SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of the expression of growth hormone-releasing hormone (GHRH) and GHRH-Receptors (GHRH-R) in human endometrial carcinoma and pediatric oncohematological samples

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The Examination takes place at Department of Laboratory Medicine Faculty of Medicine, University of Debrecen,
2025. February 17. 11:00

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2025. February 17. 13:00

1. Introduction

Growth hormone-releasing hormone (GHRH) is a peptide neurohormone secreted by the hypothalamus. Generally, it is known to act on the pituitary gland to stimulate the production and release of growth hormone (GH) through its binding to GHRH receptors (GHRH-R). In addition to this endocrine role, the biological actions of this 44-amino acid peptide are not limited to the pituitary–hypothalamic axis, since it was demonstrated that GHRH can function as an autocrine/paracrine growth factor in various extrapituitary tissues and several human cancers. The presence of the mRNA of GHRH was detected in human prostate, breast, ovarian, endometrial, pancreatic and adrenal cancers and cancer cell lines derived from breast, endometrial, ovarian, prostatic, pancreatic, gastric, colorectal, lung and brain tissues, and bone sarcomas, lymphomas and renal-cell carcinomas The hormonal activities of GHRH and its analogs are mediated by the pituitary type GHRH receptor (pGHRH-R).

However, previous investigations have also identified splice variants (SVs) of GHRH-Rs in human cancers and other extrapituitary tissues that can mediate the effects of GHRH and its agonistic and antagonistic analogs. In many of the above mentioned tumors, the antiproliferative functions are mediated by SV-1. Although it has been known for more than 20 years that some cancers produce GHRH, it was only recently proposed that GHRH might function as an autocrine growth factor in neoplastic cells. mRNAs for GHRH or GHRH peptide were also found in surgical specimens of human endometrial, ovarian, breast and prostate cancers. mRNAs encoding four SVs of GHRH-Rs, GHRH-R protein and specific high affinity binding sites for GHRH and its antagonistic analogs have been demonstrated in several experimental cancer models and specimens of human tumors. Thus, the direct antiproliferative action of GHRH antagonists could be exerted by the disruption of an autocrine/paracrine loop of stimulation established by tumoral GHRH and its tumoral receptors.

In earlier studies, the expression and role of the GHRH ligand were already investigated in some benign and malignant gynecologic conditions, including endometrial carcinoma. However, information on the splice variants of GHRH-Rs in human endometrial cancer is rather limited. Fu et al. also demonstrated the expression of SV1 in endometriosis.

Although the expression of GHRH, GHRH-Rs and their SVs have previously been demonstrated in various human tumors and disorders to the best of our knowledge there is no, or only very limited, existing information about the presence and gene expression of GHRH and its receptors and binding characteristics of GHRH-R SVs in pediatric hematological and oncological diseases, including various neoplastic conditions. In our study we were able to collect and investigate clinical samples of the following types of human pediatric hematological and oncological disorders: Hodgkin lymphoma (HL), rhabdomyosarcoma (RMS), teratoma (TR), acute lymphoblastic leukemia (ALL), fibrous dysplasia (FD), mesenchymal hamartoma (MH), juvenile myofibromatosis (JM), chronic benign neutropenia (CBN) and hereditary spherocytosis (HS) and immune thrombocytopenia (ITP).

2. Aims

The aim of the present study was to investigate the expression of GHRH and its tumoral receptors and the presence of GHRH-R SVs in primary human endometrial carcinoma (EC) samples and in corresponding benign endometrial tissues and in various pediatric hemato-oncological diseases.

• We studied the expression of mRNA for GHRH in the above mentioned clinical samples using RT-PCR method

• We investigated the expression of mRNA for GHRH-R using RT-PCR and the presence of GHRH-R protein using Western blot and ligand competition assay

• In receptor positive samples, the binding characteristics of the receptors were analyzed (such as receptor concentration, binding affinity, specificity)

• We also studied the expression of different receptor SVs using molecular biological methods

• We analyzed the correlation between the expression pattern of the receptors and the clinicopathological characteristics of the examined patients

• In the case of pediatric patient samples, we studied the correlations between the benign and malignant pathological characteristics and the presence of GHRH-Rs.

3. Materials and methods

Tissue Samples

Human endometrial carcinoma (EC) specimens from 39 patients (mean age 62 years; range 28–82 years) who underwent surgical removal of their uterus at the Department of Obstetrics and Gynecology, Faculty of Medicine, University of Debrecen, were investigated. Approximately 5–20 mm^3 of tissue samples of the uterus removed during staging surgery were used. Histopathological examinations of each specimen were undertaken to confirm the presence of endometrial carcinoma before molecular biology studies. There were 28 endometrioid (71.8%) and 11 papillary serous (28.2%) adenocarcinomas. Among patients with endometrioid adenocarcinoma, five had grade 1, twenty had grade 2 and three had grade 3 diseases. Among patients with the papillary serous subtype, three had grade 1, five had grade 2 and three had grade 3 cancers. Normal endometrial tissues were available in seven cases. Tissue samples were frozen and stored at −80 °C until total RNA isolation and membrane preparations were performed. The collection and the use of these specimens and normal human pituitary samples in our studies was conducted in accordance with the Declaration of Helsinki and approved by the local institutional ethics committee named Regional Institutional Ethics Committee, Clinical Center, University of Debrecen (DERKEB/IKEB 2284-004). Informed consent was obtained from all patients. Five normal human pituitary reference samples used as positive controls were collected in an anonymous fashion from the paraffin tissue-archives of autopsy cases at the Department of Pathology, Faculty of Medicine, University of Debrecen.

Specimens of pediatric hematological and oncological diseases were obtained from 15 children. The average age of children was 8.03 years (range: 9 months–15 years). Human specimens were collected from children treated at the Department of Pediatric Hematology-Oncology, University of Debrecen, Hungary. In our study, solid tumor samples were

investigated from seven children, and another eight patients had malignant or benign types of hematological disorders. Seven specimens represented bone marrow aspirates, and one hematological sample was collected from peripheral blood. Solid tumor tissues were always obtained at the time of primary surgery. Each and every sample was processed for routine histopathological examination. The final pathological diagnosis was always confirmed by an expert pathologist. Our research work was conducted in accordance with the Declaration of Helsinki, the collection and use of these pediatric samples for the current study was approved by the University of Debrecen Local Institutional Ethics Committee (DERKEB/IKEB 2284-004) and informed consent was obtained.

Methods

RNA Isolation and reverse transcription polymerase chain reaction in endometrium cancer samples

Tissue samples were homogenized with a Mikro-Dismembrator-U and total RNA was extracted with a Nucleospin Total RNA Isolation Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer'instruction.

One μg of total RNA was reverse transcribed to cDNA with MMLV Reverse Transcriptase and oligo(dT)15 (Promega Co, Madison, WI, USA) according to the manufacturer 's instructions. Primers for GHRH-R, sense 5′-CACGTCTTCTGCGTGTTGAG-3′ (exon 1) and antisense 5′-GCATCTCCTCTGCTGCTTGT-3′ (exon 2), for SV1, sense 5′-GGAAGGAGTTGTGGCTAGAGAG-3′ (intron 3) and antisense 5′-GTCATGGTGGCGAA- ATGG-3′ (exon 7) were designed using primer3_www.cgi v 0.2 program. PCR products were sequenced on an ABI-PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) in both directions to confirm the specificity of the primers. Gene-specific primers for β-actin

housekeeping gene, GHRH ligand, SV2, SV3 and SV4 splice variants were used as described previously.

For β-actin, GHRH-R, GHRH and SV1 genes the PCR reaction mix contained 1 × PCR Buffer, 1U Taq Polymerase (Invitrogen, NY, USA), 1.5 mM $MgCl_2$, 0.3 μM of each primer (Invitrogen, NY, USA), 200 μM of each dNTP (Fermentas,Leon-Rot, Germany) and 1.0 μL cDNA template in a final volume of 25 μL.

After denaturation (3 min at 94 °C), cDNA was amplified for 45 cycles (45 s at 94 °C; 30 s at 62 °C and 90 s at 72 °C). β-actin was amplified with 30 cycles. Then, a final elongation step of 72 °C 10 min was applied, and finally, the samples were cooled down to 4 °C.

For SV2, SV3 and SV4 splice variants, the PCR reaction mix contained 1 × PCR Buffer, 1.25 U TruStart Taq Polymerase (Fermentas, Leon-Rot, Germany), 3 mM (SV2, SV4) or 4 mM (SV3) $MgCl_2$, 0.4 μM (SV2, SV4) or 0.5 μM (SV3) of each primer; 300 μM (SV2) or 200 μM (SV3, SV4) of each dNTP; and 1.5 μL cDNA in a final volume of 25 μL. After denaturation and enzyme activation (3 min at 95 °C), cDNA was amplified for 45 cycles (30 s at 95 °C; 30 s at 63 °C and 60 s at 72 °C). Then, a final elongation step of 72 °C 5 min was applied, and finally, the samples were cooled down to 4 °C.

The PCR products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide.

Preparation of Membranes and Radioligand Binding Studies in endometrial samples

Radioiodinated derivatives of GHRH antagonist JV-1-42 were prepared by the chloramine- T method, as previously described with some minor modifications. The preparation of tumor cell

membranes from human EC samples for the receptor binding studies was performed as reported previously. Briefly, the human cancer specimens were homogenized in 50 mmol/L Tris-HCl buffer (pH 7.4) and supplemented with protease inhibitors (0.25 mmol/L phenylmethylsulfonylfluoride, 2 μg/mL pepstatin A, and 0.4% aprotinin) using an Ultra-Turrax tissue homogenizer (IKA Works, Wilmington, NC, USA); then, the crude membrane fraction was prepared as described and stored at −70 °C until investigated in vitro. Protein concentrations were determined by the method of Bradford. GHRH-R binding assays were carried out, as reported in detail, using in vitro ligand competition assays based on the binding of [^{125}I]JV-1-42 as radioligands to membrane fractions of human EC specimens. GHRH antagonist JV-1-42 and [^{125}I]JV-1-42 as radioligand were well-characterized previously and showed high-affinity binding to rat and human pituitaries and human renal, prostate, breast and other cancers. The high affinity binding of radioiodinated JV-1-42 to SV1 was also demonstrated and reported previously. In brief, membrane homogenates containing 50–160 μg protein were incubated in duplicate or triplicate with 60,000–80,000 cpm [^{125}I]JV-1-42 and increasing concentrations (10^{-12}–10^{-6} mol/L) of nonradioactive peptides as competitors in a total volume of 300 μL binding buffer (50 mmol/L Tris-HCl, 5 mmol/L EDTA, 5 mmol/L $MgCl_2$, 1% BSA and 30 μg/mL bacitracin, pH 7.4) supplemented with protease inhibitors, as mentioned above. After 1 h of incubation and the separation, the final pellet containing the receptor bound fraction was counted in a γ-counter. The LIGAND-PC computerized curve-fitting software of Munson and Rodbard was used to determine the type of receptor binding, dissociation constant (Kd) and maximal binding capacity of the receptors (Bmax). Due to the limited amounts of membrane protein fractions, the receptor binding of GHRH was examined in only 11 specimens.

Reverse Transcription PCR (RT-PCR) in pediatric hematooncological samples

A total of 250 ng of isolated RNA from each tissue sample was reverse transcribed into cDNA in the C 1000 Touch Thermal Cycler PCR system (Bio-Rad Laboratories, Irvine, CA, USA) using a Tetro cDNA Synthesis Kit (Bioline, London, UK).

Experiments were conducted in accordance with the manufacturer's guidelines. The RT-PCR reaction was performed in a 20 µL final volume using random hexamers. The reaction for RT-PCR for one sample consisted of the following components (primer: random hexamer (1 µL), 10 mM of the dNTP mix (1 µL), 5× RT buffer (4 µL), RiboSafe RNase Inhibitor (1 µL), Tetro Reverse Transcriptase (all reagents from Bioline, London, UK), (1 µL), DEPC-treated water (20 − (n + 8)) µL, where n (for each sample is different) is the amount of total RNA used in the reaction which was calculated based on the measured RNA concentration of the samples.

The run consisted of 35 cycles (95 °C for 15 s, 60 °C for 30 s, 72 °C for 10 s, and 72 °C for two minutes). To test for contamination, RT-NTC was incorporated into the reaction.

RT-PCR Reaction for GHRH and SV1 of GHRH-R in pediatric hematological and oncological samples

The RT-PCR reaction for the detection of GHRH and SV1 in pediatric samples was performed in a 25 µL final reaction volume with gene specific primers. Normal human pituitary tissue was used as a positive control, and β-actin (ACTB) was used as a housekeeping gene. Negative samples were run for testing the clarity of the RT-PCR reaction. The RT-PCR reaction consisted of 35 cycles (95 °C for 15 s, 60–67 °C Tm annealing for 30 s, 72 °C for 10 s) and the last extension at 72 °C was for 2 min. PCR products were separated in a 1.5% agarose gel containing

GelRed (Bioline, London, UK) and detected with UV light and digitalized with AlphaDigiDocTM RT (Alpha Innotech, Santa Clara, CA, USA). To determine the size of the DNA, 25 or 50 bp DNA marker (Bioline, London, UK) was used.

Western Blot Analysis of GHRH-R Protein in pediatric hematological and oncological samples

Pediatric tissue samples were homogenized and lysed in ice-cold protein lysis buffer (M-PER, Thermo Fisher Scientific, Waltham, MA, USA), which was supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). The Bradford assay was applied for protein quantification. Before Western blotting, proteins were diluted with 4× Laemmli buffer (Bio-Rad Laboratories, Irvine, CA, USA) and 40 μg of each protein sample in equal volumes were denatured at 95◦C for 8min. Afterwards, protein lysates were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis (SDS-PAGE). To define the size of the separated proteins, using Precision Plus Dual Color Protein Standard as molecular weight marker (Bio-Rad Laboratories, Irvine, CA, USA) was also loaded on the SDS-PAGE. Then, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Burlington, MA, USA). Non-specific binding sites were blocked with 5% milk-TBS-Tween, followed by the incubation of the membranes with the following primary antibodies overnight, at 4 ◦C: anti-GHRH-R antibody (1:1000 dilution, GHRH-R polyclonal antibody: PA3-117, Thermo Fisher Scientific, Waltham, MA, USA) and anti-HPRT antibody (1:1000 dilution, P00492 rabbit monoclonal; Biol. Technology, Carlsbad, CA, USA). HPRT as a housekeeping protein was measured to ensure equal loading of all samples. After extensive washing steps with TBS-Tween, membranes were incubated with anti-rabbit IgG secondary antibody (1:5000, Thermo Fisher Scientific, Waltham, MA, USA—diluted in 5% skim milk in TBS-Tween) for two hours at room temperature. Then, after washing membranes with TBS-Tween several

times, the signal was detected by chemiluminescence using Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA). The intensity of each band was normalized to HPRT. Quantification was carried out using the Bio-Rad Image Lab 5.2.1 software (Bio-Rad Laboratories, Hercules, CA, USA).

Preparation of Membranes and Radioligand Binding Studies in pediatric hematological and oncological samples

For the radioreceptor binding studies, GHRH antagonist JV-1-42 was radioiodinated by the chloramine-T method, as previously reported, and then purified by HPLC. Cell membrane preparation from human pediatric solid tumor specimens for the receptor binding studies using radioligand competition assays was carried out as described earlier. Protein lysate preparation from the pediatric tissue samples was performed according to the next steps: the pediatric solid tumor samples were first defrosted and then homogenized using a tissue homogenizer (Ultra-Turrax, IKA Works, Wilmington, NC, USA) in 50 mmol/L Tris-HCl buffer (pH 7.4), supplemented with protease inhibitors (0.25 mmol/L PMSF: phenylmethylsulfonylfluoride, 2 µg/mL pepstatin A, and 0.4% apro- tinin) (Merck, Darmstadt, Germany). The final crude membrane fractions were prepared and stored at −80 ◦C until further in vitro investigations, as described earlier in the literature. The amount of protein was determined by the Bradford assay. GHRH-R binding investigations were accomplished, as reported in our protocols previously. In vitro ligand competition assays were performed based on the binding of [^{125}I]JV-1-42 as radioligand to membrane fractions of human pediatric samples. High affinity binding properties of the radioligand [^{125}I]JV-1-42 GHRH antagonist to rat and human pituitaries were reported earlier. This GHRH antagonist also has a high binding affinity to various human cancers, such as renal, endometrial, prostate and breast. The specific, high affinity binding of radiolabeled

JV-1-42 to SV1 was also demonstrated and reported in the literature. In detail, membrane homogenates were incubated with radioligand [^{125}I]JV-1-42 and increasing concentrations (10^{-12}–10^{-6} mol/L) of non-radioactive competitor peptides in binding buffer. After a one-hour incubation and separation, the receptor bound fraction in the final pellet was counted in a γ-counter. A curve-fitting computer program (LIGAND-PC) of Munson and Rodbard was used to calculate the binding characteristics (Kd and Bmax) of GHRH-Rs. Due to the very small sample size and limited amounts of membrane fractions, the GHRH ligand competition studies were performed in only seven specimens.

4. Results

New primers were designed for the PCR amplification of GHRH-R and SV1. PCR products were sequenced in both directions, and the specificity of the primers was confirmed. For GHRH-R, a 121 base-pair-long product was amplified from exon 1 to exon 2, which is present only in the full-length receptor mRNA and absent in the splice variants. This product could be detected in none of the endometrial tumor specimens or normal endometrial tissues. However, as expected, the expression of mRNA for the full-length GHRH-R was found in all five pituitary samples used as positive controls (data not shown). Accordingly, only the GHRH-R PCR product obtained from these samples was used for sequence analysis. In the case of the SV1 receptor variant, the 415-bp long PCR products (from intron 3, absent in the full-length receptor; to exon 7, present only in SV1 and the full-length receptor but not in the other variants) of the endometrial tumor samples were identical to that of the pituitaries. The SV2, SV3 and SV4 splice variants were detected as 523-, 245- and 120-bp long PCR products, respectively. As a positive control, we have investigated five human pituitary tissues, which expressed the four splice variants and the full length GHRH-R. Twenty-four of the investigated thirty-nine tumor samples (61.5%) and three of the seven corresponding normal endometrial tissues

(42.9%) expressed mRNA for GHRH ligand The expression of mRNA for GHRH was also detected in the five human pituitary tissues investigated.

In patients with endometrial carcinoma, SV1 is the most functional form in the view of a potential cancer therapy and could be shown in nine cancer samples (23%). The second most frequent variant was SV4, which was detected in 8 of 39 malignancies (20.5%). The incidence of SV2 could be observed only in three cancer specimen (7.7%), and the expression of SV3 variant was absent in the tumor samples. The presence of the GHRH-R splice variants could not be revealed in any of the normal endometrial tissues investigated.

Altogether, we were able to detect splice variants of GHRH-Rs in 14 of the 39 EC specimens (35.9%). The co-expression of mRNA for GHRH ligand and splice variants for GHRH-Rs was also found in 14 of 39 (35.9%) patients. Our results show that all GHRH-R splice variant positive specimens expressed mRNA for the GHRH ligand. Ten of thirty-nine endometrial cancer specimens exhibited mRNA expression for GHRH but not for splice variants for GHRH-Rs. In five cases, only SV1or SV4 were expressed among the four splice variants of GHRH-Rs. In one case, SV1 and SV2 or SV1 and SV4 co-expression, and in other two cases, SV1, SV2 and SV4 coexpressions were observed.

The presence and binding characteristics of GHRH-Rs and specific binding of radioiodinated GHRH analog JV-1-42 to membrane homogenates of human EC samples were determined using radioreceptor assays. Of the eleven tumor specimens examined by ligand competition assays, nine samples (81.8%) showed GHRH binding. The concentrations and binding affinities of GHRH-Rs in EC membranes were also investigated. The analyses of the displacement curves of [^{125}I]JV-1-42 and the Scatchard plots of the specific binding data in the 9 receptor positive cancer specimens revealed that GHRH-Rs had a mean dissociation constant (Kd) of 5.28 nM (range, 1.63 to 8.81 nM). The mean concentration of GHRH-Rs (maximal binding capacity,

Bmax) was 385.0 fmol/mg membrane protein in crude membranes derived from human EC cells (range, 249.5 to 509.5 fmol/mg protein). Based on our receptor binding results, the one-site model could provide the best fit representing a single class of high affinity GHRH-Rs in human EC specimens. Biochemical specifications and parameters crucial to characterize specific binding sites were also defined. Thus, the in vitro receptor binding of [^{125}I]JV-1-42 was detected to be specific, reversible, temperature dependent and time dependent, and linear with protein concentrations in the human endometrial tumor specimens examined. The binding of radiolabeled JV-1-42 was displaced completely by increasing the concentrations (10^{-12}–10^{-6} M) of hGHRH(1-44) or hGHRH(1-29)NH2, whereas none of the structurally and functionally different and unrelated peptides analyzed, such as somatostatin, luteinizing hormone-releasing hormone (LHRH), epidermal growth factor (EGF), [Tyr4]bombesin, and insulin-like growth factor I (IGF-I), inhibited the binding of radioiodinated JV-1-42 at concentrations as high as 1 μM . Our results also showed that ligand binding was accompanied by the expression of mRNA for SV1 subtype of GHRH-Rs in all endometrial cancer specimens examined. A comparative analysis of the results of radioreceptor assays and SV1 subtype mRNA studies demonstrated that the expression of the SV1 subtype was 100% consistent with the presence of specific binding sites for GHRH antagonist [^{125}I]JV-1-42. In our study, no correlation was found among clinicopatholological features and receptor findings.

In our other study RT-PCR analyses revealed the presence of 150 bp products corresponding to GHRH peptide ligand in pediatric specimens investigated. Of the 15 specimens studied eleven pediatric samples (73%) showed the expression of mRNA for GHRH. CBN, HS, ITP and only one of the ALL specimens did not express GHRH. Similarly to the GHRH mRNA studies, of the 15 specimens studied eleven pediatric samples (73%) showed the expression of mRNA for SV1. CBN, HS, ITP and only one of the ALL specimens did not express mRNA for SV1.

Negative controls yielded no detectable signals, indicating that PCR products were generated from cDNA and not from genomic DNA or other contaminations.

According to Western blot analysis, the GHRH-R protein was found to be expressed all of the seven human pediatric solid tumor samples examined. In our Western blot analyses using anti-GHRH-R antibody major bands at a molecular weight of 40 kDa were demonstrated, which, according to the literature, correspond to SV1 of GHRH-R in the examined pediatric samples. We were able to examine 7 samples and our study showed that all specimens investigated, including two benign tumor samples and five malignant tumors, showed GHRH-R binding. The computerized analyses of the GHRH-R binding points in the seven pediatric samples showed that the single class of GHRH-Rs had a mean dissociation constant (Kd) of 4.57 nM (range, 1.35–8.99 nM). The mean receptor concentration of GHRH-Rs (Bmax, maximal binding capacity) was 375.7 fmol/mg membrane protein (range, 222.1–733.0 fmol/mg membrane protein). The results of the ligand competition assays correlated well with the Western blot findings, demonstrating that the presence of GHRH-R protein was 100% consistent with the expression of specific binding sites of ^{125}I-labeled GHRH analog JV-1-42.

Our results also showed that the receptor protein findings by Western blot and ligand binding assays were accompanied by the expression of mRNA for SV1 subtype of GHRH-Rs in all pediatric specimens examined. Based on the comparative analysis of the results of GHRH-R protein analyses and SV1 subtype mRNA studies we found strong correlation investigating seven specimens. These findings showed that the expression of mRNA for the SV1 subtype was 100% consistent with the presence of GHRH-R proteins studied by Western blot and specific, high affinity binding of GHRH antagonist [^{125}I]JV-1-42 investigated by radioreceptor assays.

5. Discussion

In our work, we found that about one-third (35.9%) of EC specimens, but none of the normal endometrial tissues, were positive for one or more splice variants (SV1-4) of GHRH-R and 23% showed positivity for expression mRNA for SV1. In an earlier study, 43% of endometrial cancer tissues were found to be positive for SV1 protein expression by immunohistochemistry. This slight discrepancy could be explained by the fact that the antisera used for the detection of SV1 protein in this study was directed against the first 25 amino acids at the N terminus of the SV1 protein, which is also present in SV2 and SV4 subtypes. While SV1, SV2 and SV4 can be distinguished by size based on Western blotting, immunohistochemistry provides positive signals for all three GHRH-R isoforms. In addition, positive immunohistochemical signals were detected only in the cytoplasm of the epithelial cells of the glands of the endometrial adenocarcinomas but not on the cell's surface. We found that the second most frequently expressed splice variant in our tissue series was SV4 (20.5%). The presence of the remaining two splice variants, SV2 and SV3, could be detected in only three or none of the samples, respectively. GHRH-R isoforms derived from SV3 and SV4 imply that they probably do not represent mature receptor proteins to be manifested on the cell's surface. SV2, possessing the truncated N-terminal extracellular domain of SV1 but containing only two transmembrane domains, might be transported to the cell's surface.

We could not detect mRNAs for pituitary type GHRH-R either in endometrium carcinoma or in normal endometrial tissues. In previous studies, the expression of pituitary GHRH-R was shown by real-time quantitative PCR in different cancer cell lines, including non-Hodgkin's lymphoma, pancreatic cancer, glioblastoma and small-cell lung carcinoma, but the level of expression was low in extrapituitary normal tissues. Our results are in agreement with previous

findings, where the expression of classic pituitary type GHRH-R on different human tumor tissues could not be detected or was found to be less frequently present than SV1.

In eleven cases, we were able to prepare crude membrane protein fractions for radioligand binding studies to demonstrate the presence of specific GHRH binding sites. Using ligand competition assays, we demonstrated the presence of specific, high affinity receptors for GHRH. Molecular biology analyses and radioligand binding studies clearly demonstrated that the expression of mRNA for SV1 subtype of GHRH-Rs was 100% consistent with the presence of specific receptors for radiolabeled GHRH analog JV-1-42. However, the expression of mRNA for the pituitary type of GHRH-Rs was not detected. It is also important to note that all receptor positive human EC specimens examined by ligand competition assay expressed a well-detectable amount of the SV1 GHRH-R gene. Furthermore, the PCR products for GHRH ligand were found in 24 of 39 (61.5%) human EC specimens. In 14 samples (35.9%), mRNA for both GHRH and GHRH-R splice variants was detected. While the most probable functional receptor splice variant SV1 was present in only 23% of the EC specimens investigated, the GHRH ligand could be detected in more than 60% of tumoral and 40% of normal endometrial tissues. In an earlier study, GHRH mRNA was detectable in normal endometrial tissue and EC; however, no changes in endometrial GHRH mRNA were shown between normal and neoplastic tissues obtained from the same patient. However, the levels were higher than those found in myometrial tissues obtained from other patients from benign gynecologic diseases. Thus, it was suggested that GHRH may promote endometrial proliferation and be involved in the pathogenesis of EC and endometriosis. In the present study, using RT-PCR, we demonstrated that mRNAs for GHRH and SVs, but not the pituitary type GHRH-R, are expressed in human EC tissues, suggesting the existence of an autocrine/paracrine GHRH loop.

Of the 15 human specimens studied in our other major experiments, eleven pediatric samples (73%) showed the expression of mRNA for GHRH. These 11 samples also expressed mRNA for GHRH receptor SV1. GHRH-R protein was found to be expressed in two benign tumor samples and five malignant tumors examined by Western blot. The presence of specific, high affinity binding sites on GHRH-R was demonstrated in all of the seven human pediatric solid tumor samples investigated. To the best of our knowledge, our investigation represents the only publication about the expression of GHRH-R and a potential role of GHRH-R signaling in the pathophysiology of a neoplastic hematological disorders. In this context, it is worth mentioning that the investigated single sample of pediatric HL and 4/5 samples of childhood ALL, expressed both GHRH and SV1 in contrast to samples of CBN, HS, and ITP, which contain non-transformed blood and bone marrow cells.

Our results, showing a marked incidence of GHRH and SV1 of GHRH-R in neoplastic hematological and oncological disorders in children, support the merit of further investigation of GHRH-Rs as potential molecular targets for diagnosis and therapy. This problem is particularly prominent if we consider the number of different subsets of samples studied. However, despite the limited number of investigated samples we were able, for the first time, to demonstrate the presence of GHRH and GHRH-Rs in neoplastic lesions of children, both at the RNA and protein levels. Although the size of the investigated cohort is rather small and its composition is heterogenous, it is worth noting that the examined specimens displayed marked expression of GHRH and GHRH-R SV1. Therefore, in the near future we would like to extend our investigation and we are trying to collect a reasonable number of samples. Hopefully, based on the findings in this study investigating 15 specimens, additional human specimens from children will be able to clarify our further questions and can provide novel data about the potential clinical significance of GHRH and GHRH-Rs for diagnostic and therapeutic applications in children.

These new findings may offer a novel innovative therapeutic approach for these malignancies including EC and pediatric hematological and oncological disorders based on potent GHRH receptor antagonists and suggest a possible function of GHRH-R signaling in the pathology of various pediatric oncohematological and proliferative disorders and also in human endometrial cancer. This proposal appears to be supported by our present results that in the case of non-proliferative pediatric disorders (HS, CBN, ITP), neither GHRH mRNA nor mRNA for SV1 could be detected.

1. Summary and conclusion

1. We demonstrated the expression of mRNA for GHRH and GHRH-R SVs, but the mRNA for the pituitary type of GHRH-R was not expressed in human EC tissues. This may indicate the existence of an autocrine/paracrine GHRH regulatory loop. 23 % of the endometrial carcinoma samples expressed mRNA for GHRH-R SV1.
2. Specific, high-affinity GHRH-R were detected by radioligand assay in a cohort of EC samples and the binding characteristics of the GHRH receptors were also determined.
3. Based on our knowledge this is the first study demonstrating the expression of mRNA for GHRH and GHRH-R SV1 in various childhood tumors and other neoplastic conditions.
4. Expression of mRNA for GHRH and GHRH-R SV1 was found in 73% of the pediatric hemato-oncology tissue samples examined. Our results show that the occurrence of GHRH and GHRH-R SV1 is frequent in malignant hemato-oncology disorders in children.
5. GHRH-R protein and high-affinity, specific GHRH binding sites were also detected in pediatric hemato-oncology tissue samples examined by Western blot and radioreceptor assay.
6. Overall, our results suggest that SV1 can function as a hypoxia-induced oncogenic promoter that can serve a potential therapeutic target for GHRH-R antagonists.

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List of publications related to the dissertation

1. **Juhász, É.**, Szabó, Z., Schally, A. V., Király, J., Fodor, P., Kónya, G., Dezső, B., Szabó, E., Halmos, G., Kiss, C.: Expression of Growth Hormone-Releasing Hormone and Its Receptor Splice Variants in a Cohort of Hungarian Pediatric Patients with Hematological and Oncological Disorders: a Pilot Study.
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List of other publications

3. Gomena, J., Modena, D., Cordella, P., Vári, B., Ranđelović, I., Borbély, A., Bottani, M., Vári-Mező, D., Halmos, G., **Juhász, É.**, Steinkühler, C., Tóvári, J., Mező, G.: In vitro and in vivo evaluation of Bombesin-MMAE conjugates for targeted tumour therapy.
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