The expression of ghrelin in somatotroph and other types of pituitary adenomas

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Abstract

OBJECTIVES: It has been suggested that ghrelin synthesized locally in pituitary regulates the function and growth of pituitary cells in autocrine/paracrine way and might be an important factor of pituitary tumorogenesis. The expression of ghrelin receptor in neoplastic cells of pituitary adenomas has also been demonstrated. *In vitro* studies confirmed that ghrelin stimulates the proliferation of somatotroph cells in GH_3 cell line. The presence of both ghrelin mRNA and protein was detected in a number of benign and malignant neoplasms as well as in neoplastic cells of the tissues which do not express ghrelin in physiological conditions. This study showed the presence of ghrelin mRNA and its protein in different types of pituitary adenomas.

DESIGN: The samples of 37 pituitary adenomas were obtained during standard neurosurgical tumor removal. The study tissues included 20 somatotroph tumors (15 patients treated and 5 patients untreated with octreotide LAR before the surgery), 12 nonfunctioning adenomas, 4 prolactinomas and 1 ACTH-secreting tumor. The control included samples of normal mucous membrane of the stomach and normal pituitaries. Expression of ghrelin mRNA was studied in 28 pituitary adenomas by RT-PCR. Immunohistochemical evaluation of ghrelin presence was performed in 34 tumors.

RESULTS: The presence of ghrelin gene transcripts was demonstrated in 10 out of 15 examined somatotroph tumors (obtained from patients treated with octreotide LAR before the surgery) and also in 2 out of 4 samples of prolactinomas, 7 out of 8 of nonfunctioning tumors and in 2 samples of normal pituitary. Immunohistochemical analyses revealed the presence of the protein in all 5 examined somatotroph tumors obtained from patients not treated prior to the surgery and in 10 out of 15 tumors obtained from patients treated with octreotide LAR. The

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peptide was detected also in 10 out of 12 examined nonfunctioning tumors and in 2 examined PRL-secreting tumors. The immunostaining for ghrelin was not shown in normal pituitaries.

CONCLUSIONS: The study demonstrated that ghrelin gene is expressed in somatotroph adenomas, both treated and untreated with octreotide LAR before the surgery, and also in other types of pituitary adenomas (prolactinomas and nonfunctioning adenomas).

Abbreviations:

GHSR – growth hormone secretagogue receptor
GHSR 1a – growth hormone secretagogue receptor transcript variant 1a
GH – growth hormone
PRL – prolactin
GHRH – growth hormone releasing hormone
GH_3 – rat pituitary tumor cell line
ACTH – adrenocorticotropic hormone
LAR – long-acting release
RT-PCR – reverse transcription polymerase chain reaction
RT – reverse transcription
PCR – polymerase chain reaction
bp – base pairs
DTT – dithiotreitol [(*2S,3S*)-1,4-Bis-sulfanylbutane-2,3-diol]
dNTPs – mixture of four deoxyrybonucleotides triphosphates
RNA – ribonucleic acid
cDNA – complementary to RNA DNA
DNA – deoxyribonucleic acid
RNasin – rnase inhibitor
GHRL – ghrelin gene
ACTB – beta actin gene (house keeping gene)
MOPS – 3-(N-morpholino)propanesulfonic acid
EDTA – 2-[2-(Bis(carboxymethyl)amino)ethyl-(carboxymethyl)amino]acetic acid (ethylenediaminetetraacetic acid)
TBS – tris buffered saline
TBS-T – tris buffered saline with tween 20
CRH – corticotrophin releasing hormone
TRH – thyrotropin-releasing hormone
LHRH – gonadotropin releasing hormone type 1 (GNRH)/ luteinizing hormone releasing hormone
PIT-1 – pituitary specific transcription factor

INTRODUCTION

Ghrelin is a 28-aminoacid hormone which was identified as an endogenous ligand for growth hormone secretagogue receptor (GHSR) [1, 2, 3, 4]. The first recognized function of ghrelin was a strong stimulation of growth hormone (GH) secretion from anterior pituitary [5, 6, 7]. It has been postulated that ghrelin, together with growth hormone releasing hormone (GHRH) and somatostatin, might be an integral element of hypothalamo-pituitary system which regulates GH release [8, 9].

Apart from being a natural GH secretagogue, ghrelin was also found to exert multiple functions, not only in endocrine system. They include influence on energy balance, stimulation of appetite, regulation of glucose and lipid metabolism, influence on exocrine and endocrine pancreatic function, gastric acid secretion and motility, the effects in cardiovascular system as well as modulation of cell proliferation [2, 10, 11, 12].

Ghrelin is produced predominantly by the stomach and it circulates in plasma in measurable concentrations [12, 13, 14]. However, its synthesis was also described in other organs and tissues, including: small intestine, kidneys, pancreas, placenta, lungs, adipose tissue, immune system and many others [14, 15, 16]. Ghrelin was also found to be produced locally in hypothalamus and anterior pituitary [16, 17]. The expression of ghrelin receptor (GHSR) was confirmed in the cells of anterior pituitary, predominantly in somatotroph cells [15, 18, 19, 20, 21]. Thus, it has been suggested that locally produced ghrelin might regulate the function and growth of pituitary cells in autocrine/paracrine manner. The role of ghrelin synthesized within the pituitary is still unclear. It is suggested, that ghrelin might be an important factor connected with pituitary tumorogenesis [18, 19].

It has been shown that ghrelin can modulate cells proliferation, and this activity was either stimulatory or inhibitory, depending on the cells type [2, 11, 22, 23, 24, 25, 26]. *In vitro* studies demonstrated that ghrelin stimulates the proliferation of somatotroph cells of GH_3 cell line [27]. In addition to this data, the presence of both mRNA and protein of ghrelin was detected in a number of benign and malignant neoplasms, including different neuroendocrine tumors, endocrine pancreatic tumors, breast tumors and thyroid carcinomas [28, 29, 30, 31, 32]. Ghrelin expression was described also in neoplastic cells of those tissues which do not express ghrelin in physiological conditions [11, 31].

It was demonstrated that neoplastic cells of pituitary adenomas also express ghrelin receptor [33, 34, 35].

The data mentioned above suggest that ghrelin, locally synthesized within pituitary adenomas, might be associated with pituitary tumorogenesis. It might be assumed that the presence of ghrelin within pituitary adenomas is responsible for the tumor growth, its local invasiveness, the tendency for regrowth and resistance to the treatment.

In this study we confirm ghrelin expression both on mRNA and protein level in different types of pituitary adenomas.

MATERIAL AND METHODS

Pituitary adenomas tissues were obtained from 37 patients during standard neurosurgical tumor removal. The type of the tumor was determined before the surgery on the basis of clinical examination and hormonal findings. The diagnosis was confirmed postoperatively during routine histopathological proof and histochemical evaluation. The pituitary tumors tissue samples included 20 somatotroph tumors, 12 nonfunctioning adenomas, 4 prolactinomas and 1 adrenocorticotropic hormone (ACTH)-secreting tumor. The samples of GH-secreting adenomas were obtained from 15 patients treated with long acting octreotide (octreotide

LAR) before the surgery and from 5 patients who did not receive presurgical pharmacotherapy. All 4 subjects with prolactin (PRL)-secreting tumor were treated with dopamine agonists before the tumor removal.

A total of 28 pituitary adenomas, including 15 somatotropinomas (all tumors obtained from patients pretreated with somatostatin analogue), 4 prolactinomas, 8 nonfunctioning tumors and 1 adrenocorticotropinoma, were studied for ghrelin mRNA expression

Immunohistochemical evaluation of ghrelin synthesis was performed in 15 somatotroph tumors pretreated with octreotide LAR and 5 somatotropinomas from subjects with acromegaly who did not receive presurgical pharmacotherapy (archive paraffin sections collected when somatostatin analogues were not used routinely) and also in 2 lactotroph and 12 nonfunctioning adenomas.

The control consisted of samples of normal mucous membrane of the stomach, collected at gastroscopy or during gastrectomy performed for other reasons. The second control group inlcuded three normal pituitaries collected at autopsy from patients with no endocrine abnormalities.

Tumor tissue samples collected during the surgery were directly placed either in RNA*Later* (for RT-PCR analysis) or in 4% buffered paraformaldehyde and then in paraffine (for immunohistochemical examination).

The study was approved by the ethics review board of University of Medical Sciences in Poznan, Poland (No 202/05). All patients gave their written consent to participate in the study.

The assessment of ghrelin mRNA expression (RT-PCR)
RNA extraction and cDNA synthesis (reverse transcription)

Total RNA was isolated from the tissue with TriPure Isolation Reagent (Roche Diagnostic) according to manufacturer protocol. The quality of isolated RNA was analyzed by examining ribosomal RNA bands after agarose gel electrophoresis of 1μg of the probe in 1,2% agarose gel in 1xFA buffer (20 mmol/l MOPS (Sigma-Aldrich), 5 mmol/l sodium acetate (Sigma-Aldrich), 1 mmol/l EDTA (Fluka), pH 7.0) containg 1.2 ml of 37 % formalin (POCh) pro 100 ml of buffer, and 200ng/ml ethidium bromide (Fluka). Electrophoresis was performed in the same buffer in which agarose was diluted for 30 minutes at 80V. The quality and the amount of RNA was also measured using NanoDrop™ 1000 UV/Vis Spectrophotometer (Thermo Scientific) at A260 nm wavelength.

2 μg of total RNA (DNase treated) was used for ghrelin cDNA synthesis. Mixture of RNA, universal oligo(d)T_{10} primer and RNase-free water was placed in PTC-200 thermocycler (MJ Research) at 65°C for 10 minutes in order to denature RNA secondary structure. Then the mixture was placed on ice to let the primer anneal to RNA template. Furthermore, other components were added to the reaction mixture including: 500 mmol/l dNTPs, 10 nmol/l DTT, 20 U RNasin, 5 x reverse transcriptase buffer and 50 U of Transcriptor Reverse Transcriptase (Roche Diagnostic).

mRNA was reversely transcribed in thermocycler at 42°C for 60 minutes. cDNA was placed on ice or stored at the temperature of -20°C until PCR reaction was performed.

Universal primer for β-actin (*ACTB)* and ghrelin (*GHRL*) cDNA synthesis is commercially available oligo(d)T_{10} (Roche Diagnostics).

PCR

cDNA was used as a template for PCR amplification using gene specific primers for ghrelin. 1 μl of cDNA was used for 25 μl PCR reaction. The PCR amplification was performed in a reaction mixture containing: 1 × Taq DNA polymerase buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.25 μM of each, sequence specific primer and 1 U Taq DNA polymerase (Bioline, London, UK) with a thermal profile as follows: 5 min at 95°C, 1 min at 95°C, 45 sec at the temperature specific for the primers' set, 45 sec at 72°C for 30 cycles, followed by final 7 min elongation.

Sequence specific primers for ghrelin *(GHRL)* were designed to amplify a 200 bp of ghrelin (GenBank AC No: NM_016362).

The presence of the 509 bp fragment corresponding to β-actin gene nucleotides 434-942 [GenBank AC No: X00351] in the tissues lacking ghrelin served as a control and confirmed the integrity of isolated RNA

The sequence of primers and nucleotides complementary in mRNA sequence are shown in Table 1. All primers used in reactions were synthesized in TIBMolBiol, Poznan, Poland.

Thermal profile and number of cycles of ghrelin gene amplification are shown in Table 2.

Analysis of PCR products

The products of PCR amplification were analyzed by electrophoresis of 12.5μg of PCR reaction mixture in 1% agarose (BMA) gel diluted in 1xTBE buffer (89mmol/l TRIS (Sigma-Aldrich), 89mmol/l boric acid (Fluka), 2mmol/l EDTA, pH 8,0) stained with ethidium bromide (final concentration 200ng/ml; Fluka). Electrophoresis was performed in the same buffer in which agarose was diluted for about 45 minutes at 80V. As a mass marker the 100bp or 200bp HypperLadder was used (HyperLadder, Bioline).

DNA bands were detected using transluminator Herolab UVT-20LE and Scion Image, version 4.02 image analysis system.

Immunohistochemistry

5 μsections of analyzed tissue fixed in 4% buffered paraformaldehyde, embedded in paraffin were used for immunohistochemical study. The tissues were deparafinized and rehydrated. After washing with TBS (100 mmol/l Tris, 65 mmol/l NaCl (POCh); pH 7,5)

Table 1. The primers and complementary nucleotides in mRNA sequence for *GHRL* i *BACT* synthesis

primer	PCR product lenght	primers sequences	complementary nucleotides
GHRL sense	200 bp	5′ – GGTTCAGTACCAGCAGCACA – 3′	303-312
GHRL antisense		5′ – TGTTCGAGTCCTCCGCTTAT – 3′	484-503
ACTB sense	509 bp	5′ – CATGTACGTTGCTATCCAGGCTG – 3′	434-454
ACTB antisense		5′ – CAGACAGCACTGTGTTGGCG – 3′	924-942

Table 2. The stages and thermal profile of PCR reaction.

lp.	step	temperature	time
1	initial denaturation	95°C	5 min.
2.	denaturation	95°C	1 min.
3.	primers annealing	54°C	45 sec.
4.	elongation	72°C	45 sec.
5.	final reaction of elongation	72°C	7 min.

The steps 2. to 4. were repeated 35 times.

commercial reagent from DakoCytomation (EnVision™ Detection Systems Peroxidase/DAB, Rabbit/Mouse) was used for blocking the activity of endogenous peroxidase. The procedure included 5 minutes incubation and two washings in TBST, 5 min each. Antigens were retrieved by microwave activation (2×10minutes, 250W) in citrate buffer (13mmol/l citrate acid (POCh), 37mmol/l trisodium citrate (POCh), 15mmol/l NaCl, pH 6,0) and cooled down. Endogenous peroxidase activity was stopped with blocking reagent for endogenous peroxidase provided with the kits. After being blocked in blocking buffer – TBS (pH 7.5, containing 100 mM TRIS-HCl, 0.9% NaCl, 0.05% Tween-20 (TBS-T) and 3% BSA (Sigma-Aldrich)) overnight in 4°C, the sections were incubated with primary rabbit polyclonal antibodies against human ghrelin, diluted 1:50 in blocking solution 30 minutes in a room temperature. After two washings in TBS-T, 5 min each, the incubation was continued with the EnVision™ reagent, a peroxidase-conjugated polymer backbone, carrying secondary antibody molecules directed against rabbit and mouse immunoglobulins for 30 minutes. Subsequent steps were performed according to the manufacturer's manual for the DakoCytoamtion kit. Final staining step included application of diaminobenzidine (DAB) substrate chromogen solution for horseradish peroxidase.

Controls included detection reactions carried out under identical conditions as the experiment except that the primary antibodies were replaced by nonimmune serum.

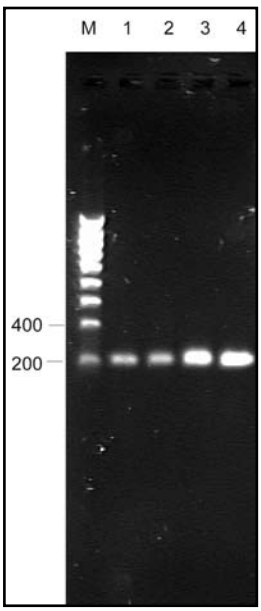

Figure 1. Electrophoretic separation showing representative RT-PCR results performed for ghrelin gene amplification in somatotroph tumors. A 200 bp fragment of *GHRL* was amplified for the samples (lanes 1 – 4, respectively patients: JS, KC, TM, DJ). Molecular size marker is given in lane M.

RESULTS

The evaluation of active ghrelin gene by RT-PCR

To verify at the molecular level the presence of ghrelin in pituitary tumors total RNA was isolated from adenoma tissue, reverse transcribed, and a 200 bp fragment was amplified. The presence of ghrelin gene transcripts was confirmed in 10 out of 15 examined somatotroph tumors (obtained from patients treated with octreotide LAR before the surgery) (Fig.1). Ghrelin mRNA was also detected in 2 out of 4 samples of prolactin-secreting tumors, as well as in 7 out of 8 samples of nonfunctioning tumors and in 2 samples of normal pituitary (Fig.2).

In the remaining tissues (ACTH-secreting tumor, a nonfunctioning tumor and 2 prolactin-secreting adenomas) the presence of ghrelin active gene was not detected (Fig.3, lines 3-4). Amplification of a control β-actin gene demonstrated the presence of integral RNA isolated from those tumors (Fig.3, lines 1-2). Thus, the amplification of house keeping gene demonstrated that examined tumors do not expressed ghrelin

Immunohistochemical evaluation of ghrelin gene expression

The fragments of mucous membrane of the stomach were used as a positive control of immunohistochemical study. Very strong positive immunostaining of ghrelin in the cytoplasm of the cells of human stomach tissue was detected (Fig.4).

Immunohistochemical analysis of tumor tissue revealed that ghrelin is synthesized in neoplasticaly transformed cells. The positive staining was showed in the cytoplasm of cells of all 5 examined somatotroph tumors obtained from patients not treated with a somatostatin analogue before the surgery. The presence of immunoreactive protein was also revealed in 10 out of 15 tumors obtained from patients pretreated with octreotide LAR (Fig.5)

Ghrelin was also present in the cells of 10 out of 12 examined non-functioning tumors (Fig.6) and in 2 examined PRL-secreting tumors.

Positive immunostaining was observed in the cytoplasm of cells, in majority of them predominantly in perinuclear area (Fig.6)

The immunostaining of ghrelin was not shown in normal pituitaries (Fig.7)

No labeling was also observed in the control reactions in which the primary antibodies were omitted (Fig.8).

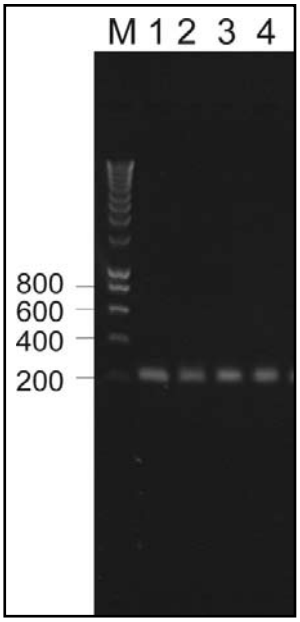

Figure 2. A representative example of electrophoretic analysis of RT-PCR reaction products for the ghrelin gene amplification in pituitary tumors and normal pituitary. Products of 200 bp representing the fragment of an active ghrelin gene in: PRL-secreting tumors (lines 1&2, patients AT and MK respectively), nonfuctioning pituitary tumors (lines 3&4, patients SK, MS respectively); and in normal pituitary (line 5). Molecular size marker is given in lane M.

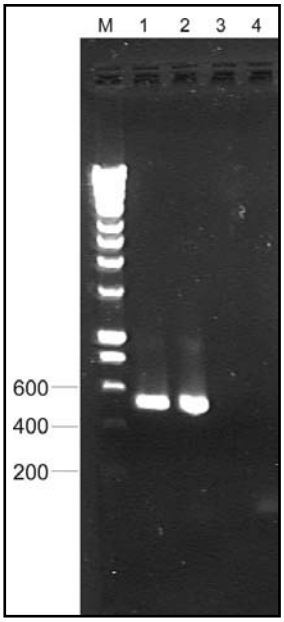

Figure 3. A representative example of electrophoretic analysis of RT-PCR reaction products of 509 bp beta actin gene amplification (lines 1&2, patients: MD – prolaktinoma and BA – adrenokortykotropinoma). In those tumors the lack of ghrelin gene expression was confirmed (lines 3&4). M – molecular size marker.

DISCUSSION

This study was performed to assess the expression of ghrelin gene in somatotroph and other types of pituitary tumors as well as in normal pituitary. The majority of somatotroph adenomas analyzed during this study were obtained from subjects with acromegaly who were treated with a long-acting somatostatin analogue (octreotide LAR) before the surgery. In those adenomas both the presence of ghrelin active gene (by RT-PCR method) and and its protein (by immunohistochemistry) were determined. Ghrelin immunoreactivity in samples of adenomas which were resected without previous pharmacotherapy was also analyzed. These samples were collected before somatostatin analogues were used as a routine standard preparation of acromegalic patients for neurosurgical removal of the tumor.

The results of the study showed the expression of ghrelin in somatotroph tumors. Both ghrelin mRNA and protein were present in the majority (10 ot of 15) of examined (previously treated) somatotroph adenomas. A positive ghrelin immunostaining was detected in all examined by us somatotropinomas resected without presurgical pharmacotherapy.

Since ghrelin gene expression was found to be present in somatotroph adenomas, it might be postulated that ghrelin may be one of the factors involved in pituitary neoplastic transformation.

Somatotroph cells are the main target cells for ghrelin in pituitary, strongly stimulated by ghrelin to secrete GH [5, 6, 20]. It has been postulated that locally synthesized ghrelin in hypothalamo-pituitary area is a factor influencing pituitary function (in terms of GH secretion) in an autocrine and paracrine manner [7, 8, 9, 20, 21, 36]. The presence of ghrelin in the pituitary seems to be essential for the optimal secretory response of somatotroph cells to GHRH stimulation [21]. It might also be hypothesized that ghrelin is an important factor of the complex pathogenesis of pituitary adenomas which stimulates, in an auto- and paracrine way, the growth and proliferation of somatotroph cells [37].

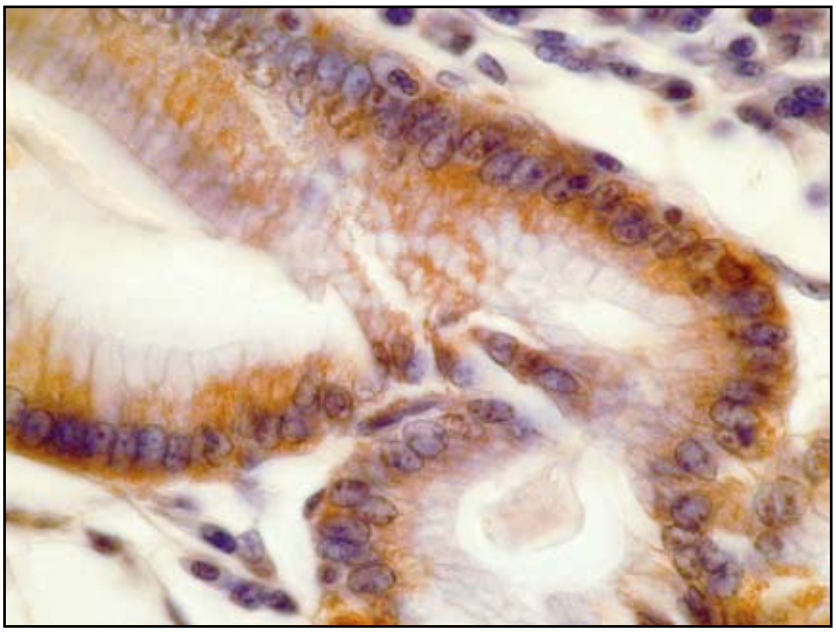

Figure 4. Positive control for immunohistochemical reactions - a strong positive ghrelin immunostaining in the cytoplasm of cells of mucous membrane of the stomach.

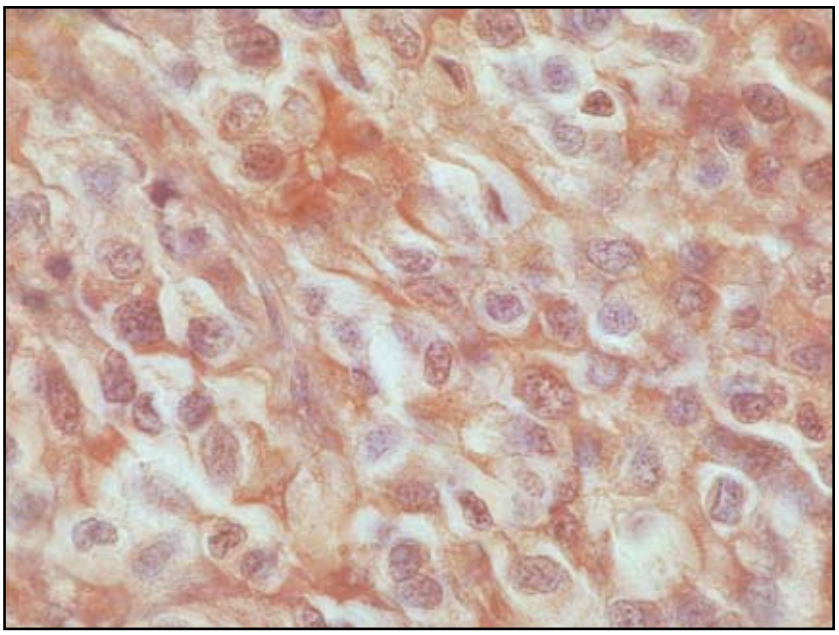

Figure 5. Positive immunohistocemical staining for ghrelin in the sample of somatotroph adenoma (patient treated with somatostatin analogue before the surgery).

As an analogy, it has been demonstrated that other neuropeptides (GHRH, CRH, TRH, LHRH, somatostatin) that regulate pituitary function are also synthesized in different types of pituitary adenomas [38, 39, 40, 41]. GHRH was shown to be produced locally in somatotroph adenomas [38, 42, 43, 44, 45]. A positive correlation between the level of GHRH expression and the size of the adenoma as well as the serum concentration of GH was demonstrated [38]. Overexpression of GHRH gene was shown mostly in large tumors, with aggressive growth and bad clinical prognosis [38]. It has also been demonstrated that the production of somatostatin is significantly lower in cases of big invasive somatotroph adenomas compared with normal pituitary [42, 43, 46].

Our study confirmed that ghrelin is expressed in somatotroph adenomas. Since ghrelin was shown to stimulate the proliferation of somatotroph cells in studies *in vitro* [30], it might be postulated that ghrelin plays a role in the development of somatotroph adenomas. The overexpression of ghrelin receptor (GHS-R1a) in this type of pituitary tumors was also shown, compared with normal pituitary and other types of pituitary tumors [18, 22, 23, 47, 48]. Korbonits and co-workers demonstrated that somatotroph adenomas showed the highest level of GHS-R1a expression, which was 2-10 times higher compared with normal pituitary and other types of adenomas [23]. Similar results were obtained by Kim and co-workers [22]. Skinner and co-workers demonstrated that GH-producing adenomas express GHS-R mRNA at levels 200 times higher than the normal pituitary [48]. This findings together with the evidence of ghrelin expression suggest that the interaction between the ligand and receptor, co-expressed in

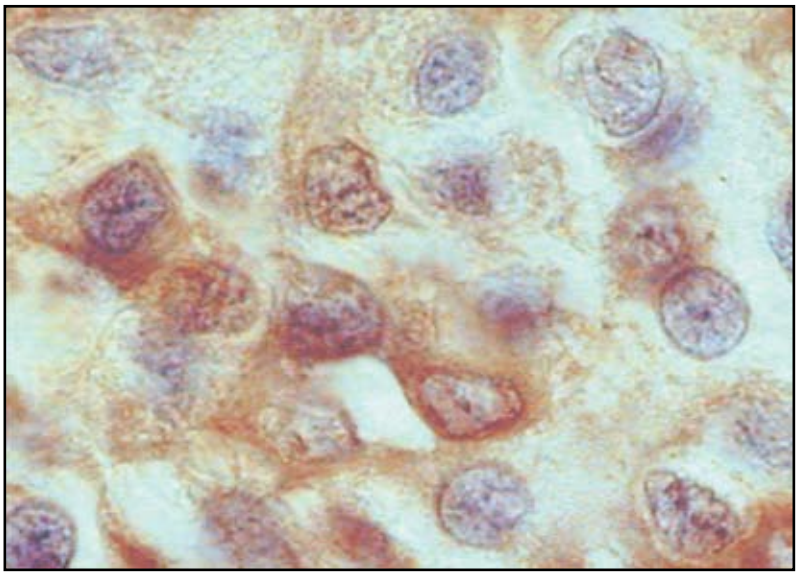

Figure 6. Positive immunostaining in perinuclear area of the cytoplasms of cells (non-functioning adenoma).

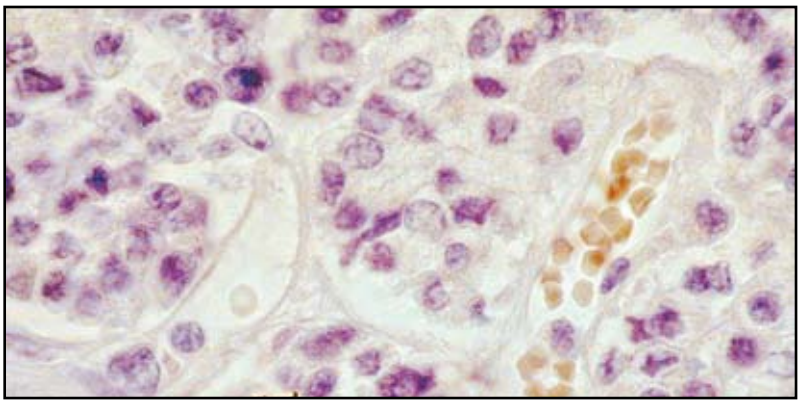

Figure 7. Negative immunohistochemical reaction for ghrelin in cells of normal pituitary.

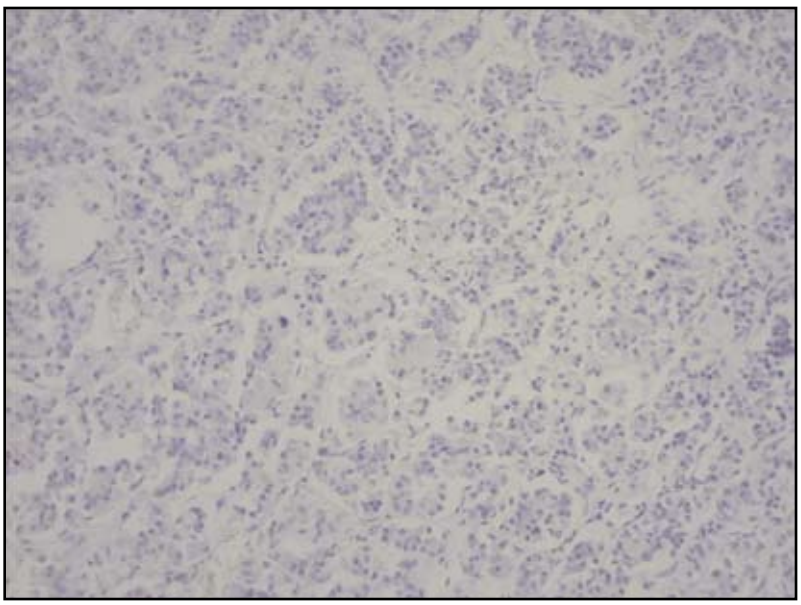

Figure 8. Negative control for immunohistochemical reactions – incubation without primary antibodies.

the same tissue, may be involved in the pathogenesis of somatotroph adenomas.

It was already demonstrated that many neoplastic cells of endocrine and non-endocrine organs express ghrelin [11, 18, 31, 34, 49, 50, 51, 52]. Furthermore, they were also shown to express ghrelin receptor GHS-R1a and binding sites for unacylated ghrelin. Co-expression of ghrelin and its receptor in different, other than pituitary, neoplasms propose the role of ghrelin in the growth of different tumors.

The pathogenesis of pituitary adenomas is still not fully recognized [53, 54]. A lot of information regarding the role of intra-pituitary mutations, transcription as well as growth and hormonal factors has been collected [55]. It has been demonstrated that the majority of pituitary tumors are monoclonal neoplasms that develop from a single mutant cell [54, 55]. It has also been proven that many growth factors synthesized in hypothalamus and within the pituitary are connected with the development of pituitary adenomas [56, 57]. It is possible that ghrelin is also one of growth factors that stimulate tumor growth and progression. Nanzer and co-workers demonstrated that ghrelin significantly increased the proliferation of pituitary somatotroph cells in GH_3 cell line [30]. Ghrelin was also shown to inhibit apoptosis [58, 59] and stimulate angiogenesis [60] in different cell lines, and these processes are known to be integral elements of tumor development and growth. It might be hypothesized that there is a relationship between ghrelin gene expression within different organs and the proliferation of cells as well as the rate of tumor development and progression.

The results of our study demonstrated that ghrelin mRNA and its protein were not present in all examined somatotroph adenomas. Such result might indicate that ghrelin gene expression is not typical for this type of adenomas. It should also be considered that in those tumors in which we didn't detect ghrelin, ghrelin gene was also expressed, however, the level of expression was much lower and the sensitivity of methods used in the study was too low to detect products of this expression. As known, RT-PCR and immunohistochemistry are qualitative methods of gene expression evaluation and few copies of gene expression products might have not been detected. Another reason might

be the influence of pharmacological treatment with a somatostatin analogue that was administered before the tumor removal. Somatostatin and its analogues exert mostly inhibitory effects [61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71]. They were also shown to influence the expression of genes [61, 72, 73]. It might be hypothesized that they inhibit the expression of ghrelin gene in the pituitary. In our study in 5 out of 15 GH-secreting adenomas, pretreated with a somatostatin analogue, ghrelin mRNA and peptide were not detected. The use of quantitative methods might reveal a lower level of ghrelin gene expression in adenomas previously treated, compared with tumors not treated before the surgery.

The hypothesis that somatostatin and its analogues influence ghrelin gene expression is more probable if one considers the fact that GHRH - hormone antagonizing somatostatin actions – increases ghrelin gene expression in the pituitary [21]. Kamegai *et al*. demonstrated that infusions of GHRH and stimulation of GHRH synthesis in hypothalamus increased pituitary levels of ghrelin mRNA [21]. Similarly, pituitary ghrelin gene expression was decreased when hypothalamic GHRH synthesis was decreased. Analogically, pituitary ghrelin gene expression might also be somatostatin-dependent and it would be inhibited when somatostatin analogues were used in the presurgical pharmacotherapy. Thus, the inhibition of GH secretion, cell proliferation and adenoma growth would result from local changes of ghrelin content in adenoma cells after somatostatin analogues administration. Qualitative methods should be used to compare the level of ghrelin gene expression in adenomas treated and untreated before the surgery.

In our study we also demonstrated ghrelin gene expression in other types of pituitary adenomas. Ghrelin mRNA was present in 7 out of 8 examined non-functioning adenomas and 2 out of 4 examined PRL-secreting tumors. Positive ghrelin immunostaining was detected in 10 out of 12 tumors with no secretory activity and 2 examined prolactinomas. In the fragment of ACTH-secreting tumor ghrelin mRNA was not detected.

The result of our study confirms that ghrelin gene expression is not specific only for somatotroph adenomas. Other types of pituitary tumors also express ghrelin. However, the number of examined PRL- and ACTH-secreting tumors was too small to draw any conclusions as for the presence or the lack of ghrelin gene expression in these types of adenomas. The results of our study demonstrating the presence of ghrelin mRNA in different types of pituitary tumors are in agreement with the data from the literature [18, 19, 22]. Korbonits *et al*. demonstrated that different types of pituitary adenomas express ghrelin [18, 19]. They showed that the level of ghrelin mRNA expression in somatotroph adenomas was higher compared with normal pituitary but lower then in non-functioning pituitary adenomas. Corticotroph adenomas presented with significantly low level of ghrelin mRNA expression and one ACTH-producing tumor presented with no ghrelin mRNA at all [18]. Similar results were obtained by Kim and co-workers [22]. The immunohistochemical evaluation of ghrelin gene expression was performed only by Korbonits *et al* [18]. The results of our study demonstrating the positive immunostaining in somatotroph, lactotroph and non-functioning tumors are in agreement with data presented by them.

In our study we also demonstrated the presence of ghrelin mRNA in normal pituitaries. However, immunohistochemistry demonstrated negative staining for ghrelin. Presumably in normal pituitary the messenger RNA is not translated into peptide. It would be an interesting hypothesis to explain and might confirm the role of ghrelin in the development of pituitary adenomas.

The results of our study confirming the presence of ghrelin in different types of pituitary adenomas indicate that ghrelin might regulate the function and proliferative activity of neoplastic cells. Ghrelin was shown to influence the expression of different genes, which products are responsible for stimulation of cell proliferation. Garcia *et al*. demonstrated that ghrelin regulates *Pit-1* gene transcription [74]. Pit-1 is a transcription factor responsible for differentiation of anterior pituitary cells and defining their secretory phenotype [41]. It was found that Pit-1 is also involved in somatotroph cell proliferation [74] and one of the factors that contributes to pituitary tumorigenesis [41, 75, 76]. Ghrelin was shown to stimulate the transcription of *Pit-1* gene in the pituitary. Furthermore, Caminos *et al*. confirmed the presence of ghrelin in those cells of anterior pituitary which differentiation is Pit-1-dependent (somatotrophs, lactotrophs and thyreotrophs) [20]. These observations might confirm the role of ghrelin in pathogenesis of pituitary adenomas. Increased expression of ghrelin gene would result in the increased expression of *Pit-1* gene and increased cell proliferation, and in cases of GH-secreting tumors – increased synthesis and secretion of GH.

CONCLUDING REMARKS

We demonstrated the presence of ghrelin mRNA and peptide in the majority of examined somatotroph adenomas. Ghrelin gene expression was also shown in lactotroph – and non-functioning pituitary tumors. It might be suspected that ghrelin synthesized in pituitary tumors might play a role in their pathogenesis and be a local factor influencing the tumour growth, the size of the tumor, the tendency for regrowth after the surgery and the resistance to aplied treatment. Further studies should be undertaken to determine whether ghrelin contributes to pituitary tumorigenesis and can be used as a marker in diagnosis and treatment of pituitary adenomas.

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