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Ghrelin and obestatin in thyroid gland — immunohistochemical expression in nodular goiter, papillary and medullary cancer

Edyta Gurgul[*1], Aldona Kasprzak[*2], Agata Blaszczyk[3], Maciej Biczysko[4], Joanna Surdyk-Zasada[2], Agnieszka Seraszek-Jaros[5], Marek Ruchala[1]

*Both authors contributed equally to this work

[1]Department of Endocrinology, Metabolism and Internal Medicine, Poznan University of Medical Sciences, Poznan, Poland

[2]Department of Histology and Embryology, Poznan University of Medical Sciences, Poznan, Poland

[3]Department of Clinical Pathomorphology, Poznan University of Medical Sciences, Poznan, Poland

[4]Department of General Surgery, Oncologic Gastroenterological and Plastic Surgery, Poznan University of Medical Sciences, Poznan, Poland

[5]Department of Bioinformatics and Computational Biology in the Chair of Clinical Pathomorphology, Poznan University of Medical Sciences, Poznan, Poland

Abstract

Introduction. Previous studies analyzing ghrelin and obestatin expression in thyroid gland tissue are not unanimous and are mostly related to ghrelin. The role of ghrelin and obestatin in the thyroid gland appears very interesting due to their probable involvement in cell proliferation. Furthermore, since the thyroid gland is associated with the maintenance of energy balance, the relationship between ghrelin, obestatin and thyroid function is worthy of consideration. The aim of the study was to assess ghrelin and obestatin immunocytochemical expression in nodular goiter (NG), papillary cancer (PTC) and medullary cancer (MTC).

Material and methods. Analyzed samples included 9 cases of NG, 8 cases of PTC and 11 cases of MTC. The analysis of ghrelin and obestatin expression was performed by use of the immunohistochemical (IHC) EnVision system and evaluated with filter HSV software (quantitative morphometric analysis).

Results. Quantitative ghrelin expression in MTC cells was higher than in NG ($p = 0.013$) and correlated negatively with the size of the tumor ($r = -0.829$, $p < 0.05$). We did not observe any differences in ghrelin expression neither between MTC and PTC nor between NG and PTC. Obestatin immunoexpression pattern in all analyzed specimens was irregular and poorly accented. The strongest immunoreactivity for obestatin was demonstrated in NG. In MTC obestatin expression was significantly weaker than in NG and PTC ($p < 0.05$ in both cases). In NG the intensity of obestatin immunostaining was significantly higher than that of ghrelin ($p = 0.03$). Conversely, ghrelin expression in MTC was definitely more evident than obestatin immunoreactivity ($p < 0.01$). There was no statistically significant difference between ghrelin and obestatin expression in PTC. No correlations were detected between reciprocal tissue expressions of ghrelin and obestatin in the analyzed specimens of NG, PTC or MTC.

Conclusions. The differences between ghrelin expression in NG and MTC suggest that ghrelin may be involved in thyroid cell proliferation. The differences between ghrelin and obestatin immunoreactivity in benign and malignant thyroid tumors could support the theory of alternative transcription of the preproghrelin gene and independent production of ghrelin and obestatin. *(Folia Histochemica et Cytobiologica 2015, Vol. 53, No. 1, 19–25)*

Key words: ghrelin; obestatin; thyroid; nodular goiter; papillary thyroid cancer; medullary thyroid cancer; IHC

Correspondence address: E. Gurgul, M.D., Ph.D.
Department of Endocrinology, Metabolism and Internal Medicine
Przybyszewskiego St. 49, 60–355 Poznan
tel.: +48 61 869 13 30, fax: +48 61 869 16 82
e-mail: liberius@interia.pl

Introduction

Ghrelin and obestatin are two peptides encoded by the same gene, both postranslationally cleaved from their precursor — preproghrelin [1–3]. Ghrelin was initially reported as the first endogenous ligand for growth hormone secretagogue receptor (GHS-R) and potent stimulator of growth hormone release [1, 4]. However, further studies revealed that it is a multifunctional hormone and an essential regulator of metabolic processes [3]. Ghrelin is released mainly from gastric mucosa [5], but to a lesser extent it was demonstrated also in most other central and peripheral tissues [6]. Previous studies indicated that the active form of ghrelin is uniquely modified (octanoylated at Ser^3 residue) [7, 8] and its biological effects are transduced by ghrelin receptor type 1a (GHS-R1a) [3]. Recently, some biological functions have also been relegated to des-acyl ghrelin [9]. The role of GHS-R1b receptor has not been established yet [3]. The role of the second peptide, obestatin, is still controversial. Studies demonstrating its function (related *e.g.* to food intake or gastric emptying) conflict to those that reveal no biological activity of obestatin [10, 11].

Previous studies analyzing ghrelin and obestatin expression in thyroid gland tissue are not unanimous and are mostly related to ghrelin. Ghrelin has been demonstrated in parafollicular cells and medullary thyroid cancer cells [12–14]. Ghrelin binding sites have been shown in the thyroid [6] and exogenous ghrelin uptake in the thyroid gland was demonstrated to be notably higher than in other tissues [15].

The role of ghrelin and obestatin in the thyroid appears very interesting for their probable involvement in cell proliferation [16–18]. The relationship between thyroid cancer pathogenesis and ghrelin and obestatin expression has not been clearly defined. Furthermore, since the function of thyroid gland is associated with the maintenance of energy balance, the relationship between ghrelin, obestatin and thyroid function is worthy of consideration [19–24].

The aim of the study was to assess both ghrelin and obestatin immunocytochemical expression in both benign and malignant thyroid tumors (nodular goiter, papillary and medullary thyroid cancer) using quantitative morphometry. We assumed that ghrelin and obestatin expression may be altered in different types of thyroid tumors. Furthermore, the potential differences may reflect the influence of both peptides on the pathogenesis of nodular goiter and thyroid cancer.

Material and methods

Material. Tissue samples were collected from thyroids operated due to therapeutic indications. The study was approved by the local Ethics Committee and the informed consent was obtained from each patient.

Analyzed samples included 9 cases of nodular goiter (NG), 8 cases of papillary thyroid cancer (PTC) and 11 cases of medullary thyroid cancer (MTC). The clinical data of analyzed cases are presented in Table 1. In 4 patients with MTC complete result of TNM Classification of Malignant Tumors could not be obtained. In 5 patients with MTC and 3 patients with PTC, the precise information on the size of tumors could not be obtained.

Tissue processing and microscopy image analysis. We used the archival material that was fixed in buffered 10% formalin or Bouin's fluid and embedded in paraffin using the routine procedure. Paraffin sections 5 μm thick were placed on the SuperFrost/Plus microscope slides. Staining with hematoxylin and eosin (HE) was performed. Patterns of HE-stained histological preparations were examined using Olympus BH-2 light microscope (Olympus, Tokyo, Japan) by two histopathologists.

Immunocytochemical studies. The analysis of ghrelin and obestatin expression in thyroid gland was performed by use of the new polymer-based immunohistochemical (IHC) technique [25]. Rabbit polyclonal antibodies directed against human ghrelin (Phoenix Pharmaceuticals, Inc., catalog no. H-031-30, Burlingame, California, USA) were employed in dilution 1:1,500 and against human obestatin (Phoenix Pharmaceuticals, Inc., catalog no. H-031-92) in dilution 1:8,000. The sections were incubated with these primary antibodies overnight at 4°C, then incubated with dextran backbone (attached to peroxidase — HRP) and secondary biotinylated link anti-rabbit and anti-mouse IgG (Dako REAL™EnVision™ Detection System peroxidase/DAB+, Rabbit/Mouse, Dako, Glostrup, Denmark) was applied. After subsequent deparaffination and rehydration the preparations were boiled in

Table 1. The clinical characteristics of analyzed patients with different thyroid gland diseases

	Female/Male	Age	TNM scale	
	(No.)	(years, mean ± SD)	T1/T2/T3/T4	N0/N1
Nodular goiter	6/3	52.2 ± 12.2	–	–
Papillary thyroid cancer	8/0	48.9 ± 16.4	4/1/1/2	5/3
Medullary thyroid cancer	5/6	48.6 ± 16.7	1/3/1/2	4/4

10 mM sodium citrate buffer (pH = 6.0) in a 700 W microwave oven for 18 min (for sections to be stained with anti-ghrelin antibodies), washed in phosphate buffer saline (PBS) and then subjected to the reaction according to the standard procedure. Internal negative control reactions were based on substituting specific antibodies with a normal serum of a respective species in 0.05 M Tris-HCl, pH 7.6, supplemented with 0.1% bovine serum albumin (BSA) and 15 mM sodium azide (Sigma-Aldrich, St. Louis, MO, USA). Histological slides with ghrelin and obestatin expression were examined under an optical Olympus BH-2 microscope coupled to a digital camera. Color microscope images were recorded using 40 × objective (at least 10 fields in every microscope slide with an IHC positive reaction), using LUCIA Image 5.0 computer software (Nikon, Tokyo, Japan). The positive control staining was done on human stomach tissue.

Microscopy image analysis. Quantitative morphometric analysis of ghrelin and obestatin tissue expression was done by use of HSV Filter software, originally developed and described in Department of Bioinformatics and Computational Biology, Poznan University of Medical Sciences, Poland [26]. We measured the area of IHC reaction and then we calculated the percentage of the reaction in the relation to the area of the whole image (analyzed area of thyroid gland). Such evaluation was performed in 10 microscopic fields in each patient. Then we calculated the mean value in every patient and each studied group of patients (NG, PTC and MTC).

Statistical analysis. The statistical analysis was performed using the Statistica PL *vs.* 9 software (Statsoft, Inc., Tulsa, OK, United States) and appropriate tests. The Kruskal-Wallis test was used to compare the peptides' IHC expression. In case of significant differences, additional analysis was performed with a multiple comparison test (Dunn test). The Wilcoxon test was used for non-parametric dependent data. Correlations between data rows were determined with Spearman's rank correlation index. The results were accepted as significant at the threshold of $p \leq 0.05$.

The results of positive control staining in human stomach tissue were not analyzed statistically.

Results

Cellular localization of ghrelin and quantitative analysis of ghrelin immunoexpression

Ghrelin immunoreactivity in NG was observed in cytoplasm of single thyroid cells and single groups of cells (Figure 1A). In PTC cells ghrelin expression was observed also in cytoplasm (Figure 1B). The intensity of the reaction was irregular by means of unequal intensity of IHC reaction in papillary and follicular structures, as well as irregular expression in apical and basal poles of the cancer cells. The lumen of several follicular structures contained parts of cancer cells that were also ghrelin-positive. No reaction for ghrelin was observed in the stroma. MTC presented strong intensity of cytoplasmic expression of ghrelin (Figure 1C). The stroma of MTC was ghrelin-negative.

The comparative quantitative analysis (Table 2) showed that ghrelin immunoexpression was significantly higher in MTC than in NG ($p = 0.013$). No differences were demonstrated in ghrelin expression neither between MTC and PTC nor between NG and PTC.

The immunoexpression of ghrelin in MTC correlated negatively with the size of the tumor ($r = –0.829$, $p < 0.05$).

There was no correlation between ghrelin expression and PTC tumor size ($r = 0.4$, $p > 0.05$) (data not shown).

Cellular localization of obestatin and quantitative analysis of obestatin immunoexpression

Obestatin expression in all analyzed specimens was irregular and poorly accented. Cells presenting obestatin immunoreactivity (cytoplasmatic reaction) were accompanied by obestatin-negative regions. The strongest immunoreactivity for obestatin was demonstrated in NG (Figure 1E). The reaction was also observed in the colloid; however, it was not taken into statistical analysis.

In PTC, the immunohistochemical reaction for obestatin was also irregular and observed in some groups of cancer cells (Figure 1F). In MTC obestatin expression was barely noticeable (Figure 1G).

The results of quantitative analysis of obestatin immunoexpression in the studied tissues showed that it was significantly lower in MTC than in NG and PTC (Table 2).

No correlations were found between obestatin expression and MTC tumor size ($r = –0.49$, $p > 0.05$) nor between obestatin expression and PTC tumor size ($r = –0.1$, $p > 0.05$) (data not shown).

Ghrelin versus obestatin expression

In nodular goiter the immunoreactivity of obestatin was significantly higher than that of ghrelin. Conversely, in MTC ghrelin immunoexpression was definitely more evident than that of obestatin (Table 2). There was no statistically significant difference between ghrelin and obestatin immunoexpression in PTC.

Correlation between ghrelin and obestatin expression

Spearman's rank coefficients analysis for correlation between ghrelin and obestatin reciprocal immuno-

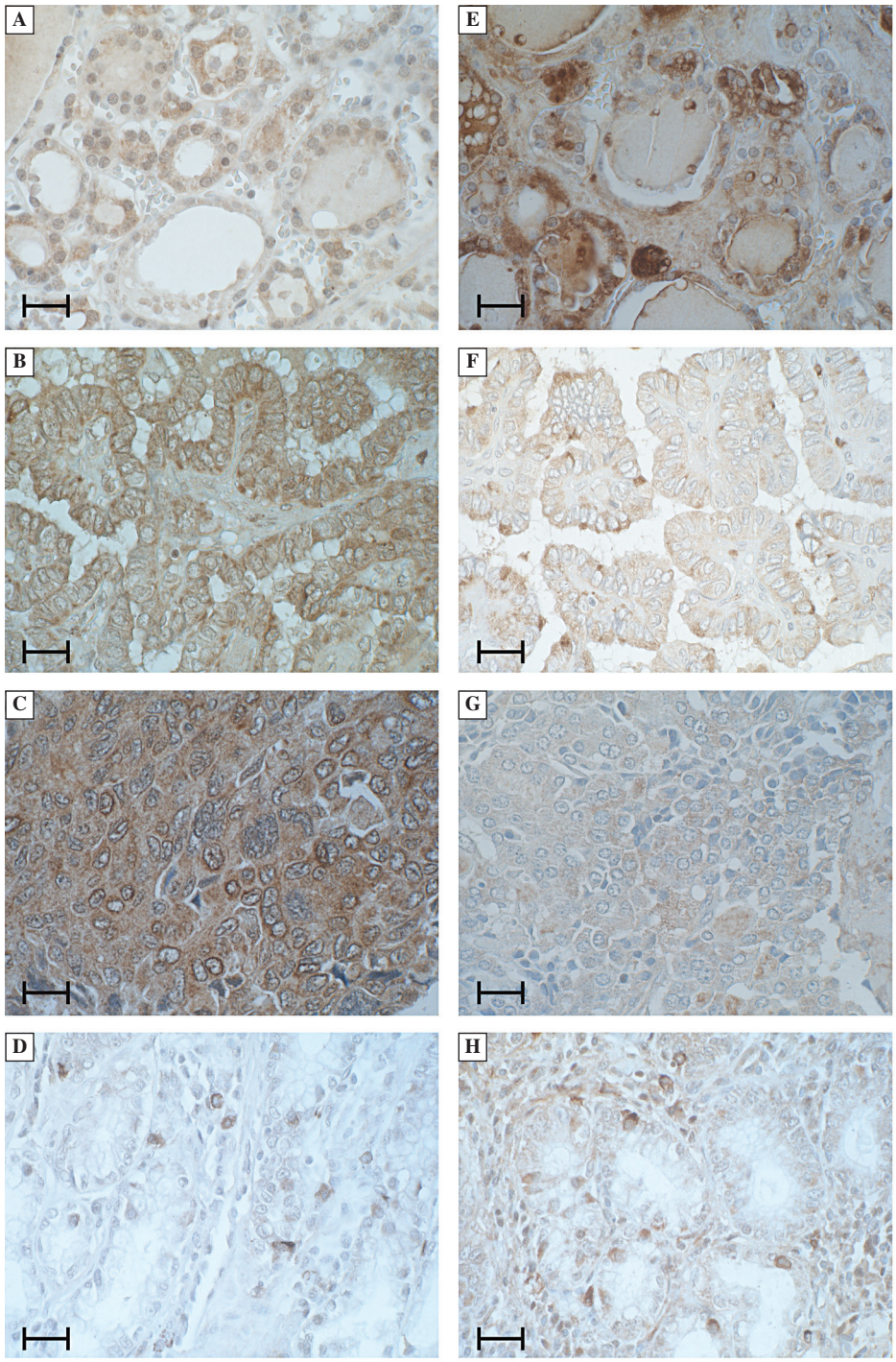

Figure 1. Immunohistochemical expression of ghrelin in nodular goiter (**A**), thyroid papillary cancer (**B**), and thyroid medullary cancer (**C**). Immunohistochemical expression of obestatin in nodular goiter (**E**), papillary cancer (**F**), and medullary cancer (**G**). Human stomach tissue was used as positive control for ghrelin (**D**) and obestatin (**H**) immunostaining. Sections were counterstained with hematoxylin. Bar, 40 μm

expression in patients with NG, PTC and MTC revealed that tissue expression of ghrelin and obestatin did not correlate in any of the analyzed groups (NG: $r = 0.209$, $p > 0.05$; PTC: $r = -0.238$, $p > 0.05$; MTC: $r = -0.009$, $p > 0.05$).

Cellular localization of ghrelin and obestatin in positive and negative control samples

In positive control samples from the human stomach there were many cells immunopositive for ghrelin and obestatin (Figure 1D and 1H).

Table 2. Comparison of quantitatively assessed ghrelin and obestatin immunoexpression in nodular goiter (NG), medullary (MTC) and papillary thyroid cancer (PTC)

		No.	Mean	Median	Min	Max	SD	Statistical significance
Ghrelin	**NG**	9	2.04	1.39	0.01	4.95	1.87	NG *vs.* MTC: $p = 0.013$
	MTC	11	15.74	16.29	0.86	43.50	11.94	NG *vs.* PTC — NS
	PTC	8	11.47	5.03	0.97	33.38	11.81	MTC *vs.* PTC — NS
Obestatin	**NG**	9	6.06*	4.36	0.91	13.24	4.51	NG *vs.* MTC: $p < 0.001$
	MTC	11	0.74**	0.10	0.00	3.27	1.20	NG *vs.* PTC — NS
	PTC	8	3.22	3.03	0.30	5.66	2.00	MTC *vs*. PTC: $p = 0.04$

Data show area fraction (%) of the IHC reaction in analyzed area of thyroid gland. Abbreviations: min and max — minimal and maximal values, respectively; NS — statistically non-significant. The Kruskal-Wallis test was used to compare the peptides' immunohistochemical expression. In case of significant differences, additional analysis was performed with a multiple comparison test (Dunn test) (see Material and methods). *significantly higher immunoexpression of obestatin than ghrelin in NG, $p < 0.05$; **significantly lower immunoexpression of obestatin than ghrelin in MTC, $p < 0.01$

No staining was observed in negative controls of NG, PTC and MTC (not shown).

Discussion

The first reports of ghrelin expression in the thyroid were related to parafollicular cells. In 2001 Kanamoto et al. suggested that MTC cells produce ghrelin [12]. The authors demonstrated by Northern blot analysis that TT cells (stabilized cell lines derived from MTC) express preproghrelin mRNA. Radioimmunological analysis revealed that ghrelin is present in the extracts from TT cells and immunoreactivity was shown in cell cytoplasm [12]. Ghrelin was even proposed as a new diagnostic marker of MTC [27]. However, a comparison of plasma ghrelin levels between patients with MTC and control subjects did not reveal significant difference [27]. Further studies showed that ghrelin is also present in parafollicular cells of a healthy thyroid [13]. In our study immunocytochemical expression of ghrelin in MTC was significantly higher than in benign NG. Nevertheless, its immunoreactivity was not significantly stronger than the IHC reaction observed in PTC.

Ghrelin expression in follicular cells is still controversial. Raghay et al. reported that follicular cells are ghrelin-negative [13]. The authors noticed that there was a strong ghrelin immunoreactivity in parafollicular cells, and GHS-R1a expression on follicular cells may indicate that ghrelin produced in parafollicular cells influences follicular cells in a paracrine manner [13]. Study by Karaoglu et al. demonstrated ghrelin expression in follicular cells of Hashimoto thyroiditis [28]. Furthermore, it was even considered that ghrelin may be present only in follicular cells of fetal thyroid glands with no expression in adult thyroid glands, as reported by Volante et al. [29]. In the same study ghrelin was re-expressed in follicular-derived thyroid tumors.

The differences between ghrelin expression in benign and malignant thyroid tumors have not been thoroughly characterized. Zhang et al. [30] revealed that ghrelin is expressed in thyroid cancer, but not in benign thyroid diseases. Volante et al. demonstrated ghrelin in papillary, follicular and medullary cancer [29]. On the other hand, Karaoglu et al. [28] described poor ghrelin expression in PTC in contrast to intense ghrelin immunoreactivity in non-cancerous thyroid tissues.

In this study ghrelin expression was noted in nodular goiter. However, the intensity of the reaction was low. In comparison to benign nodular goiter ghrelin immunoreactivity was more noticeable in PTC and MTC cells (with statistically significant differences in case of MTC). The discrepancies between ghrelin expression in healthy thyroid, benign thyroid diseases and thyroid cancer are worthy of consideration, especially in view of ghrelin influence on cell proliferation. The results of most of the previous studies show that ghrelin stimulates the proliferation of normal cell lines [18, 31, 32]. However, in the matter of cancer cell lines some authors described pro-proliferative properties of ghrelin and some demonstrated its anti-proliferative action [3, 18, 29]. Cassoni et al. showed that synthetic growth hormone secretagogues induce growth-inhibitory effects on cell lines derived from follicular thyroid cancers [33]. Similarly, Volante et al. demonstrated anti-proliferative effects of ghrelin in the cells lines of papillary and anaplastic thyroid cancer analyzed *in vitro* [29]. While inhibition of proliferative processes in anaplastic cancer cell lines required higher ghrelin concentrations than PTC, the authors suggested that ghrelin receptors in anaplastic cancer are either modified or their ability to bind ghrelin is disturbed.

Interestingly, several studies revealed that ghrelin expression in cancer cells is decreased in comparison to healthy tissue. This was observed in thyroid, stomach, esophagus, pancreas and renal cell carcinoma [28, 34–36]. Therefore, if pro-proliferative properties of ghrelin should be considered, it could be discussed if its absence or poor expression in cancer tissue provides protection from further growth.

The second peptide — obestatin was demonstrated in our study mainly in NG and PTC. The reaction for obestatin in MTC was poor. The expression of obestatin and its potential role in the thyroid gland still needs to be defined. In previous studies Volante et al. [37] demonstrated obestatin in medullary, papillary, follicular and poorly differentiated thyroid cancer. The authors observed obestatin immunoreactivity mostly in ghrelin-positive areas, which showed more intense and a diffuse pattern of staining. There was no obestatin expression in normal thyroid parenchyma. On the contrary, Karaoglu et al. demonstrated obestatin expression in healthy thyroid, Hashimoto thyroiditis and PTC without evident differences in the intensity of reaction [28].

In this study obestatin immunoreactivity was observed in benign NG, as well as cancer cells. However, the intensity of immunohistochemical expression was poor. Obestatin-positive cells were accompanied by regions without any immunoreactivity.

The differences between ghrelin and obestatin immunoreactivity in benign and malignant thyroid tumors could support the theory of alternative transcription of pre-proghrelin gene and thus of independent production of ghrelin and obestatin [38]. In this study there was no correlation between ghrelin and obestatin immunoreactivity in any of the analyzed groups. Moreover, a strong reaction for ghrelin was accompanied by poor obestatin expression in MTC and, on the other hand, obestatin immunoreactivity in NG was significantly higher than ghrelin expression.

The relationship between ghrelin, obestatin and thyroid dysfunction (hyperthyroidism, hypothyroidism) is also worth further studies. Recently, the new peptide of probable influence on body metabolism — irisin has been studied in different thyroid functional states [39]. Hopefully the association between thyroid hormones and their modulators will be soon identified.

Conclusions

Ghrelin is expressed in PTC and MTC cells and, to a lesser extent, in NG. The differences between ghrelin expression in NG and MTC suggest that ghrelin may be involved in thyroid cell proliferation. The immunohistochemical reaction for obestatin in NG and thyroid cancer cells is poor and irregular. In light of the common origin of ghrelin and obestatin, it is interesting that in MTC and NG the intensity of reaction for both peptides was different. The potential role of obestatin in thyroid gland needs further investigation.

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