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Ghrelin and gastric cancer

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Ghrelin is a recently discovered peptide, described predominantly in gastric endocrine cells. Gastric ghrelin – positive cells were studied in chronic atrophic gastritis, H. Pylori-related gastritis and gastric carcinoids mainly. Presence of ghrelin- positive cells in gastric cancer was less investigated. The aim of the present study was to describe ghrelin-positive cells in gastric cancer of diffuse and intestinal types and in surrounding mucosa from antral, fundic and corpus regions. Endocrine cells were revealed immunohistochemically with antibodies against chromogranin (Cha), gastrin (Gas), somatostatin (Som), serotonin (Ser) and ghrelin (Ghr). Ghrelin positive cells were found in all cancers (diffuse type gastric cancer), (1,93±1,76 cells/mm^2). In antral mucosa Ghr$^+$ cells were between 42,37±4,8 cells/ mm^2 followed by corpus mucosa between 27,6±1,27 cells/ mm^2 and by fundus mucosa between 25,2±6,3 cells/ mm^2. Co-localization studies showed that some of the Cha$^+$cells, Gas$^+$ cells, and Som $^+$cells were also Ghr $^+$. In conclusion we may state that in gastric cancer from the diffuse type there could be detected Ghr $^+$ECs. Ghrelin could be secreted not only by separate Ghr $^+$ ECs but also by ECs positive for gastrin and somatostatin.

Keywords: ghrelin, endocrine cells, gastric cancer

INTRODUCTION

Ghrelin structure and production

Ghrelin was described by Kojima et al. [1] as a novel growth-hormone-releasing peptide, which was originally isolated from rat and human stomachs. It was also reported that human ghrelin is homologous to rat ghrelin exept for two amino acids. This ligand for growth hormone secretagogue receptor (GHS-R) is a peptide consists of 28 amino acids. Its serine 3 residue is n-octanoylated. The acylated peptide was established to release growth hormone (GH) both in vivo and in vitro, and O-noctanoylation at serine 3 is very important step in its activation. Circulating ghrelin contains more than 90% of desacyl ghrelin and less than 10% acyl ghrelin [2]. However, according to Kojima et al. [1, 3] the acyl group of ghrelin is essential for its binding to GHS-R and the simultaneus activation of the inositol triphosphates-calcium pathway).

The human ghrelin gene is located on the short segment of chromosome 3 (3p25-26) and comprises 4 introns and six exons (2 are noncoding) and encodes a 511 bp mRNA [4, 5, 6]. In relation to the chemical properties of its precursors, Korbonits et al. [7] reported that preproghrelin (117 AA) contains 23 AA signal peptide and a 94AA segment corresponding to proghrelin. Proghrelin is made of the 28AA ghrelin peptide and a 66AA carboxyterminal peptide named C-ghrelin [6, 8, 9]. C-ghrelin can be transformed to a 23AA peptide colled obestatin [10]. On the other hand, some authors give information for the alternative splicing of the human ghrelin gene produces additional transcripts coding for other peptides including des-Gln14-ghrelin [6, 11]. The enzymes responsible for transforming preproghrelin into ghrelin include signal peptidase cleaving at Arg23, prohormone convertase 1/3 (PC 1/3) cleaving at Arg51 (producing ghrelin 1–28) [12**],** and carboxypeptidase-B like enzyme cleaving at Pro50 (producing ghrelin 1–27) [13].

As mentiomed above, two ghrelin peptides are described as a result of human ghrelin gene transcription and translation. The main metabolic pathway is the acylation of the hydroxyl group of the Ser3 [1]. Both ghrelin 1–28 and ghrelin 1– 27 undergo to acylation, mainly by an octanoyl group (C8:0) and more rarely by a decanoyl (C10:0) or decanoyl (C10:1) group [Hosoda et al., 2003). Enzyme involved in ghrelin acylation is ghrelin *O*-acyl transferase (GOAT), which is a member of the family of membrane *O*-acyl transferases (MBOAT) [14, 15]. Gutierrez et al. [14] found out that ghrelin and GOAT are co-expressed in X/A like cells of gastric mucosa. The addition of C8 medium-chain or C10 medium chain triglycerides in diets

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modificate the proportions of octanoyl or decanoyl ghrelin involved in the same granules in gastric X/A like cells, suggesting that GOAT uses the most available substrate to catalize ghrelin acylation [16]. It is known that ghrelin acylation is performed in the endoplasmic reticulum prior to the processing of proghrelin by proteases on either the preproghrelin or the proghrelin precursors [12, 15, 17, 18]. GOAT acylates ghrelin with fatty acids ranging from C:7 to C:12 [14]. Yang et al. (2008) described acyl-CoA as donors of acyl group. After that, Ohgusu et al. (2009) proved that *in vitro* GOAT prefers *n*-hexanoyl-CoA to *n*-octanoyl- CoA as an acyl donor.

Biological activities of ghrelin depend on the presence of acyl group on Ser3 [19, 20]. C8:0 Ser3 defines maximal ghrelin activity, which is maintained by C10:0 Ser3, C12:0 Ser3, and C16:0 Ser3 but decreased by C4:0 Ser3 orC2:0 Ser3 [19]. The replacement of Ser3 by Trp3 maintains the activity of ghrelin, but its replacement by aliphatic AA (such as Val, Leu, or Ile) inhibits its activity [19]. The N-terminal positive charge and Phe4 are necessary for ghrelin activity and recognition by GHS-R1A [21]. Systematic C-terminal reduction of ghrelin identified the N-terminal pentapeptide of ghrelin, including C8:0 Ser3, to be the minimal peptide fragment with the same power like ghrelin [20, 22]. In addition, amidation of the C-terminus increased the activity of the ghrelin fragments [20, 22] while N-acetylation decreased it [21, 22].

In human, circulating ghrelin contains desacyl ghrelin (more tan 90%), acyl ghrelin, and C-ghrelin [2, 8, 23]. Desacyl ghrelin mostly circulates as a free peptide, while acyl ghrelin is bound to lipoproteins [24, 25]. The acyl group is needed for ghrelin interaction with lipoproteins bound with triglycerides and low-density lipoprotein, while N- and C-terminal ends of ghrelin are needed for its coupling to high-density lipoproteins and very high-density lipoproteins. In this regard, De Vriese et al. [24] hypothesized that triglyceride-rich lipoproteins predominantly transport acyl ghrelin, while high-density and very high-density lipoproteins transport both acyl and desacyl ghrelin.

Ghrelin receptor (GHS-R)

GHS-R is a type of G-protein coupled receptors (GPCR), characterized by seven transmembrane helix domains [26]. The localization of human GHS-R was established on chromosome 3 (3q26.2) consisting of 2 exons and 1 intron. Exon 1 codes for the GHS-R region from the extracellular N-terminal end to the 5th transmembrane helix, while exon 2 codes for the rest of the receptor GHS-R is represented by GHSR1A and GHS-R1B. GHS-R1A is a 366AA protein consisting of 7 transmembrane helix domains, while GHS-R1B is a 289AA protein consisting of 5 transmembrane helix domains [27].

Binding of GHS-R1A to G-protein involves the 3rd intracellular loop. The lack of a 3rd intracellular loop in GHS-R1B prevents its binding to G-proteins. GHS-R1A activation causes the activation of phospholipase C, inositols triphosphates, and intracellular calciump athways [28]. At physiological concentrations, only acyl ghrelin binds to GHS-R1A, while at supraphysiological concentrations (1 μM) desacyl ghrelin couples to the receptor as well [29, 30]. Acyl ghrelin and desacyl ghrelin are electrostatically attracted to membranes by their basic residues, but acyl ghrelin penetrates deeper by its acyl group [30]. GHS-R1A was shown to function as homodimer [31, 32] and it also forms heterodimers with members of the prostanoid receptor family such as vasodilatator prostacyclin receptor (IP), the vasoconstrictor prostaglandin E2 receptor subtype EP3-I (EP3-I), and thromboxane A2 (TPα) [33].

GHS-R1B, considered in the past to be functionally inactive, is now believed to act as an important modulator in ghrelin-induced GHS-R1A signaling. Indeed, GHS-R1B is able to heterodimerize with GHS-R1A and to decrease the constitutive activity of GHS-R1A [32, 34, 35, 36]. GHS-R1B exerts a dominant negative effect via a conformational restriction of the GHS-R1A that becomes unable to subsequently activate G protein and recruit β -arrestin [37].

GHS-R1B is unable to bind acyl or desacyl ghrelin and acts as a modulator in ghrelin-induced GHS-R1A signaling. GHS-R1B is able to heterodimerize with GHS-R1A and decrease the constitutive activity of GHS-R1A [32, 34, 35, 36]. GHS-R1B exerts a negative effect through a conformational constraint of the GHS-R1A which becomes unable to activate G protein and strengthen β-arrestin [37].

The role of ghrelin in physiological processes

Ghrelin expression is mainly detected in the digestive tract, with highest levels in the gastric mucosa [1, 38]. Gastric mucosa involves five endocrine cell types, represented by enterochromaffin cells (EC), enterochromaffin-like cells (ECL), D cells, G cells, and X/A like cells which, respectively, secrete serotonin, histamine, somatostatin, gastrin, GABA, and ghrelin. Human and rat gastric mucosa are, respectively, composed of 30%/60–70%ECL cells, 20%/20%X/A-like cells,

22%/2.5%Dcells, and 7%/0–2% of EC and G cells [39, 40]. Ghrelin is mainly located in the oxyntic mucosa of the gastric fundus in neuroendocrine cell subtype of P/D_1 cells which represent 20% of all neuroendocrine cells at this place [41, 42]. In the normal mucosa of stomach corpus, ghrelin-positive cells were located between parietal cells and chief cells in the lower part of the fundic glands. In man, ghrelin cells (147 nm) were characterised by round, electron-dense secretory granules [42]. Ghrelin has been known as a multifunctional hormone. Various studies have investigated ghrelin and its systemic effects regarding growth hormone release from the pituitary gland, appetite regulation and its impact on body weight [43, 44]. However, ghrelin is also a hormone with gastro-protective local effects. It stimulates propulsion and mucus secretion and contributes to the healing process after a mucosal injury. Therefore, ghrelin is essential for maintaining the mucosal barrier of the human stomach [45, 46]. The mechanism for the action of ghrelin on feeding, growth hormone secretion, secretion of gastric acid and the gastric contractility was studied and demonstrated that the vagal nerve was involved in the action of ghrelin [47, 48]. Yakabi et al., [49] established that ghrelin stimulates gastric acid secretion via a mechanism involving activation of vagal efferent nerve and histamine release from gastric enterochromaffin-like cells. In humans, peripheral administration of ghrelin stimulate gastric emptying [50] with no modification of orocecal and colonic transit [51]. Moreover, ghrelin strengthens the human migrating motor complex [52]. Furthermore, ghrelin has been established to be useful for the treatment of gastrointestinal motility disorders [53, 54]. Future studies are needed to study the beneficial effects of novel ghrelin receptor agonists in gastrointestinal motility disorders.

The role of ghrelin and its receptors in pathological processes of the human stomach

The role of ghrelin in pathological processes has been described in different aspects. Several studies have reported that ghrelin is able to exert anti-inflammatory actions by inhibiting the production of proinflammatory cytokines [55, 56, 57, 58]. Ghrelin anti-inflammatory actions were found out in inflammatory bowel disease, pancreatitis, sepsis, arthritis [59, 60, 61, 62].

Rau et al., [63] investigated the influence of ghrelin in several pathological situations of the stomach. For example, in autoimmune gastritis, Rau et al. [63] established a new gastrin-mediated mechanism for ghrelin suppression. In autoimmune gastritis, ghrelin-positive cells comprised up to 50% of the investigated nodules of neuroendocrine cell hyperplasia. Neoplastic ghrelinomas have also been described by several authors [64, 65, 66, 67]. Some authors claim that ghrelin plays an autocrine/paracrine role in a number of processes related to cancer progression, including cell proliferation [68, 69], cell migration [70], and apoptosis [71]. According to Duxbury et al. [72] ghrelin increases cell proliferation, migration and invasion in pancreatic cancer cell lines via PI3K/Akt pathway [72], which is associated with an increase in cell motility and invasion. The participation of ghrelin in proliferation of gastric cancer was also reported by Tian and Fan [73]. These authors found out that ghrelin and des-acyl ghrelin stimulate the proliferation of gastric cancer cells via the activation of the ERK1/2 and PI3K/Akt pathway. While a number of studies have demonstrated that ghrelin stimulates cell proliferation, some reports indicate that ghrelin may inhibit proliferation. These include thyroid [74], prostate [75] and breast cancer [76] and small cell lung carcinoma [77] cell lines. Ghrelin is expressed in a wide range of cancer tissues and plays a role in a number of key processes in cancer progression, including cell proliferation, cell migration and invasion, and apoptosis. As there have been a number of conflicting reports, it is currently unclear whether ghrelin promotes cancer or inhibits its development..

The data about ghrelin expression in gastric cancer cases is too limited, but it is different for neuroendocrine tumors. In gastric cancer, Rau et al. [63] did not detect any ghrelin-expressing cells. Papotti et al. [64] demonstrated that the majority of gastric carcinoids and a fraction of intestinal endocrine tumors show immunoreactivity for ghrelin. It was established that 75% gastric carcinoids were immunoreactive for ghrelin in a variable percentage of tumor cells. Cellular ghrelin reactivity was observed as a diffuse, finely granular cytoplasmic staining, as strong as that of peritumoral gastric endocrine cells. Intratumoral stromal or inflammatory cells were not stained. Although gastric carcinoids are rare and generally benign conditions, Papotti et al. [64] observed two aggressive cases and an additional low grade tumor with a lymph node metastasis. Ghrelin was found to be produced by two of these tumors, which meens that its expression is probably independent from the biological aggressiveness of the tumor. These findings suggest that assessment of circulating ghrelin levels may be useful in diagnosis of atrophic gastritis and associated neuroendocrine

cell growths as well as of gastrointestinal tumors of neuroendocrine nature. Tsolakis et al. [66] studied a patient with a malignant gastric neuroendocrine tumor secreting ghrelin as the main hormone. This might be a new tumor entity of the stomach, and it is suggested that patients with malignant gastric neuroendocrine tumors should be investigated for ghrelin production, for example ghrelin immunoreactivity in tumor cells as well as total and active ghrelin concentrations in the blood.

It was reported that ghrelin-expressing cells are a cell type differs from the enterochromaffin-like cells, but intermingle with them as derivatives from type A-like cells. However, the idea of enterochromaffin-like cell hyperplasia is derived from the idea of a direct gastrin influence on these cells which still has to be shown for ghrelin-positive cells [78]. Gastrin is known to have growth stimulating effects on neuroendocrine cells in the gastric fundus [78, 79] but it is unknown if it interacts with ghrelin-expressing cells. Some authors presented data that shows synergistic effects of gastrin and ghrelin on gastric acid secretion and histamine production by gastric mucosa which involves the vagal nerve [80, 45].

However, Rau et al. [64] confirmed the existence of the gastrin/cholecystokinin (CCKB) receptor for the first time on human ghrelin-positive cells, which corresponds to data from animal models [45]. The finding of this receptor on ghrelin-expressing cells suggests a possible influence by gastrin. For example, Rau et al. [63] observed dose-dependent ghrelin suppression by gastrin.

MATERIALS AND METHODS

We used specimens from two patients with intestinal type of gastric cancer who undergo of total gastrectomy in Surgical Department in University Hospital in Stara Zagora. Tissue specimens were fixed in 10% buffered formalin, embedded in paraffin and cut to 4 μm thickness. Specimens were deparaffinated and endogenous peroxidase was blocked for 5 minutes with blocking reagent according to the protocol. Then the slides were washed 3 times with PBS and incubated with primary antibody for 1 hour followed by incubation with marked polymer and washed again.

Tissue samples were incubated with DAB substrate-chromogen and after washing counterstained by Mayer's hematoxylin.

Table1 shows immunohistochemistry panel of applied antibodies and components, their manufacturer, dilution and reaction:

Table 1. Immunohistochemistry panel of applied antibodies and components

Nº	Antibody	Manifacture	Dilution	Reaction
1.	Monoclonal Mouse Anti-Human Chromogranin A	M0869, DAKO	1:100	Cytoplasmatic
2.	Polyclonal Rabbit Anti-Human Gastrin	A 0568, DAKO	1:500	Cytoplasmatic
3.	Monoclonal Mouse Anti-Human Serotonin	M0758, DAKO	1:100	Cytoplasmatic
4.	Polyclonal Rabbit Anti-Somatostatin	A0566, DAKO	1:200	Cytoplasmatic
5.	Polyclonal Rabbit Anti-Human Ghrelin	H-40 Santa Cruz Biotechnology	1:100	Cytoplasmatic
6	EnVision™ FLEX+, Mouse, High pH, (Link)	K8002, DAKO	-	-

RESULTS AND DISCUSSION

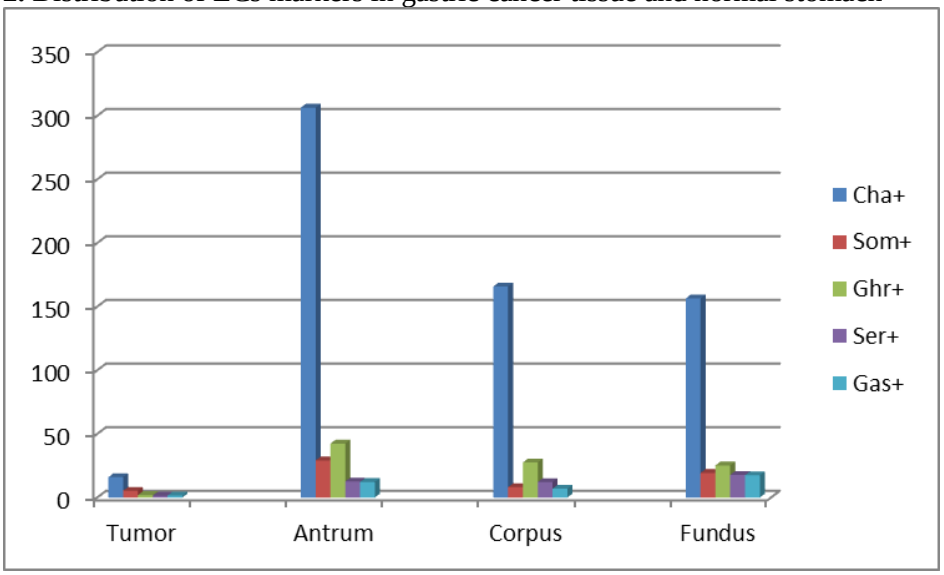
After analysis, we found endocrine positive cells (ECs) for all investigated markers in tumor parenchyma and in the overlying mucosa of gastric antrum, corpus and fundus (Table 1, Fig. 1, Fig. 2 and Fig. 3).

The most common ECs in the tumor tissue were cells positively for Chromogranin (15,9±8,5 cells/mm2), followed by somatostatin positive (5,33±1,25 cells/mm^2), Ghrelin$^+$ (1,93±1,76 cells/mm^2), gastrin positive (1,59±0,32 cells/mm^2) and serotonin positive cells (1,47±1,44 cells/mm^2). In normal tissue from gastric antrum and gastric

fundus the distribution of ECs was similar: Cha$^+$cells (306,35±6,97 and 156,5±1,6 cells/mm^2 respecively), Ghr$^+$cells (42,37±4,8 and 25,2±6,3 cells/mm^2, respectively), Som$^+$cells (29,14±5,93 and 19,4±7,2 cells/mm^2, respectivly), Ser$^+$cells (and 17,8±4,14 cells 12,1±6,9 cells/mm^2, respectively). cells/mm^2, respectivly), and ECs positively for gastrin were least (12,2±8,4 for antrum and 17,6±2,03 cells/mm^2 for fundus).

Finally after investigation of gastric corpus the result shown that the most common type of ECs was again chromogranin positive (165.76±3,13cells/mm^2), followed by ghrelin positive (27,6±1,27 cells/mm^2), serotonin + (12,6±1,4 somatostatin positive (8,1±0,04 cells/mm^2), /mm^2) and gastrin positive (6,89±1,4 cells/mm^2).

Table 2. Distribution of ECs markers in gastric cancer tissue and normal stomach



Ghrelin has a number of functions, including roles in the regulation of growth hormone release, metabolism, appetite, the cardiovascular system and insulin secretion [1]. Another function of ghrelin is that it increases the secretion of gastric acid via nitric oxide which stimulates mucosal blood flow [81**.** Ghrelin cell density was found by to be positively correlated with the degree of diarrhea and inversely correlated with the degree of constipation [82]. Therefore, changes in ghrelin cell density play a decisive role in the development of diarrhea and constipation in irritable bowel syndrome patients.

In addition the role of ghrelin and its receptor GHS-R is not clear. It was reported that GHSR 1a expression differs from that of GHSR 1b in cancer. For instance, in a number of cancers, GHSR 1a expression is downregulated or absent [76, 83, 84, 85], while the non-functional form of the receptor, GHSR 1b is widely expressed in cancer and expression may be upregulated compared to normal tissues [86]. Some autors state that gastric cancers arised after different types of mucosal injury, but today we know that ghrelin is also a hormone with gastro-protective local effects. It stimulates propulsion and mucus secretion and contributes to the healing process after a mucosal injury. Therefore, ghrelin is essential for maintaining the mucosal barrier of the human stomach [44, 45].

Ghrelin is expressed in a wide range of cancer tissues and plays a role in a number of key processes in cancer progression, including cell proliferation, cell migration and invasion, and apoptosis. As there have been a number of conflicting reports, it is currently unclear whether ghrelin promotes cancer or inhibit its development and it is possible that it could have both stimulatory and inhibitory effects.

Since in the literature data is scarce, our results suggest that ghrelin and some from the other endocrine markers in gastric cancer tissue and normal mucosa may play an important role in carcinogenesis, and its expression will be a valuable prognostic marker for prognosis and prediction in gastric cancer patients.

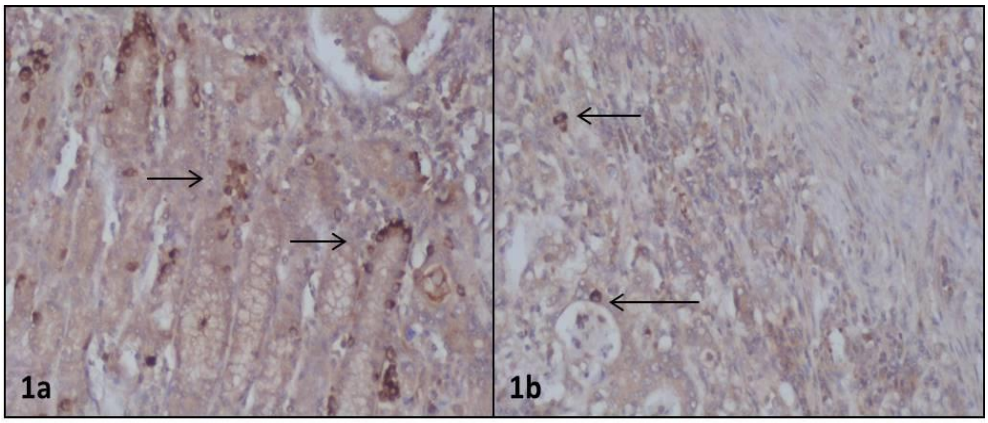


Fig. **1a)** Ghrelin positive ECs (arrows) hyperplasia in transitional mucosa and in tumor (x200); **b)** Ghrelin positive ECs (arrows) in tumor (x200).

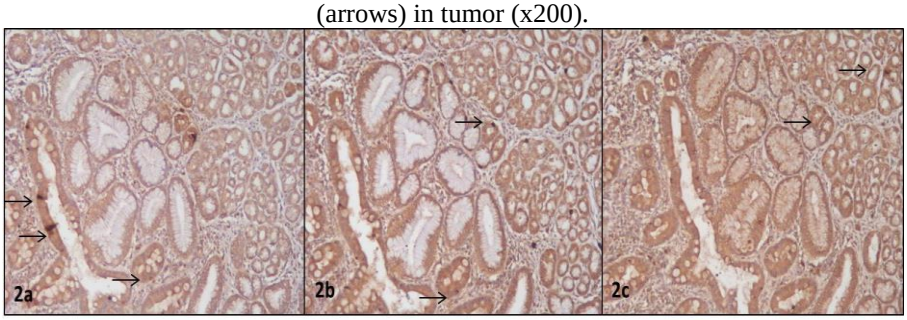


Fig. 2a) Gastrin positive ECs (arrows) in gastric fundus (x200); **b)** Ghrelin positive ECs (arrows) ingastric fundus (x200); **c)** Somatostatin positive ECs (arrows) in gastric fundus (x200)

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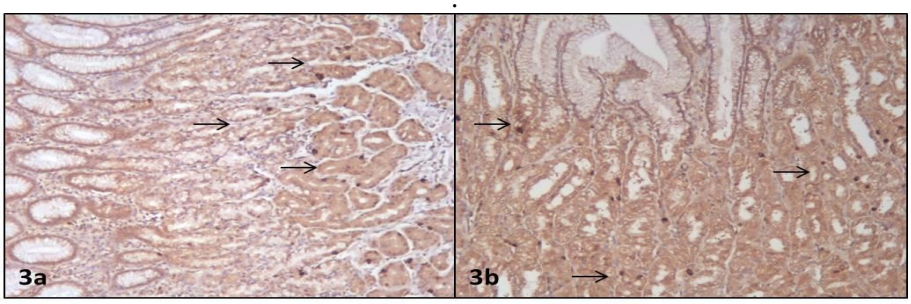

Fig. 3. **a)** Ghrelin positive ECs (arrows) in pylor (x100); **b)** Ghrelin positive ECs (arrows) in corpus (x100).

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(Резюме)

Грелинът е наскоро установен пептид, описан предимно в ендокринните клетки на стомаха. В стомаха, грелин позитивни клетки са описани основно при хроничен атрофичен гастрит, H. Pylori- свързан гастрит и стомашни карциноиди. Присъствието на грелин позитивни клетки при рак на стомаха е слабо проучено. Целта на настоящото изследване е да опишем грелин позитивните клетки при дифузен и интестинален тип рак на стомаха и в околната лигавица на антрума, фундуса и тялото на стомаха. Ендокринните клетки са установени имунохистохимично с антитела срещу хромогранин (Cha), гастрин (Gas), соматостатин (Som), серотонин (Ser) и грелин (Ghr). Грелин позитивни клетки са наблюдавани във всички видове стомашен карцином **(**дифузен тип) (1,93±1,76 cells/mm^2). Най-много Ghr$^+$ клетки 42,37±4,8 cells/ mm^2 са открити в антралната лигавица на стомаха, последвани от тези (27,6±1,27 cells/ mm^2) в лигавицата на тялото и най-малко - 25,2±6,3 cells/mm^2 в лигавицата на фундуса на стомаха. Извършената колокализация с използваните антитела показа, че някои от Cha$^+$ клетки , Gas$^+$ клетки и Som$^+$ клетки са едновременно и Ghr $^+$. В заключение, Ghr $^+$ECs могат да бъдат идентифицирани в стомашния карцином от дифузен тип. Грелинът може да бъде секретиран не само от определени Ghr $^+$ ECs, но също от позитивни за гастрин и соматостатин ендокринни клетки.