Expression of ghrelin and ghrelin functional receptor in human pituitary adenomas

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INTRODUCTION

Pituitary adenomas are heterogenous lesions commonly observed in the central nervous system. Signal transduction of ghrelin, an endogenous ligand specific for growth hormone secretagogue receptor (GHSR), has been reported to be involved in the development of endocrine tumors. However, there are limited data concerning the role of ghrelin and its functional receptor in pituitary adenomas.

OBJECTIVES

The aim of the study was to establish the expression pattern of GHRL and its functional receptor GHSR1a in human pituitary adenomas.

PATIENTS AND METHODS

Tissue specimens, including somatotropinomas (n = 20), prolactinomas (n = 5), and nonfunctioning adenomas (n = 52) were obtained from 77 patients. Thirteen normal pituitaries served as controls. The expression pattern of GHRL and GHSR1a mRNAs was established using reverse transcription followed by quantitative polymerase chain reaction.

RESULTS

Ghrelin mRNA was detected in 92.2% of the samples including controls, while GHSR1a transcripts were detected in 54.4% of the cases. Significant differences were found among subgroups in the GHSR1a expression (P < 0.0001) but not in that of GHRL (P = 0.7). The relative GHSR1a expression level was significantly lower for nonfunctioning tumors than for the control group or somatotropinomas. Controls revealed a strong positive correlation between the expression of both genes (r = 0.8; P < 0.0001), unlike adenomas, which showed a weak negative correlation (r = –0.3; P > 0.05). The maximum tumor diameter for nonfunctioning adenomas was higher than that for somatotropinomas (mean [SD], 31.4 [76] mm vs 24.8 [10.9] mm; P = 0.01). Neither the GHRL nor GHSR1a expression showed a significant correlation with tumor size in the subgroups.

CONCLUSIONS

The presence of GHRL and GHSR1a in the neural system indicates their effect on pituitary function regulation and suggests their possible role in adenoma pathogenesis.
The biological function of ghrelin is mediated by interaction with G-protein coupled family GHSR, highly expressed in neuroendocrine tissues. There are 2 isoforms of GHSR, type 1a (GHSR1a) and type 1b (GHSR1b), as a result of alternative splicing of a single gene located on chromosome 3q26.2.2,11,12 Ghrelin binds to GHSR1a, while GHSR1b seems to be biologically inactive itself; however, it may interact with other proteins and modulate their functions.3,13 The signal transduction disruption in the case of GHSR1b may result from its structural alteration comprising the lack of 6 and 7 transmembrane domains.11

Stimulation of GH secretion from the anterior pituitary was the first known function of ghrelin, demonstrated in both in vitro cultured rat pituitary tumor cell line, and in vivo after intravenous administration of ghrelin, in both rats and humans. The activity of ghrelin administered exogenously was dose dependent. It was confirmed that ghrelin stimulates the release of GH and demonstrates even stronger effect than GH-releasing hormone (GHRH).14,15 It is suggested that GHRL, present in the hypothalamus–pituitary axis, may be synthesized in the anterior pituitary cells. Thereby, it could be one of the factors involved in tumor formation and development of clinical acromegaly symptoms, and therefore it could determine the amount of GH released by the tumor. What is more, GHSR1a was identified within the cells of somatotropic adenomas, suggesting that ghrelin might be the factor involved in the above pathological processes mediated by the modulated GH level.16 As a consequence, it cannot be excluded that ghrelin may be one of the factors stimulating tumor-cell growth.

The expression of the GHSR mRNAs has been identified in different types of pituitary tumors, including the majority of somatotropic tumors, but the results are divergent.7,8,11,12,18 Furthermore, the role of ghrelin in pituitary tumor development has not been explained so far and previous studies have provided conflicting results. Thus, to investigate the role of GHRL and GHSR1a in pituitary tumor development, we examined the presence of ghrelin and GHSR mRNA in a full spectrum of human pituitary adenoma subtypes and in normal pituitary tissue.

The aim of the study was to establish the expression pattern of GHRL and its functional receptor GHSR1a in subtypes of human pituitary adenoma in relation to the suggested role of ghrelin and GHSR in pituitary lesions. Therefore, we assessed whether the relative levels of GHRL and GHSR1a mRNA differ between the various types of human pituitary adenomas. We also evaluated whether there was a correlation between the expression levels of ghrelin and GHSR mRNA and the size of pituitary adenomas.

PATIENTS AND METHODS Patients Human pituitary specimens were obtained during transsphenoidal surgery from 77 patients (41 women and 36 men). The tumor size ranged between 10 mm and 55 mm at the largest diameter. Twenty patients were diagnosed with acromegaly (somatotroph tumors), 5 patients had prolactinoma, and 52 tumors showed no hormonal activity. Patients with acromegaly were treated with somatostatin analogues before the neurosurgical procedure, in accordance with current standards. Patients with prolactinoma were referred for surgical treatment because of the compression of the optic nerve or intolerance to treatment with dopamine agonists. Patients with nonfunctional tumors underwent surgery owing to occurrence of a tumor mass. The maximum tumor diameter was measured on the basis of preoperative magnetic resonance imaging. Thirteen sphenoid bones containing pituitary glands included in the study were obtained at autopsy (6–24 hours postmortem) from patients with no history or evidence of pathological endocrine processes in the pituitary. All tissue specimens were immediately immersed in RNA-protective medium RNA Later (Sigma-Aldrich, Saint Louis, Missouri, United States) and stored in ultra-low freezer at a temperature of –80°C until nucleic acid isolation.

Written informed consent was obtained from all patients, and the study was approved by a local institutional review board.

Methods RNA isolation Total cellular RNA was isolated by liquid-to-liquid guanidinium thiocyanate–phenol–chloroform phase separation method with TRI Reagent (Sigma-Aldrich), according to the manufacturer’s protocol. The whole procedure was conducted twice. The quantity and purity of total RNA was determined with spectrophotometry (NanoDrop ND-1000, Thermo Fisher Scientific, Waltham, Massachusetts, United States). The absorbance at 230, 260, and 280 nm was measured, and the ratios 230/280 and 260/280 were calculated. Standard denaturing agarose gel electrophoresis (1.2%; LabEmpire, Rzeszów, Poland) was used to check the integrity (confirmed throughout visible 18S and 28S ribosomal RNA bands).

Reverse transcription Complementary DNA (cDNA) was synthesized in 4 thermal reaction steps in a PTC-100 MJ Research thermal cycler (Bio-Rad, Hercules, California, United States) according to the manufacturer’s protocol, using 50 ng/µl of total RNA, 5 pm/µl of universal oligo(d)T18 primer, 10 U/µl of transcriptor reverse transcriptase, 1× reverse transcriptase buffer, 10 U/µl of RNase inhibitor, and 1 pm/µl of each deoxinucleotide triphosphates (Roche Diagnostics, Basel, Switzerland). In the first step, the RNA, water, and the primer were incubated for 10 minutes at 65°C to ensure secondary structure denaturation, followed by 5-minute cooling on ice. After addition of the remaining reaction’s components, the incubation proceeded for 10 minutes at 25°C (short primer’s annealing step), followed by cDNA synthesis at 55°C for
Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) with sequence-specific primers (Genomed S.A., Warsaw, Poland) and TaqMan® hydrolysis probe (Roche Diagnostics) were used to assess the expression level of genes of interest (GOI): ghrelin (GHRL) (NCBI: AB029434.1 referring to mRNA transcripts' variants 1-4 and 8-12: NM_016362.4, NM_001134941.2, NM_001134944.1, NM_001134945.1, NM_001302821.1, NM_001302822.1, NM_001302823.1, NM_001302824.1, NM_001302825.1, respectively), its receptor (GHSR), transcript variant 1a, mRNA [NCBI: NM_198407.2], and reference HPRT1 gene encoding the hypoxanthine phosphoribosyltransferase 1 (Human HPRT Gene Assay cat. N°05046157001; Roche Diagnostics). TaqMan® hydrolysis probe #36 (Roche Diagnostics, cat. N°04687949001) for GOIs and reference assay were purchased from the collection of Universal Probe Library (Roche Diagnostics). The GHRL sense and antisense primers' sequences were as follows: 5’-gaagaagccaccaaggaag-3’ and 5’-gcttgattccaacctaaagg-3’ (amplicon 134bp; covering AB029434.1 template position 156–189 nt), and for amplification of GHSR1a: 5’-accaacagcaacgctgga-3’ and 5’-aatatgccctagttgag-3’ (amplicon 85bp; flanking NM_198407.2 template position 813–897 nt). The primers were designed using in silico analysis (UPL assay design center; http://lifescience.roche.com; Roche Diagnostics), and primer-to-probe position adjustment was subsequently verified by blast software (NCBI; https://blast.ncbi.nlm.nih.gov/). Primers were synthesized by Genomed S.A.

qPCR reactions were performed in a total volume of 20-µl LightCycler® TaqMan® Master (Roche Diagnostics) according to the manufacturer’s protocol, with the use of an LC® 2.0 glass Capillary-Based thermal cycler (Roche Diagnostics). The reaction mixture in the final concentration was as follows: 1× FastStart Light-Cycler® TaqMan® Master mix, 100-nm hydrolysis probe #36 (200 nm in the case of HPRT) and 200 nm of each primer, and 5 µl of cDNA. qPCR reactions were performed using the preincubation step at 95°C for 10 minutes, followed by 45 quantification cycles consisting of denaturation at 95°C for 10 seconds, and the annealing/extension step at 60°C for 30 seconds, and the final step at 72°C with 1 second for fluorescence data acquisition. Then the reaction was cooled down to 40°C.

To calculate qPCR efficiencies, standard curves were constructed for each gene with decimal dilution of 10 pooled cDNA samples, beginning from undiluted cDNA and ending with the dilution of 10⁻⁵. In each reaction set, there was no template control included (negative control, pooled RNA reverse transcription reaction without reverse transcriptase pretreated as the same way as normal cDNA synthesis reaction). Because no contamination was observed, the uracil-DNA glycosylase incubation step was omitted.

Data collection

The PCR results were assembled using the LightCycler® Data Analysis Software version 4.0.5.415 dedicated for the LightCycler® 2.0 instrument (Roche Diagnostics). Baseline and threshold values were automatically set by the software. The number of qPCR cycles required to reach the fluorescence over the background was defined as the threshold cycle. Each sample was analyzed in triplicates with independently synthesized cDNA. The average threshold cycle values were normalized to the HPRT gene expression level, and efficiency correction with standard curves of each gene was performed. As a result, the concentration ratio values were obtained and used in statistical analyses.

Statistical analysis

All statistical analyses were performed using the STATISTICA computerized statistics package (version 12.0; StatSoft Inc., Tulsa, Oklahoma, United States). Data were expressed as mean (SD). The nonparametric analysis
of variance Kruskal–Wallis test with the Dunn post hoc test, Spearman rank correlation, and Pearson χ² test were used as appropriate. The significance level was set at a P value of less than 0.05 for all tests.

RESULTS The HPRT expression was detectable in all samples and confirmed by electrophoretic separation (FIGURE 1A). The median threshold cycle values for controls, somatotropinomas, prolactinomas, and nonfunctioning tumors were 28.1 (range, 26.1–35.6), 28.1 (range, 21.7–35.1), 28.9 (range, 21.0–32.5), and 20.9 (range, 20.9–37.7), respectively. There were no significant differences in the median threshold cycle values of the HPRT housekeeping gene between controls and pituitary tumor tissues.

GHRL and GHSR1α mRNAs were shown to be expressed in the pituitary tissue, using real-time qPCR (FIGURE 1B and 1C). In 83 of the 90 examined tissue samples (92.2%), the GHRL expression was detected in 92.3% of controls, 90% of somatotropinomas, 100% of prolactinomas, and 92.3% of nonfunctioning tumors (P = 0.9). No GHRL gene expression was detected in 1 control tissue, in 2 patients with somatotropinoma, and in 4 patients with nonfunctioning tumors (TABLE 1). No difference in the relative GHRL expression level among the groups was detected. The concentration ratio values for controls, somatotropinomas, prolactinomas, and nonfunctioning adenomas were 1.5 × 10⁻⁷ (2.1) × 10⁻⁴, 1.1 × 10⁻⁴ (1.4) × 10⁻⁴, 8.8 × 10⁻⁵ (8.1) × 10⁻⁵, and 2.3 × 10⁻⁴ (4.1) × 10⁻⁴, respectively (P = 0.7; FIGURE 2A).

In 49 of the 90 examined tissue samples (54.4%), the GHSR1α expression was detected in 84.6% of the controls, 85.0% of somatotropinomas, 40.0% of prolactinomas, and 35.6% of nonfunctioning tumors (P = 0.0002). No GHSR1α expression was detected in 2 control tissue samples, in 3 patients with somatotropinoma, 3 patients with prolactinoma, and 33 patients with nonfunctioning tumors (TABLE 1). There was a significant difference in the relative GHSR1α expression level among the groups (P < 0.0001; FIGURE 2B). The concentration ratios for controls, somatotropinomas, prolactinomas, and nonfunctioning tumors were 1.6 × 10⁻⁴ (2.1) × 10⁻⁴, 3.4 × 10⁻⁴ (4.9) × 10⁻⁴, 1.4 × 10⁻⁴ (2.1) × 10⁻⁴, and 6.5 × 10⁻⁵ (2.7) × 10⁻⁴, respectively. Patients with somatotropinoma had the highest level of the GHSR1α expression; however, its values were highly variable. The relative GHSR1α gene expression level in nonfunctioning tumor was significantly lower than in the control group (P = 0.013) and in somatotropinomas (P = 0.004; FIGURE 2B).

A strong positive correlation between relative GHRL and GHSR1α expression levels (r = 0.8; P < 0.0001) was found in the control group. A weak and negative correlation between GHRL and GHSR1α was found in patients with pituitary adenomas (r = −0.3; P > 0.05).

The mean (SD) maximum tumor diameter measured on preoperative magnetic resonance imaging was 24.8 (10.9) mm, 26 (11.9) mm, and 31.4 (76) mm for somatotropinomas, prolactinomas, and nonfunctioning tumors, respectively (P = 0.018). The maximum tumor diameter was significantly higher for nonfunctioning tumors than for somatotropinomas (P = 0.018). No significant correlation between the GHRL or GHSR1α expression levels and the maximum tumor diameter was found for any of the subgroups.

DISCUSSION Ghrelin induces GH secretion from the anterior pituitary by binding to the GHSR. It is suggested that ghrelin together with GHSR may be involved in the neoplastic progression of GH-producing pituitary adenomas.18

In the present study, ghrelin and GHSR1α expression was assessed at the mRNA level using real-time qPCR. The results showed that GHRL and GHSR1α mRNA were expressed in normal pituitary and in various pituitary adenomas; however, the expression levels varied between different types of adenomas.

The ghrelin mRNA expression was found in more than 92% of the analyzed tissue samples. The highest expression level was observed in nonfunctioning adenomas, while moderate level—in controls and somatotropinomas. The lowest level was noted in prolactinomas. Differences in the expression level were not significant. Our results are consistent with those of Kim et al9 as regards the expression of ghrelin in various pituitary adenoma tissues. The ghrelin expression was observed in all cases.9 Similarly to our study, the authors detected the highest mean level of GHRL mRNA in nonfunctioning tumors, a moderate level in GH-producing adenomas and gonadotropin-producing adenomas, and the lowest level in prolactinoma. Wang et al10 detected the highest mean expression of ghrelin mRNA in GH-producing adenomas, moderate levels in clinically nonfunctioning adenomas, and the lowest level in adrenocorticotropic hormone-producing tumors.11 In contrast to our study, differences observed by Kim et al9 and Wang et al10 were significant. Those authors did not reveal whether acromegaly patients had been treated before surgery with somatostatin analogues reducing cell proliferation. In our study, all patients with somatotropinoma were treated with somatostatin analogues before surgery.

### Table 1: GHRL and GHSR1α mRNA-positive samples in the control group and different types of pituitary adenomas

<table>
<thead>
<tr>
<th>Type of adenoma</th>
<th>GHRL</th>
<th>GHSR1α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Controls</td>
<td>12</td>
<td>92.3</td>
</tr>
<tr>
<td>Somatotropinoma</td>
<td>18</td>
<td>90.0</td>
</tr>
<tr>
<td>Prolactinoma</td>
<td>5</td>
<td>100.0</td>
</tr>
<tr>
<td>Nonfunctioning tumors</td>
<td>48</td>
<td>92.3</td>
</tr>
</tbody>
</table>

**DISCUSSION**

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Studies of Kim et al. and Wang et al. confirmed that the level of ghrelin mRNA found in GH-producing adenomas correlated negatively with the tumor size. Moreover, Kim et al. observed a significantly lower GHRL mRNA level in high-grade GH-producing adenomas than in low-grade adenomas.

In our study, we noted a negative correlation between ghrelin mRNA levels and the size of somatotropinomas; however, it was not significant. It could be a consequence, among other factors, of the effectiveness of the preoperative therapy with somatostatin analogues, which indirectly confirms our results, indicating that the maximum tumor size was significantly higher for nonfunctioning adenomas than for somatotropinomas.

In this study, in contrast to common ghrelin presence, GHSR1a mRNA was detected only in approximately 54% of tissue specimens. Receptor

The hypothesis of the inhibition of the GHRL expression by somatostatin seems possible, considering that GHRH, which acts as ghrelin agonist, increases pituitary GHRL expression. Using rat models, Kamegai et al. demonstrated that both the administration of GHRH and an increased expression of its gene in the hypothalamus enhance pituitary GHRL expression.

It is known that somatostatin and its analogues primarily show inhibitory effects on proliferation in normal and tumor cells as well as reduce hormone secretion in adenomas. Somatostatin analogues decrease the release of local growth factors, cause cell cycle arrest, and inhibit angiogenesis. Pleiotropic effect of somatotropin and its analogues influences the expression of different genes. It is possible that somatostatin and its analogues decrease GHRL expression, which seems to be consistent with our results.

Studies of Kim et al. and Wang et al. confirmed that the level of ghrelin mRNA found in GH-producing adenomas correlated negatively with the tumor size. Moreover, Kim et al. observed a significantly lower GHRL mRNA level in high-grade GH-producing adenomas than in low-grade adenomas. In our study, we noted a negative correlation between ghrelin mRNA levels and the size of somatotropinomas; however, it was not significant. It could be a consequence, among other factors, of the effectiveness of the preoperative therapy with somatostatin analogues, which indirectly confirms our results, indicating that the maximum tumor size was significantly higher for nonfunctioning adenomas than for somatotropinomas.

In this study, in contrast to common ghrelin presence, GHSR1a mRNA was detected only in approximately 54% of tissue specimens. Receptor
transcripts were present in 85% of somatotrophinomas and 84.6% of normal pituitaries, while over 60% of prolactinomas and nonfunctioning tumors did not show the GHSR1a mRNA expression. The different expression pattern of the ghrelin receptor in various tumor types and normal pituitaries confirmed data reported by other authors. Those authors also indicated the highest mean level of GHSR mRNA in GH-producing adenomas.16,18,21 These results confirm earlier suggestions that the GHRL/GHSR1a signal transduction system plays an important role in the pathogenesis of pituitary tumors.26

In this study, we observed a highly variable GHSR1a mRNA level in somatotropinomas. On the one hand, it might have resulted from biological tumor differences, but on the other hand it may have been caused by the pleiotropic effects of somatostatin or its analogues (lanreotide or octreotide). Both medications are effectively used in the therapy of acromegaly. Octreotide shows the highest affinity for somatostatin receptor type 2 (SSTR2). Lanreotide interacts most strongly with SSTR2, and in addition it shows a high affinity to SSTR5.27 Our in vitro studies (unpublished data) regarding cultured rat pituitary GH3 tumor cell line showed a significant dose- and ligand-dependent modulation of GHSR1a mRNA caused by these 2 somatostatin analogues.

It was reported that somatostatin analogues may be useful for the inhibition of plasma ghrelin levels. This phenomenon seems to be mediated via SSTR2 receptors in animal models. Octreotide caused dose-dependent inhibition of ghrelin secretion, and this effect was increased by ghrelin inhibitors. Moreover, the analysis of different subtypes of somatostatin receptors in rat models showed highly differentiated expression patterns, starting from mRNA predominantly occurring for SSTR1 and SSTR2, less often for SSTR3 and SSTR4, and hardly detectable for SSTR5.28 It might also explain the ghrelin action in human pituitary adenomas. Investigators indicate a possible connection of GHRH, somatostatin, and ghrelin on the hypothalamic–GH axis and thus its influence on the GH level.29

In summary, our findings regarding ghrelin and its receptor variant 1a in the neural system highlight their role in pituitary function. Alterations of the GHRL/GHSR1a signaling pathway could be involved in the development of adenomas.

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**Contribution statement** MK and RW conceived the idea for the study, MA, HK, and IS contributed to the design of the research and were involved in data collection. MC, MJ Ś, WL, and HK contributed to data collection. MK, MA, HK, and IS analyzed the data. MK coordinated funding for the project. All authors (except RW) edited and approved the final version of the manuscript.

**REFERENCES**


