). Another study has also suggested the co-localization of nesfatin-1 and ghrelin, the 'hunger hormone', in gastric tissue (11). Combined with the results of the current study, it can be concluded that the loss of appetite in patients with early GC may be associated with the high expression of nesfatin-1 in GC tissues.

The expression of Ki67 varies greatly during the cell cycle and is increased in a variety of tumor types (35). It has also been reported that Ki67 protein expression in GC tissues was significantly higher compared with that in normal gastric mucous tissues (36). Furthermore, in Greek patients with GC, a stronger expression of Ki67 was found to be correlated with a higher ratio of metastatic lymph nodes to the total number of dissected lymph nodes, as well as with advanced stage disease, indicating that the level of Ki67 was identified as an independent prognostic factor of survival (18). In the present study, Ki67 protein expression in GC tissues was significantly higher compared with that observed in normal gastric tissues, suggesting that the detection of Ki67 expression in GC may provide useful prognostic information for patients with this disease.

A positive correlation was observed between the plasma nesfatin-1 concentrations and the protein expression of Ki67 in patients with GC in the present study, suggesting that the abnormally elevated levels of plasma nesfatin-1 in these patients may be associated with the expression of Ki67 in GC tissues. Similarly, a previous study reported that the NUCB2/nesfatin-1 status was positively associated with Ki67 expression in human endometrial carcinoma (37). However, the mechanism behind this correlation has yet to be determined. Previous studies have demonstrated that NUCB2 knockdown using specific siRNA resulted in decreased cell proliferation and migration of the endometrial carcinoma cell lines Ishikawa and Sawano cells, as well as reduced the levels of nesfatin-1, a derivative form of NUCB2 that significantly stimulated cell proliferation and migration in Ishikawa cells (37). These findings are supported by a previous study performed by Kan *et al* (31), which indicated that nesfatin-1/NUCB-2 enhanced the migration, invasion and EMT in colon cancer cells *in vitro* and *in vivo*. Therefore, NUCB2 and/or nesfatin-1 are considered to be associated with the invasiveness of endometrial cancer by promoting the proliferation and migration of endometrial cancer cells (37). As Ki67 is a well-established marker for the evaluation of proliferation in GC cells, it can be assumed that plasma nesfatin-1 may also serve as a potent biomarker for the progression of GC, due to the close association between the plasma nesfatin-1 concentration and the protein expression of Ki67 in GC tissues.

A number of studies have identified potential serum/plasma biomarkers in the diagnosis of GC. Recently, numerous GC serum biomarkers have been revealed, including carcinoembryonic antigen, cancer antigen (CA) 19-9 and CA 72-4 (38-40). However, compared with other types of cancer, the sensitivity of these serum markers in the diagnosis of gastric adenocarcinoma is lower, at 20-30% (38-40). Furthermore, although microRNAs are promising biomarkers for cancer detection and prognosis, these novel methods often require specific technology and expensive instruments, and cannot be used in conventional screening tests (41,42). The variety of methodologies, types of carcinomas assessed, analysis software and normalization strategies used in the studies in the published literature have led to a considerable amount of variability and inconsistency among the reported findings (42). Therefore, the identification of novel biomarkers for early GC diagnosis is a currently major research focus.

It has been suggested that nesfatin-1 may be a new biological marker that can be used in the diagnosis of a number of diseases (43). In addition, NUCB2/nesfatin-1 has been reported to be capable of distinguishing patients from the healthy population in non-alcoholic fatty liver disease (44), major depression (45) and epilepsy (46). Therefore, in the present study, the potential of nesfatin-1 as a biomarker for GC diagnosis was investigated. Based on ROC analysis, the plasma nesfatin-1 cut-off point of 1.075 ng/ml was found to exhibit 70.0% sensitivity and 95.0% specificity, indicating that plasma nesfatin-1 has a superior diagnostic value (AUC=0.857) for GC. A previous study has demonstrated that serum nesfatin-1 levels decreased in patients with lung cancer compared with healthy subjects (30). This is inconsistent with the elevated levels of nesfatin-1 in the plasma of patients with GC that are reported in the present study, indicating that the biological function of nesfation-1 may vary among tissues. Additionally,

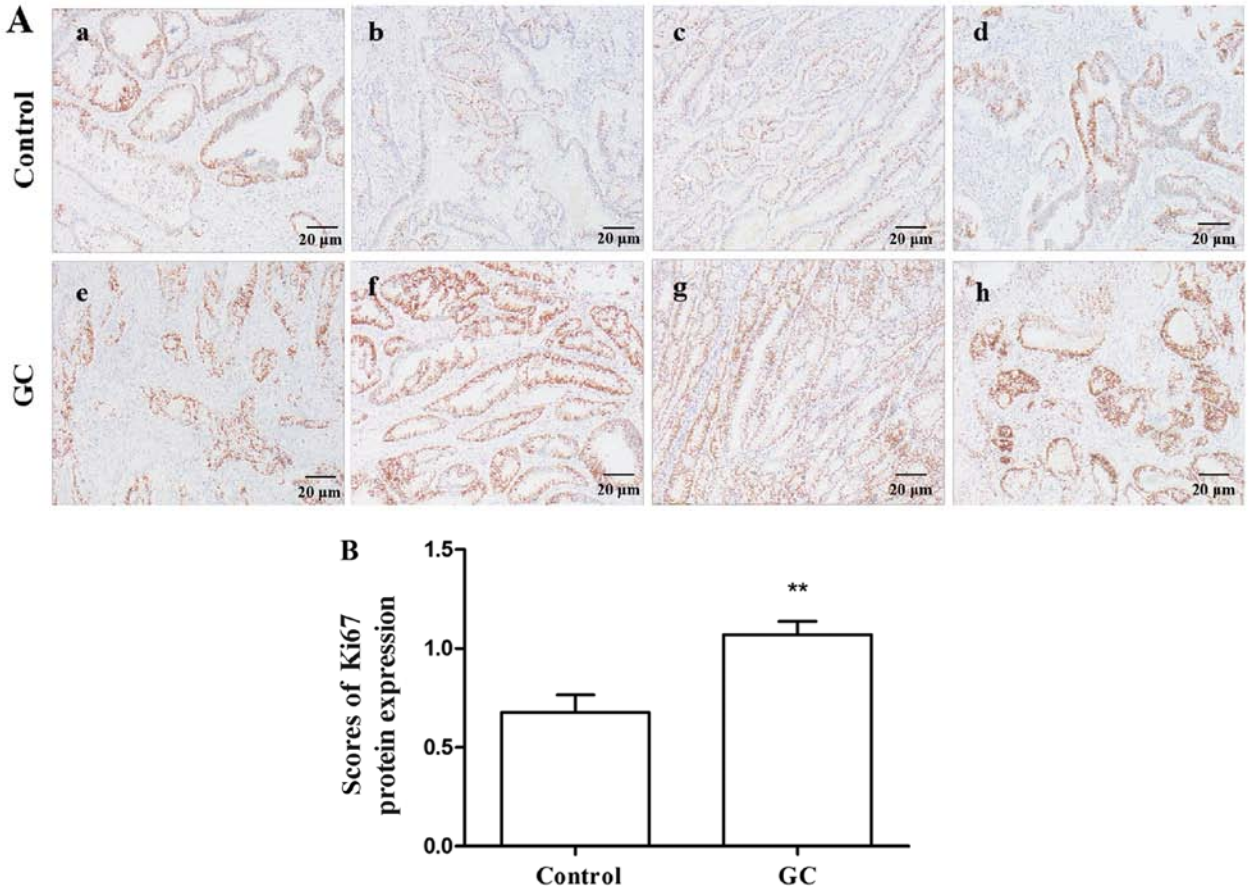

Figure 3. Immunostaining of Ki67 in the tumor tissues of GC patients and in normal gastric tissues of healthy controls. (A) Typical immunostaining images of the Ki67 protein expression (magnification, x40; scale bar, 20 μm). The scores and percentage of immuno-positive cells in the representative control group samples (a-d) were 1 (42.3%), 0 (8.4%), 0 (9.1%) and 1 (32.9%), respectively. The scores and percentage of immuno-positive cells in the representative GC group images (e-h) were 1 (47.5%), 2 (88.3%), 2 (78.6%) and 2 (85.6%), respectively. (B) Quantitative analysis of Ki67 protein expression. The data are presented as the mean ± standard deviation, with n=40 in each group. **P<0.01 vs. control group. GC, gastric cancer.

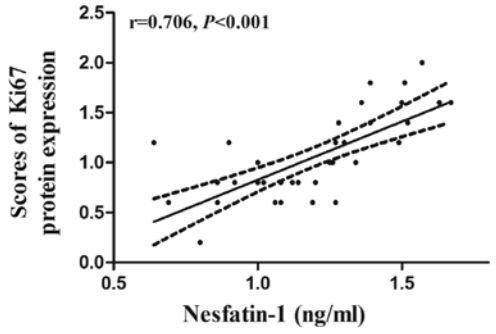

Figure 4. Correlation between the scores of Ki67 protein expression and plasma nesfatin-1 concentrations. The solid line represents the linear fit line for the presented data, while the dashed lines indicate the 95% confidence interval for the trend line slope.

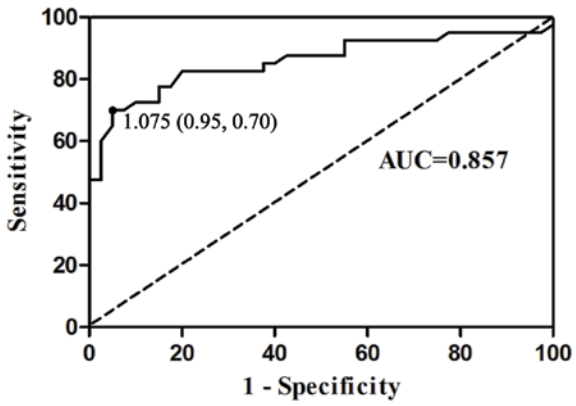

Figure 5. Receiver operating characteristic curve analysis of plasma nesfatin-1 in discriminating patients with GC from healthy controls. AUC, area under the curve.

the difference in the plasma levels of nesfatin-1 observed in these two types of cancer may be associated with the loss of adipose tissue. Nesfatin-1 gene and protein are expressed in human and murine adipose tissue (47). Therefore, loss of fat mass may decrease serum nesfatin-1 level in patients with lung cancer (30). In the present study, BMI values were not significantly different between the patients with GC and the healthy controls, indicating that no loss of body weight and adipose tissue occurred in patients with GC.

The limitations of the present study include the relatively small sample size examined and the recruitment of all subjects from a single hospital. Additionally, due to the cross-sectional study design, the causal association between nesfatin-1 and GC was not determined. Therefore, multicenter and longitudinal studies are required to validate the potential of nesfatin-1 as a novel biomarker fo0r GC.

In conclusion, the results from the present study suggest that the plasma concentrations of nesfatin-1 were significantly increased in patients with GC. Moreover, the protein expression of Ki67 in the tissues of patients with GC was also upregulated. Furthermore, plasma nesfatin-1 concentration was positively correlated with Ki67 protein expression in GC tissues. Additionally, the ROC analysis revealed an AUC value of 0.857, with 70.0% sensitivity and 95.0% specificity of nesfatin-1 in distinguishing patients with GC from healthy controls. These results indicate that the detection of plasma nesfatin-1 may be of clinical value for the diagnosis of GC.

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Availability of data and materials

All data generated and analyzed during the present study are included in this published article.

Authors' contributions

XQW and XBS designed the experiments. XQW, YZ and PFF performed the experiments. XQW, PFF and XBS analyzed data and assisted in the experiments. XQW drafted the manuscript. All authors approved the final version of this manuscript.

Ethics approval and consent to participate

All procedures performed in the present study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee, and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This study was approved by the Ethics Committee of Chaohu Hospital Affiliated to Anhui Medical University. Informed consent was obtained from all individual participants included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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