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ORIGINAL ARTICLE

The role of serum total ghrelin level elevation in colon cancer patients

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Summary

Purpose: *Many studies have pointed out a possible role of ghrelin, in the pathogenesis and natural history of gastrointestinal tract malignancies. The objective of this study was to estimate serum total ghrelin levels (STGL) in patients with colon cancer (CC) and to evaluate the value of this assay in research and clinical practice.*

Methods: *STGL were measured pre-operatively in 95 CC patients and in 39 healthy controls, and were correlated with patients' age, gender, body mass index (BMI), tumor location, Dukes stage, grade of differentiation and patients' survival.*

Results: *STGL were significantly elevated in patients with CC (127.6±34.5 pmol/L) vs healthy controls (89.3±19 pmol/L, p<0.05). STGL were significantly higher in end-stage (180±10 pmol/L) vs initial-stage CC (94.2±22.6 pmol/L; p<0.05). Furthermore, patients with poorly differentiated tumors had statistically significantly higher STGL (144.5±30.7 pmol/L) compared to those with well (91.8±23.6 pmol/L) or moderately well (126±34 pmol/L) differentiated CC cases (p<0.05). No statistically significant differences in the patients' STGL were noticed with respect to their demographic/clinical characteristics, tumor location and survival (p>0.05).*

Conclusions: *The obtained results showed a link between elevated STGL and CC, suggesting that colon tumors contribute to ghrelin's production.*

Key words: *cell differentiation, cell proliferation, colon cancer, ghrelin*

Introduction

Ghrelin is a 28-amino acid peptide, with a n-octanoylation indispensable for its biologic activity, originally isolated in the stomach as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) [1,2]. It is predominantly produced and secreted by the endocrine cells of the gastric mucosa, but it is also widely expressed in a variety of tissues, in both normal and malignant conditions; including the small intestine, colon, pancreas, lung, breast, thyroid, adrenals, hypothalamus, and pituitary [3-5].

Ghrelin has a plethora of physiological functions in the gastrointestinal, cardiovascular, pulmonary and immune system, and also exerts a variety of roles, ranging from increasing food intake (orexigenic effect) to affecting cell proliferation [6]. Nevertheless, despite its widespread and important physiologic functions, its precise regulatory mechanisms remain ambiguous [6]. The role of ghrelin in the control of cell proliferation and cancer has received considerable attention in the last decade. Several endocrine and non-endocrine tumors, such as gastro-entero-pancreatic carcinoids, colorectal neoplasms, pituitary adenomas, pulmonary and thyroid tumors, as well as lung, breast, and pancreatic carcinomas express ghrelin at both mRNA and protein levels [4,5].

Furthermore, STGL pathophysiologic changes have been measured and analysed in patients with esophageal [7,8], gastric [9-13] and colorec-

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tal cancer [9-11,14], gastrointestinal and pancreatic neuroendocrine tumors [15], hepatocellular [16,17] and thyroid carcinomas [18], breast [19], lung [20], prostate [21-23] and ovarian cancer [24], pituitary adenomas [25] and recently in lymphoblastic leukemia [26]. The results from these studies appear to be inconsistent: for instance, STGL were elevated in patients suffering from colon [14] or prostate cancer [23], whereas they were decreased in the D'Onghia et al. colorectal cancer study [11] or remained unchanged in the Huang et al. study [10]. These contradicting findings suggest that ghrelin's levels vary, depending on the type of neoplastic tumor. Moreover, research evidence shows that ghrelin promotes rather than protects against carcinogenesis [5,9]. Several researchers, including Murata et al. [16], Waseem et al. [14] and Nanzer et al. [27] attempted to answer the question whether ghrelin exerts a possible role in promoting or protecting against carcinogenesis. In fact, this question still remains unanswered.

Due to the unresolved ghrelin's possible regulatory mechanism in cellular proliferation, and the contradictory results of the existing research CC patients, we decided to focus on the changes of STGL in CC patients and to analyze potential clinicopathological factors that could possibly influence STGL.

Methods

Patients

Ninety-five patients (52 male-43 female) with CC participated in the study. Their mean age was 66 years (range 33-86) and they underwent colon resection in the 2nd Department of Propaedeutic Surgery of the University of Athens Medical School at Laikon General Hospital in Athens between February 2002 and August 2007.

Exclusion criteria

Exclusion criteria included treatment with chemotherapy, radiotherapy, or a major surgical operation within 6 months of recruitment. Patients with endocrine diseases, such as diabetes mellitus, hyper or hypothyroidism or pituitary deficiency were also excluded. Further exclusion criteria included acute or chronic infection, congestive heart failure, chronic obstructive pulmonary disease, cirrhosis, chronic renal failure, eating disorders (primary or secondary cachexia with BMI < 16 or morbid obesity with BMI > 30 and gastrectomy.

The clinical parameters assessed in the study included age, gender, BMI, cancer localization, stage and survival. The patients' height and weight were measured at the time of recruitment. Their BMI was calculated as their body weight divided by their height squared (kg/m^2). All pathology reports were evaluated and data on tumor histology were recorded. Tumor stage was assessed using the Dukes classification system. The type of surgery, tumor size and cell differentiation status were also evaluated.

The control group included 39 healthy subjects matched for age, gender, and BMI. The demographic and anthropometric measurements of the control and patient groups are summarized in Table 1.

Table 1. Characteristics of colon cancer and the control groups

Characteristics	*Colon cancer group (N=95) N (%)*	*Control group (N=39) N (%)*
Age, years, median (range)	67 (33-86)	59 (43-75)
Gender		
Male	52 (54.7)	23 (59)
Female	43 (45.3)	16 (41)
BMI (kg/m^2), median (range)	21 (18-25)	21 (18-26)
Primary tumor location		
Cecum-ascending	22 (23.2)	
Transverse to left splenic flexure	4 (4.2)	
Descending colon – Sigmoid	32 (33.7)	
Rectal	27 (28.4)	
Rectosigmoid	10 (10.5)	
Surgical intervention		
Right hemicolectomy	23 (24.2)	
Left hemicolectomy	5 (5.3)	
Transverse colectomy	2 (2)	
Sigmoidectomy	30 (31.6)	
Low anterior resection	16 (16.9)	
Abdominoperineal resection	18 (19)	
Subtotal colectomy	1 (1)	
Tumor size (cm)		
1-2	5 (6.3)	
2-4	59 (62.1)	
4-6	22 (23.1)	
>6	8 (8.5)	
Macroscopic tumor appearance		
Exophytic type	38 (40)	
Ulcerative type	41 (43.2)	
Both	14 (14.7)	
Dukes stage		
A	15 (15.8)	
B	36 (38)	
C	41 (43)	
D	3 (3.2)	
Histopathologic grade		
Well differentiated	3 (3.2)	
Moderately well differentiated	76 (80)	
Poorly differentiated	16 (16.8)	
Serum total ghrelin levels (pmol/L), median (range)	130 (60-190)	89.3 (65-130)

The study protocol was approved by the Research Ethics Committees of the National and Kapodistrian University of Athens Medical School, in Greece, according to the principles of the Declaration of Helsinki. Each participant signed an informed consent form before joining this study.

Ghrelin's level assays

After overnight fasting (at least 12h), with the patient in supine position at rest for at least 20 min, blood samples were drawn from each subject between 8 and 9 AM and placed into ice-cold EDTA/aprotinin polypropylene tubes, and after centrifugation at 14000g for 5 min at 4 ºC, the samples were stored in aliquots at -80 ºC, until assayed. Plasma total ghrelin was measured with a commercially available ELISA kit (Phoenix Pharmaceuticals, Inc., Belmont, CA) [11]. The intra- and inter-assay coefficients of variation were 4.8% and 3.5%, respectively. The ELISA kit sensitivity (minimum detectable concentration) was 0.1 ng/mL, with a range of 0.01 to 100 ng/mL.

Statistics

Data analyses were conducted with SPSS 17 (SPSS, Inc., Chicago, IL). The data were expressed as mean ± standard deviation for continuous variables and frequencies or percentages for discrete variables. The linear relationship of two variables was investigated with the use of correlation coefficients (Pearson's r or Kendal's τ; respectively for continuous and discrete variables). For comparisons of the groups, the two-tailed unpaired Student's t test and the Mann-Whitney U test were used (parametric and non-parametric test, respectively), whereas for comparisons of more than two groups the Kruskal-Wallis test was employed. Kaplan-Meier curves were generated to describe the patient survival and were compared with log rank test. Multivariate Cox regression analysis was carried out to investigate the prognostic value of ghrelin serum levels with respect to the patient survival. A p-value of <0.05 was considered statistically significant.

Results

STGL were found to be significantly higher [t(121)=-8.19, p<0.05, r=0.60] in CC patients (127.6±34.5 pmol/L) than in the controls (89.3±19 pmol/L).

STGL elevation in the CC group was significantly correlated with tumor size (p<0.05). With regard to Dukes stage, STGL were significantly higher in end-stage vs initial-stage disease (p<0.05). Furthermore, in poorly differentiated tumors, STGL were significantly elevated in comparison with moderately differentiated tumors (p<0.001), whereas the lowest ghrelin levels were measured in the well differentiated CC cases. Tumor location, type of surgery and histopathologic type did not reveal any statistically significant relationship with STGL in the CC group.

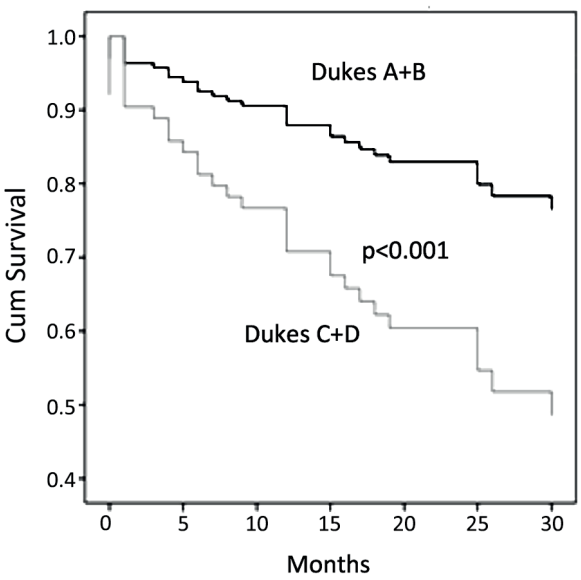

Figure 1. Kaplan-Meier overall survival curves according to Dukes stages in colon cancer patients.

The patient follow-up period ranged from 1 to 43 months (mean 20.6). STGL were not significantly correlated with the patient survival (r=-0.023, p>0.05). Nevertheless, considering the positive statistically significant relationship between STGL and Dukes stages, we investigated the patient survival by considering two groups: Dukes stage A and B vs Dukes stage C and D (D_{AB} and D_{CD}, respectively). The D_{AB} patients were found to have statistically significantly lower STGL [t(89)=-4.11, p<0.001, r=0.40)] (115.02±31.023) in comparison with the D_{CD} patients (142.14±32.984). Moreover, Kaplan-Meier survival curves and the log-rank test displayed statistically significant lower survival (x^2=4.6, p<0.05) of D_{CD} patients (26.9 months, 95% CI: 22.460–31.390) in comparison with the D_{AB} patients (35.5 months; 95% CI: 31.359–39.718) (Figure 1).

Multivariate Cox regression analysis was conducted to investigate the predictive value of STGL with survival. Initial analysis showed that Dukes stage is a significant factor predicting the patient survival. Nevertheless, a model including both Dukes stages and the STGL in the CC group, did not predict patient survival (x^2=5,623, p >0.05, df=2) with the STGL being a not statistically significant predictor (Wald's criterion, p>0.05, 95% CI: 0.983–1.005).

Finally, no statistically significant relationships were identified between STGL in CC group

Table 2. Serum total gherlin levels colon cancer patients with respect to tumor size, histopathologic grade, Dukes stage, age, gender and BMI

Disease characteristics	*Serum total ghrelin levels (pmol/L; mean±SD)*	*p-value*
Tumor size (cm)		
0-2	120±14.15	<0.05
2-4	123.8±35.3	
4-7	122.3±35.6	
>7	156.0±24.3	
Histopathologic grade		
Well	91.8±23.6	<0.05
Moderate	126±34.0	
Poor	144.5±30.7	
Dukes stage		
A	94.2±22.6	<0.001
B	123.7±30	
C	127±32.4	
D	180±10.0	
Histopathologic type		0.117
Type of operation		0.681
Primary tumor location		0.284
Age		0.285
Gender		
Male	127.7±31.9	0.977
Female	127.5±37.9	
BMI (kg/m^2)		0.300

BMI: Body mass index

and the considered demographic characteristics (age, gender and BMI; Table 2).

Discussion

Colorectal cancer is common across the globe and the leading cause of cancer-related deaths after lung cancer, but detailed understanding of its pathophysiology still remains far from complete [28]. Nowadays a large number of studies have been published that have proved immunoreactivity for ghrelin and its receptor in endocrine and non-endocrine tumors of the stomach and the intestine [5,9]. Hence, ghrelin-producing tumors have been added to the list of hormone-producing gastrointestinal endocrine tumors, with special reference to gastric carcinoids. The question that always arises - but it is not clearly answered until today - is whether ghrelin axis contributes to GIT carcinogenesis or it acts protectively against malignancy.

Recently, Waseem et al. [14] reported that colorectal cancer cells excessively secrete ghrelin *in vitro* and then use it in an autocrine and paracrine manner to promote their proliferative and invasive behavior. In malignant colorectal tissue samples overexpression of ghrelin was noted in a stage-dependent manner as opposed to normal colorectal tissue samples [5,14]. Waseem et al. [14] further observed that well differentiated (SW-48) and poorly differentiated (RKO) malignant colorectal cells, which produce more ghrelin, exhibited significant proliferative and invading/migrating activity even in the absence of growth factor addition in the culture medium [9,14]. Lastly, Lawnicka et al. demonstrated in 2012, the antineoplastic effect of ghrelin and the biphasic activity of D-Lys-GHRP-6 (strong inhibition and weak stimulation) on the growth of MC38 colon cancer cells *in vitro* [29].

By exploring the expression and the distribution of ghrelin receptor in human gastrointestinal tract cancers and investigating the possible involvement of the ghrelin-GHS-R system in human digestive cancers, Wang et al. [30] detected inverse correlation between the expressions of ghrelin receptor and the GIT cancer histological grade and TNM stage, whereas patients with more weight loss showed downregulated receptor expression. More specifically, higher expression levels were observed in the well-differentiated (I-II) group compared with the poorly differentiated (III-undifferentiated) group of colorectal cancer patients. Similarly, higher ghrelin expression levels were found in the early TNM stage (I-II) group than in the late stage (III-IV) group in colorectal cancer [30]. Following these observations, these researchers hypothesized that the decreased ghrelin receptor expression seems to be associated with other poor prognostic factors in digestive system cancer patients, such as poor differentiation, advanced stage, and malnutrition [30].

In this study, STGL were found to be significantly higher in the CC patients compared with the control subjects. Moreover, STGL elevation were positively significantly correlated with increased tumor size, while they were found to be significantly higher in end-stage disease. In our study, poorly differentiated tumors had statistically significant higher STGL compared with other histopathologic grades of CCs. Lastly, this study showed that the STGL are not a statistically significant predictor of CC patients' survival as Dukes stage is. Nevertheless, this study showed the relationship between high STGL with the poorly differentiated tumors and the end-stage CC disease. In our study the changes in STGL after surgical intervention were not assessed. A study comparing postoperative STGL with the preoperative lev-

els would be of interest, as well as comparing the changes of STGL after chemotherapy. There are so many factors which influence the STGL after surgery, such as malnutrition, cachexia, and chemotherapy; this makes it difficult to evaluate such results and their interpretation in research and clinical practice. It is considered that further *in vitro* investigations should be particularly useful.

Our results coincide with the relevant literature except the following two publications. In 2007, Huang et al. [10] reported no significant difference in STGL between 20 colorectal cancer patients (cachectic and non-cachectic) and the control group. In that study, 40% of the patients were cachectic, a clinical condition that affects ghrelin's levels [31-35]. These authors did not correlate STGL with tumor location and size, disease stage and the histopathologic grade; they only correlated some demographic characteristics (age, gender, BMI), the nutritional status and other hormones (leptin, insulin, GH, cortisol and glucagon) with STGL.

The same year, D'Onghia et al. [11] showed ghrelin levels to be significantly lower in 21 CC patients compared independently of tumor location compared to healthy subjects. They found a progressive significant decrease in STGL from earlier (Dukes A or B) to later stages, which was linked with progressive loss of cells' differentiation during the disease. In that study only 30% of the healthy control individuals had normal BMI, whereas 54% were overweight (BMI 25-30) and 16% obese; no reference was made about BMI of CC patients [11].

In contrast, in our study both the CC group and the control subjects had a BMI from 18 to 26 (exclusion of primary or secondary cachexia or morbid obesity). The regulation of ghrelin secretion, as is already known, occurs at several levels and is connected to many factors. Fasting, low BMI, leptin, thyroid hormones, testosterone, GHRH are amongst the factors that upregulate circulating STGL vs food intake, high BMI, glucose, insulin, somatostatin and GH that downregulate ghrelin levels [4]. The exclusion criteria applied in our study aimed at diminishing the factors that can affect the measured STGL.

Considering all the aforementioned studies and the findings reported in this paper, the same questions are posed: Is ghrelin carcinogenic or is it protective against malignancy? Can ghrelin promote cell proliferation and differentiation in malignant tissues or can it inhibit proliferation and protect against cancer? There are several studies that deal with ghrelin and the expression of its receptor in carcinomas and in endocrine tumors, but no study has yet determined whether ghrelin's administration promotes or inhibits carcinogenesis, so we cannot attribute any specific action of ghrelin in all of these tumors. We can only declare our strong belief that ghrelin under certain circumstances acts as a basic or complementary growth factor on cell growth and cell proliferation and also possibly contributes to the mechanisms of carcinogenesis. Moreover, the present study showed a link existing between the STGL elevation and CC. There is a strong possibility that CC cells exert some special effects on the production of ghrelin. Therefore, in the future, if the exact role of ghrelin in malignancy will be clarified and the mechanism of its action be discovered it might be used as a new therapeutic target or at the very least as a neoplastic prognostic factor [5].

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