American Journal of Pathology, Vol. 151, No. 3, September 1997

Overexpression of the Growth-Hormone-Releasing Hormone Gene in Acromegaly-Associated Pituitary Tumors

An Event Associated with Neoplastic Progression and Aggressive Behavior

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The clinical behavior of growth hormone (GH)-producing pituitary tumors is known to vary greatly; however, the events underlying this variability remain poorly understood. Herein we demonstrate that tumor overexpression of the GH-releasing hormone (GHRH) gene is one prognostically informative event associated with the clinical aggressiveness of somatotroph pituitary tumors. Accumulation of GHRH mRNA transcripts was demonstrated in 91 of a consecutive series of 100 somatotroph tumors by *in situ* hybridization; these findings were corroborated by Northern analysis and reverse transcriptase polymerase chain reaction, and protein translation was confirmed by Western blotting. By comparison, transcript accumulation was absent or negligibly low in 30 normal pituitary glands. GHRH transcripts were found to preferentially accumulate among clinically aggressive tumors. Specifically, GHRH mRNA signal intensity was 1) linearly correlated with Ki-67 tumor growth fractions ($r = 0.71$; $P < 0.001$), 2) linearly correlated with preoperative serum GH levels ($r = 0.56$; $P = 0.01$), 3) higher among invasive tumors ($P < 0.001$), and 4) highest in those tumors in which postoperative remission was not achieved ($P < 0.001$). Using multivariate logistic regression, a model of postoperative remission likelihood was derived wherein remission was defined by the single criterion of suppressibility of GH levels to less than 2 ng/ml during an oral glucose tolerance test. In this outcome model, GHRH mRNA signal intensity proved to be the most important explanatory variable overall, eclipsing any and all conventional clinicopathological predictors as the single most significant predictor of postoperative remission; increases in GHRH mRNA signal were associated with marked declines in remission likelihood. The generalizability of this outcome model was further validated by the model's significant performance in predicting postoperative remission in a random sample of 30 somatotroph tumors treated at another institution. These data indicate that overexpression of GHRH gene is an event associated with the neoplastic progression and clinical aggressiveness of somatotroph adenomas. More generally, these data merge essential elements of the hypothalamic and pituitary hypotheses of pituitary tumorigenesis, providing for a more unified concept of neoplastic progression in the pituitary. *(Am J Pathol 1997, 151:769–784)*

The pituitary tumors underlying acromegaly, although unified by their pathological hypersecretion of growth hormone (GH), are an otherwise heterogeneous group of lesions. From biological, clinical, and prognostic standpoints, the behavior of these tumors tends to be highly variable and generally defies reliable prediction. Whereas some somatotroph adenomas are amenable to curative resection, others will progress relentlessly, often

Supported by Physicians Services Incorporated Foundation of Ontario (K. Thapar and K. Kovacs), a Medical Research Council of Canada Research Fellowship (K. Thapar), and National Institutes of Health grant RO1-DK-32632 (M. O. Thorner).

Accepted for publication June 18, 1997.

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despite maximal surgical, pharmacological, and radiotherapeutic intervention.[1] It is recognized that some 80% of GH-secreting adenomas will have progressed to a macroadenoma stage when detected, and one-half of these will be grossly invasive of surrounding neurovascular or bony structures.[2–4] Curative resections can be achieved by experienced surgeons in only 55 to 65% of all somatotroph adenomas.[5–7] In the remainder, it is usually tumor invasiveness that precludes complete excision, and for these tumors, symptomatic regrowth and persistent hormone hypersecretion are virtually guaranteed.[8–10] The tendencies of some somatotroph adenomas toward aggressive, invasive, or recurrent growth, although neither reflected in nor predicted by the tumor's histological or ultrastructural morphology, is presumably the result of specific subcellular events that promote neoplastic progression among aggressive variants. To date, however, prognostically informative determinants of neoplastic progression remain poorly characterized in this tumor system.

GH-releasing hormone (GHRH), a hypothalamic peptide and mitogen, is the principal positive regulator for GH-producing cells (somatotrophs) of the pituitary.[11,12] After its release from hypothalamic nuclei and subsequent descent to the anterior pituitary via the portal circulation, GHRH binds to its receptor (GHRH-R) on the somatotroph cell surface, stimulating both the proliferation of these cells and their secretion of GH. A logical extension of such trophic physiological activity has been the implication that excessive GHRH stimulation may play a role in somatotroph tumorigenesis, particularly from the standpoint of neoplastic progression. In support of this possibility has been a growing body of evidence indicating that somatotroph adenomas may themselves be a local source of GHRH production. In this regard, several investigators have documented the presence of GHRH mRNA transcripts and/or immunoreactive GHRH within, as well as *in vitro* GHRH secretion by, somatotroph adenomas.[13–17] Whereas these observations raise the possibility of GHRH-mediated autocrine/paracrine stimulatory loops within somatotroph adenomas, neither the clinicopathological nor prognostic significance of such local GHRH expression has been systematically examined. Moreover, the important question of whether locally produced GHRH can promote neoplastic progression in somatotroph adenomas and account for the aforementioned variability in their clinical behavior remains unresolved.

The present work evaluates the hypothesis that tumor overexpression of the GHRH gene represents an event in the progression of GH-producing pituitary tumors, one associated with aggressive endocrinological and oncological behavior, and a poorer surgical outcome.

Materials and Methods

Overview of Research Design

A consecutive series of 100 GH-producing pituitary tumors were screened for expression of the GHRH gene by *in situ* hybridization (ISH). To determine the biological and clinical relevance of such expression, the degree of GHRH transcript accumulation was quantified and correlated with pertinent clinicopathological parameters, including tumor invasion, preoperative GH level, tumor morphology, tumor growth fraction, and postoperative remission status. To explore further the relationship between GHRH mRNA transcript accumulation and surgical outcome, particularly as it compared with other clinicopathological predictors, multivariate modeling was used to fit a logistic regression model of postoperative remission likelihood. The stability and reproducibility of this model were then tested in a second, randomly selected, and comparable cohort of 30 acromegaly-associated pituitary tumors treated at another institution.

Clinical Material

Of 114 consecutive acromegaly-associated pituitary tumors operated upon at the Wellesley Hospital (Toronto, Canada) between 1974 and 1991, tumor samples and clinical data of 100 cases were available for inclusion in this study. Included were 59 men and 41 women with a median age of 44.5 years (range, 17 to 70 years). All patients had been subject to a uniform management protocol. Each had been evaluated by a single endocrinologist (D. W. Killinger) and operated upon by a single neurosurgeon (H. S. Smyth), with each having had their tumors pathologically characterized by a single pathologist (K. Kovacs). These 100 patients and their tumors constituted the primary study set upon which ISH analysis, clinicopathological correlations, and statistical modeling were performed. The derived outcome model was then tested in a comparable cohort of 30 randomly selected acromegalic patients managed at the Mayo Clinic, Rochester, MN, between 1980 and 1987. Each patient had been subjected to a uniform endocrine evaluation, was operated upon by the same neurosurgeon (E. R. Laws), and each tumor was pathologically characterized by the same team of pathologists (B. Scheithauer and K. Kovacs).

Tumor Samples and Control Tissues

Three categories of pituitary tissue were used in this study.

The main group of tumors studied were those from the primary (Toronto) and secondary (Mayo) study populations. Consisting of 100 and 30 somatotroph adenomas, respectively, all tumors were fully characterized on the basis of their histology, immunohistochemical profile, and ultrastructure. All subtypes of GH-producing adenomas were represented. These archived tissues, all of which had been formalin fixed and paraffin embedded at the time of surgery, were used for ISH studies.

As control tissues for ISH, formalin-fixed and paraffin-embedded specimens of normal pituitary gland were used, which were obtained from two sources. The first included 20 normal autopsy pituitary glands, all of which were obtained from patients who died of nonendocrine

causes and in whom glands could be retrieved within 12 hours of death. Because of potential mRNA degradation associated with the delay in procuring autopsy pituitaries, 10 surgical nontumorous pituitary specimens were included as a secondary control group. These specimens consisted of morphologically normal peritumor tissue adjacent to either corticotroph adenomas (n = 6), somatotroph adenomas (n = 2), or prolactin cell adenomas (n = 2).

A third group of pituitary tissues, each consisting of a freshly frozen fragment and a formalin-fixed fragment, were also studied. Included in this group were 10 somatotroph adenomas and 1 autopsy pituitary gland (obtained within 2 hours of death). In these specimens, ISH, Northern analysis, reverse transcriptase polymerase chain reaction (RT-PCR), Western analysis, and GHRH immunohistochemistry were all performed in each case. The purpose of these analysis was to 1) confirm the results of ISH with other methods of detecting GHRH mRNA transcripts, 2) to verify that the probe used for ISH was identifying mRNA transcripts of appropriate size, 3) to evaluate and quantify the relationship between GHRH mRNA levels as determined by ISH and Northern analysis, and 4) to demonstrate GHRH protein, thereby confirming translation of GHRH mRNA transcripts.

ISH

GHRH Probe

The human GHRH probe used for both ISH and Northern analysis was a 30-mer antisense oligonucleotide probe (5′-GTT GGT GAA GAT GGC ATC TGC ATA CCG CCG-3′) derived from exon 3 (nucleotides 177 to 206) of the consensus cDNA sequence.[18] It was radiolabeled by the 3′-end-labeling method using ^{35}S-labeled deoxyadenosine 5′-triphosphate and a commercially available kit (Dupont, Missisauga, Canada). Radiolabeled antisense oligonucleotides were purified by affinity chromatography, from which labeled probes of high specific activity were eluted. The optimal specific activity for the probe, determined by a series of preliminary dilution experiments, was 1.0×10^6 cpm.

ISH Protocol

ISH was performed on 5-μm slide-mounted tissue sections. Details of the technique, including specifics of probe labeling and purification, prehybridization, overnight hybridization, and post-hybridization treatments have been outlined in previous reports from our laboratory.[19–21] After liquid emulsion autoradiography (Kodak NTB2 emulsion) and a 1-week exposure time, slides were developed, fixed, rinsed, stained with hematoxylin, dehydrated, and coverslipped.

To evaluate the relationship between GHRH mRNA distribution and GH content at the level of the individual tumor cell, combined ISH for GHRH and immunohistochemistry for GH were performed on the same tissue section. The streptavidin-biotin peroxidase complex method was used, being performed after post-hybridization washes.

Quantification of GHRH mRNA Signal

In all adenomas, GHRH mRNA signal intensity was quantified by manual densitometry. The number of silver grains in all tumor cells present in each of 20 randomly selected high-power fields were counted. Within each field, the silver grain content of every cell was specifically enumerated using oil immersion microscopy (×1000). A signal intensity index, representing the mean number of silver grains per cell, was determined in each case. All counts were performed by a single experienced cytotechnologist blinded to clinical or other details of the case.

In quantifying the ISH signal in the nontumorous control glands there were additional methodological considerations. The objective was to specifically enumerate the number of silver grains in normal somatotrophs. In the autopsy pituitary glands, this was facilitated by using horizontal sections and evaluating only those acidophilic cells in the lateral wings of the gland, a region primarily occupied by somatotrophs. In peritumor surgical fragments, such topographic orientation was not possible, and serial GH-immunostained sections were required to facilitate somatotroph localization.

ISH Control Procedures

To exclude the possibility of nonspecific probe binding, two standard control procedures were performed in tandem with the ISH protocol. The first involved predigestion of tissue sections in 100 μg/ml of RNAse, and the second was a competitive hybridization assay wherein 20-fold excess unlabeled probe was added to the hybridization mixture. By both methods, the hybridization signal was effectively eliminated or reduced to negligible background levels (<5 silver grains/cell). One or both of these control procedures were performed in all tumors.

In total, 171 specimens were studied by quantitative ISH (ie, 100 tumors of the Toronto series, 30 tumors of the Mayo Clinic serie, 20 autopsy pituitaries, 10 surgical nontumorous pituitaries, and 11 specimens for corroboration with Northern/Western/PCR analysis). Once the experimental conditions were established, ISH was performed in a batch fashion, with a series of nine separate batches, or runs, being required to complete all specimens. To ensure that batch-to-batch variability was not a confounding factor, six specimens (three autopsy control pituitaries and three somatotroph adenomas) served as internal controls, a slide of each having been included in all nine batches. After GHRH mRNA signal intensity was quantified for each of these specimens in each of nine batches, batch-to-batch variability was assessed in two ways. First, a coefficient of variation was determined for each control sample. Individually, the value of these coefficients ranged from 0 to a maximum of 6.9%, indicating strong consistency from one batch to the next for each of the controls. Second, when the mean GHRH mRNA sig-

nal intensity of all controls in each of nine batches were compared, no significant differences were found between batches (one-way analysis of variance (ANOVA), $P = 0.95$).

Northern Analysis

This analysis was performed primarily to verify that transcripts detected by ISH were of appropriate size. In 10 cases for which freshly frozen tumor tissue was available, total RNA was extracted using the single-step acid-guanidinium-thiocyanate-phenol-chloroform method.[22] In a similar fashion, RNA from an autopsy pituitary gland was also extracted, after removal of the posterior pituitary. RNA was fractionated on 0.8% agarose/2.2% formaldehyde gels, transferred to nylon membranes, and cross-linked by ultraviolet irradiation. The same GHRH oligonucleotide probe used for ISH was ^{32}P end labeled to high specific activity (1.0×10^6 cpm/ml) and membrane hybridized overnight at 42°C. Blots were washed under high-stringency conditions and exposed at to Kodak XAR-5 film for 5 days at −80°C.

The same blots probed for GHRH were sequentially re-probed for GHRH-R. A full-length 1.6-kb GHRH-R cDNA probe had been previously cloned by us (B. Gaylinn and M. O. Thorner)[23] and inserted into a bluescript vector. Using an *in vitro* transcription kit, ^{32}P-labeled riboprobes of high specific activity were generated and subsequently purified by affinity chromatography (Boehringer Mannheim, Indianapolis, IN). Hybridization conditions and post-hybridization treatments were identical to those described above for the GHRH probe.

As a loading control, all membranes were sequentially probed for their content of 18 S ribosomal RNA using the following probe sequence: 5′-CGG CAT GTA TTA GCT CTA GAA TTA CCA CAG-3′. Probe labeling and hybridization were identical to that described for the GHRH, although only a 6-hour exposure was required.

GHRH autoradiograms were subjected to densitometric quantification using the PDI system (Huntington Station, NY). Band densities were recorded in arbitrary densitometric units and were internally standardized for variations in the amount of RNA loaded into each lane. Specifically, the densities of the GHRH bands were divided by the densities of their respective 18 S bands; this ratio was then multiplied by a factor of 100 to achieve whole number values (ie, relative densitometric units).

RT-PCR

As a secondary means of demonstrating GHRH transcript accumulation, RT-PCR was performed in the same 11 cases studied by Northern analysis using an established protocol with minor modifications.[16] Briefly, an aliquot (5 μg) of total RNA was reverse transcribed using 10 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) from a 3′-primer derived from exon 5 of the consensus GHRH cDNA sequence. A 10-μl aliquot of the cDNA reaction mixture was used for the PCR amplification reaction, being carried out in a total volume of 100 μl with a primer pair at 1 μmol/L in 50 mmol/L KCl, 10 mmol/L Tris/HCl, pH 8.3, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTP, and 2 U of Ampli*Taq* DNA polymerase (Perkin Elmer-Cetus, Emeryville, CA). PCR of 30 cycles was performed, consisting of denaturation (94°C for 1 minute), annealing (55°C for 1 minute), and extension (72°C for 3 minutes) in an automated DNA thermal cycler. The primer pair used to generate a specific GHRH 235-bp fragment was the following: sense, 5′-TAT GCA GAT GCC ATC TTC AC-3′; antisense, 5′-TTCA-TCC-CTG-GGA-GTT-CCT-G-3′. The PCR product was phenol extracted, ethanol precipitated, and electophoresed on an ethidium-bromide-containing 2% agarose gel. As a negative control, total RNA extracted from a corticotroph pituitary adenoma was used.

GHRH Immunocytochemistry and Western Blotting

GHRH Immunostaining

Five micron sections of formalin-fixed and paraffin-embedded tissues were mounted onto glass slides. Immunostaining was performed using the avidin-biotin-peroxidase complex method of Hsu et al.[24] To enhance protein detection, antigen retrieval was performed as previously described, using a 0.01 mmol/L sodium citrate retrieval buffer (pH 6.0) and tissue microwaving.[25] Polyclonal GHRH antisera (Peninsula Laboratories, Belmont CA) was used at a 1:300 dilution.

Western Blotting

Protein extraction, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and immunodetection were performed according to a standard protocol with minor modification.[26] Briefly, snap-frozen tissues were homogenized and protein extracted in a lysis buffer consisting of 1% Nonidet P40, 20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml phenylmethylsulfonyl fluoride. The concentration of soluble protein was determined using the Bradford assay (Bio-Rad Laboratories, Richmond CA). Proteins were resolved by SDS-polyacrylamide gel electrophoresis on a 15% acrylamide separating gel at constant current (35 mA). After electrophoresis, proteins were transferred to Immobilon-PSQ polyvinylidene membranes (Millipore) using the Bio-Rad semi-dry transfer electroblotting apparatus. After incubation in a blocking solution consisting of 5% skim milk and 0.1% Tween 20 in PBS (0.01 mol/L sodium phosphate and 0.14 mol/L NaCl, pH 7.4) for 60 minutes, membranes were incubated in a rabbit-derived polyclonal GHRH antibody (Peninsula Laboratories) at a final concentration of 1:500 for 2 hours. After three successive washes in the 5% skim milk/0.1% Tween 20/PBS mixture, the membrane was incubated in a goat anti-rabbit IgG-horseradish peroxidase conjugate. Peroxidase activity was detected by chemiluminescence using a standard kit (ECL Western blotting kit, Amer-

sham, Arlington Heights, IL). As a positive control, a lane containing synthetic GHRH peptide (Peninsula Laboratories) was run with the tissue samples.

Determination of Tumor Growth Fractions

Tumor growth fractions were determined in each case by Ki-67 immunolabeling using the MIB-1 monoclonal antibody (AMAC, Westbrook, ME). Details of the technique have been described in a recent publication.[27] After immunostaining was performed, a mean tumor growth fraction was determined in each case by counting the number of Ki-67-immunostained nuclei in each of 20 high-power fields. The growth fraction, or Ki-67 labeling index, was expressed as the percentage ratio of Ki-67-labeled nuclei to total nuclei.

Statistical Analysis and Outcome Criteria

Several statistical procedures were used to evaluate these data. In comparing the mean GHRH mRNA signal intensity between tumor groups, a one-way ANOVA was used, followed by either pairwise comparisons using Bonferroni corrected *P* values or linear orthogonal contrasts, depending on the nature of the comparison. To test for linear association between continuous variables, scatterplots were constructed and the Pearson correlation coefficient (*r*) was derived. To determine the prognostic relevance of tumor GHRH mRNA signal intensity and other clinicopathological parameters in predicting remission likelihood, a logistic regression model was fitted. For this analysis, surgical outcome was considered a dichotomous outcome variable, being categorized as either remission or no remission and defined exclusively on the basis of postoperative dynamic endocrine testing. Postoperative remission was defined on the basis of the single, stringent, and widely accepted criterion of suppression of serum GH levels to less than 2 ng/ml during an oral glucose tolerance test, performed 1 month postoperatively.[6,11,28,29] Values above this threshold were considered surgical failures, regardless of the degree of lowering of basal GH levels. Given the era in which the majority of these patients had been treated, the currently preferred criterion of a normalized serum insulin-like growth factor-1 level was not routinely unavailable. Of particular methodological importance is the fact that a single surgeon had operated upon all cases in the primary study population and a single surgeon also operated upon all tumors in the secondary test population. As both surgeons have specific expertise with pituitary surgery, the occurrence of an unsuccessful outcome should be viewed as a reflection of the aggressiveness of the tumor that precluded its complete removal, rather than technical inexperience on the part of the surgeon. Once the outcome model was derived, its generalizability in predicting the remission status of the secondary (Mayo Clinic) test population was studied; a χ^2 analysis was used to compare predicted *versus* actual surgical outcomes.

For all statistical analysis, two-tailed probability values less than 0.05 were designated as significant. All mean values are reported as mean ± SEM. Statistical analyses were performed using SAS system software version 6.10 (SAS Institute, Cary, NC).

Results

GHRH mRNA Transcripts in Nontumorous Control Pituitaries

In all nontumorous control pituitaries, the GHRH mRNA signal was low (mean, 3.31 ± 0.43 silver grains/cell; range, 0 to 8.5), with most examples exhibiting only background levels of signal (Figure 1A). The mean GHRH mRNA signal was comparable in both the autopsy and surgical controls (2.72 ± 0.45 *versus* 4.52 ± 0.85), values within the range of background signal (ie, <5 silver grains/cell). In a small subgroup of nontumorous control specimens (3 of 20 autopsy glands and 4 of 10 surgical nontumorous pituitaries), the GHRH signal in somatotrophs, although still low, did exceed background levels (mean, 7.04 ± 0.54 silver grains/cell). In some of these specimens, the signal was not confined to somatotrophs only, as low-level signal could also be seen in occasional basophilic and chromophobic cells; no signal was evident in the posterior lobe.

GHRH mRNA Transcripts in Somatotroph Adenomas

In contrast to the nontumorous pituitary in which the GHRH mRNA signal was absent or low, transcript accumulation was detectable in 91 of 100 somatotroph adenomas. Moreover, the mean GHRH signal intensity among tumors was 18.17 ± 1.45 silver grains/cell (range, 0 to 56.7), a value significantly higher than that observed in the control groups (one-way ANOVA, *post hoc* linear contrast, F ratio = 15.63, $P < 0.001$; Figure 1B). Differences in the GHRH signal intensity between the tumor and normal glandular tissue were especially obvious in some surgical specimens for which the border between tumor and surrounding normal gland was present (Figure 1C).

Co-Localization of GHRH mRNA and Immunoreactive GH

When ISH for GHRH mRNA was combined with immunohistochemistry for GH, the signal was co-localized with the immunoreaction in the same tumor cells. In some cases, tumor cells having the highest GHRH mRNA signal intensity were seen to also exhibit the strongest immunoreactivity for GH (Figure 1D).

Northern Analysis

A single and appropriately sized GHRH transcript of approximately 0.75 kb was detected in 9 of 10 somatotroph

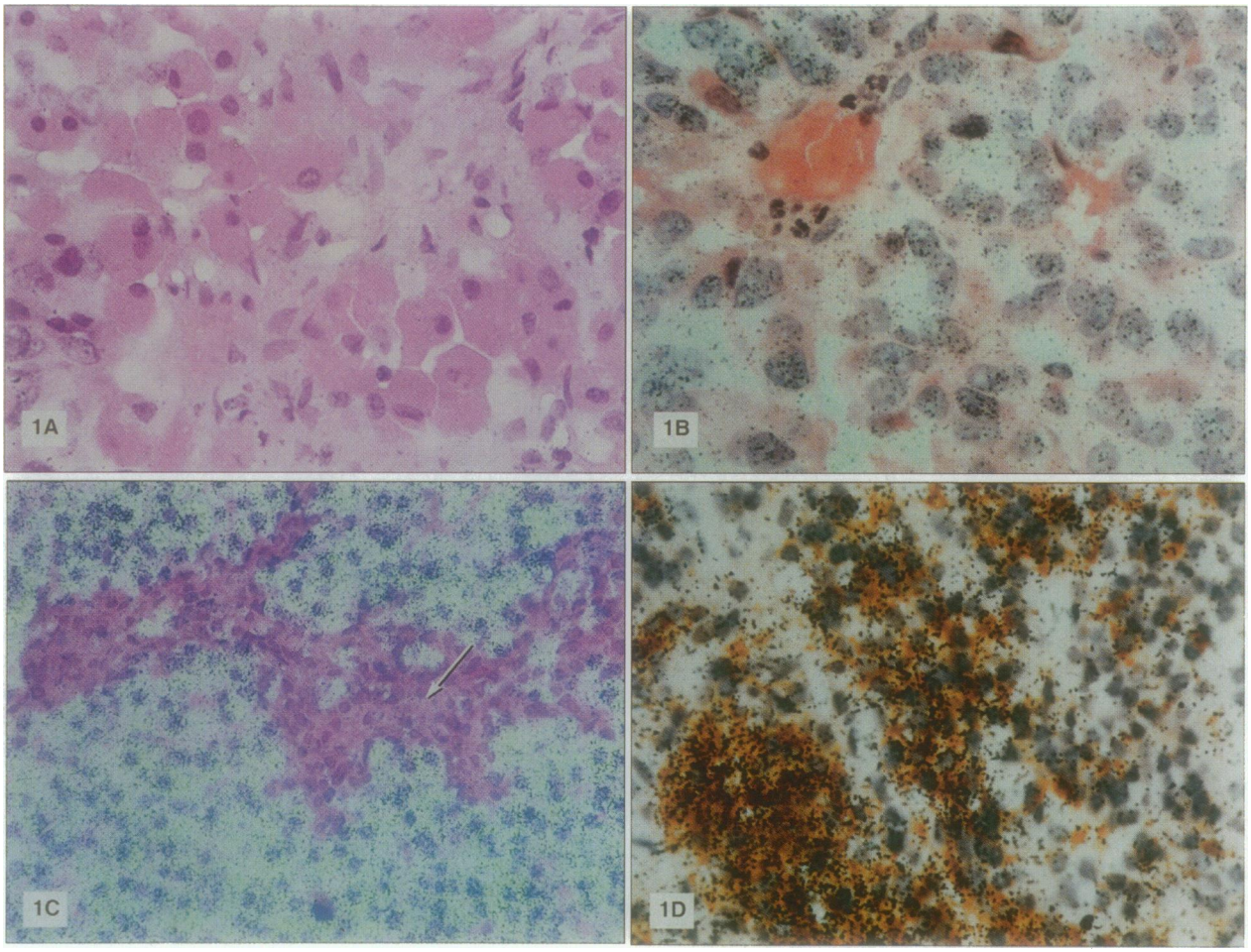

Figure 1. ISH for GHRH mRNA. **A:** In the normal autopsy pituitary gland, only a background level of signal is seen. Hematoxylin; magnification, ×400. **B:** This contrasts with the strong hybridization signal diffusely present in the somatotroph adenoma. Hematoxylin, magnification, ×400. **C:** Somatotroph adenoma containing a tongue of nontumorous tissue is seen (**arrow**). Note the selective localization of the hybridization signal only within tumor cells and not within entrapped normal gland. Hematoxylin; magnification, ×200. **D:** ISH for GHRH mRNA was combined with immunohistochemistry for GH. Note that cells having the most intense GHRH mRNA signal are also those exhibiting the strongest immunoreactivity for GH (brown color). GH immunostain; magnification, ×200.

adenomas tested, although some variability in the level of expression was noted (Figure 2A). The level of GHRH message was higher among invasive adenomas as compared with noninvasive adenomas and in those adenomas with higher growth fractions. No GHRH message could be detected in the normal gland. When blots were re-probed with a GHRH-R probe, all somatotroph adenomas also expressed the receptor mRNA. A full-sized 4.0-kb GHRH-R mRNA transcript was identified in all tumors and in the autopsy pituitary control (Figure 2B). The level of GHRH-R message was fairly similar in all tumors, suggesting a constitutive level of expression. In particular, there was no evidence of GHRH-R down-regulation even when the level of GHRH message was high.

RT-PCR Verification of GHRH Gene Expression

In 10 of 10 cases studied, RT-PCR resulted in selective amplification of the predicted 235-bp fragment of the GHRH gene, being represented as a discrete band of expected size (Figure 3). No amplification was seen in either the normal gland or in the negative control (corticotroph adenoma).

GHRH Immunohistochemistry and Western Blot Analysis

Of the 30 control nontumorous pituitaries, all of which were formalin fixed and paraffin embedded, GHRH immunoreactivity could not be demonstrated in a single instance despite application of vigorous antigen retrieval methods.[25] Even in the seven examples for which low-level GHRH transcript accumulation was demonstrated by ISH, all cells were uniformly immunonegative for GHRH. Of 20 somatotroph adenomas studied by immunohistochemistry, all of which expressed high levels of GHRH message, conclusive cellular localization of GHRH protein could be demonstrated in only two cases.

In 9 of 10 somatotroph adenomas, Western blotting revealed a dominant band of approximately 5 kd, corresponding to the size of the mature GHRH peptide and having the same migrational characteristics as synthetic GHRH peptide (positive control) (Figure 2C). In seven specimens, secondary bands between 6 and 16 kd were also seen, corresponding the size range expected of the pro-GHRH precursor protein. In the autopsy control, nei-

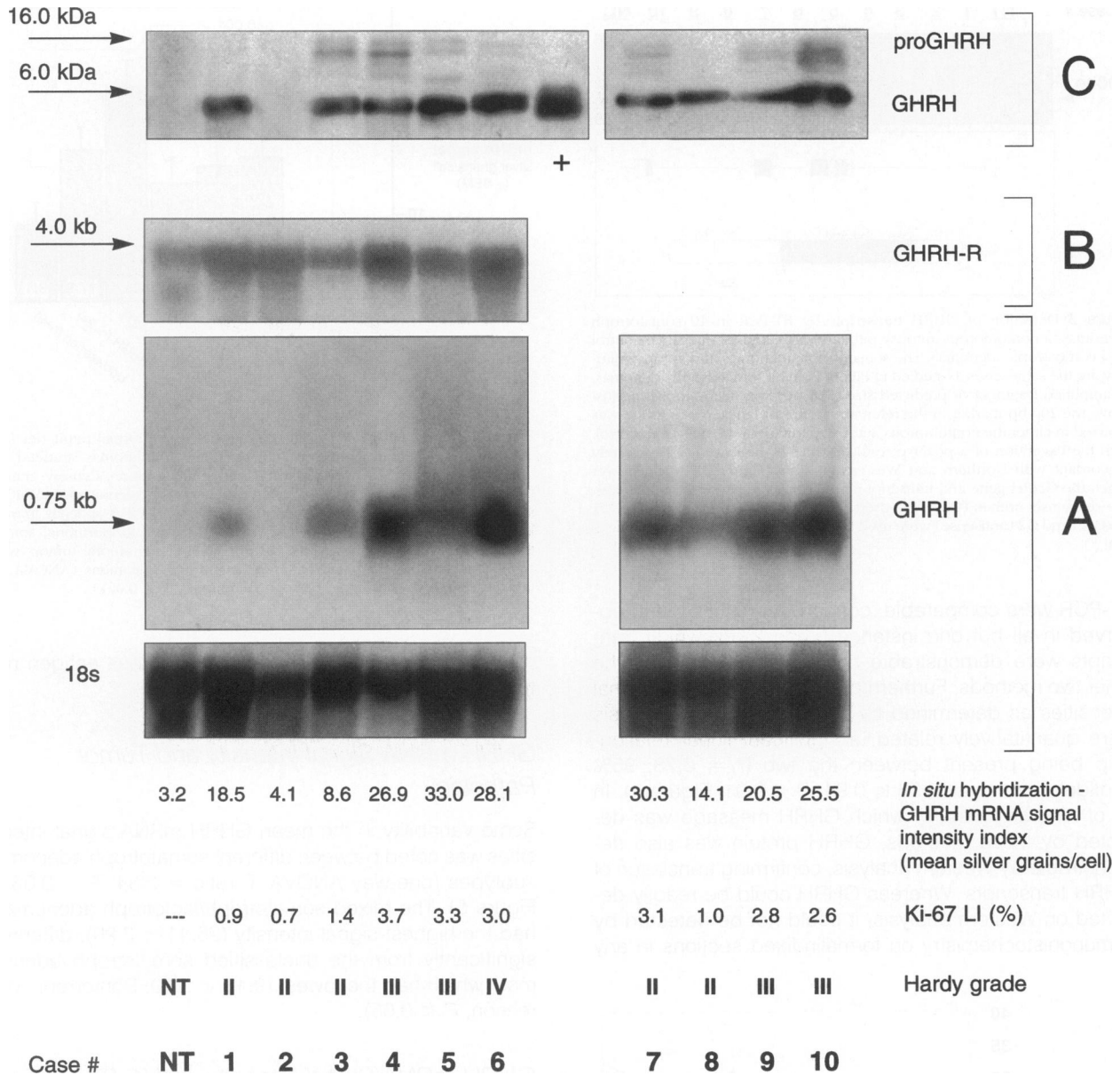

Figure 2. Northern analysis for GHRH (**A**) and GHRH-R (**B**) and Western analysis for GHRH (**C**) in 10 somatotroph adenomas and in 1 autopsy nontumorous pituitary gland (NT). For each tumor, the ISH GHRH mRNA signal intensity, Ki-67 labeling index, and the radiological (Hardy) grade are noted. **A:** Northern analysis for GHRH reveals a single, appropriately sized transcript of 0.75 kb in 9 of 10 tumors but not in the autopsy pituitary gland. The GHRH band intensity is clearly higher among invasive tumors and in those with higher Ki-67 labeling indices. As a loading control, the membrane was probed for the 18 S ribosomal RNA fraction. **B:** When reprobed for GHRH-R, a single transcript of ~4.0 kb was identified in all of 6 tumors and in the normal gland. Note that little variation is seen in the band intensity between cases, suggesting a constitutive level of expression. **C:** Western analysis for GHRH reveals the presence of an appropriately sized (~5-kd) band having the same migrational characteristics as synthetic GHRH peptide (positive control (+)) in 9 of 10 somatotroph adenomas. In addition, secondary bands in the 6- to 16-kd range are also seen in 7 of 10 cases, corresponding to the expected size of the pro-GHRH precursor protein. In the autopsy pituitary (NT), neither precursor nor mature proteins are seen. Complete concordance is seen between results of ISH/Northern analysis and Western analysis in detecting GHRH transcripts and protein, respectively. The quantitative relationship between ISH and Northern analysis in these cases is more precisely depicted in Figure 4.

ther the mature GHRH peptide nor the pro-GHRH precursor was present. Qualitatively, the amount of GHRH protein appeared higher in some invasive adenomas (cases 5, 6, and 10). As the purpose of Western analysis was purely to demonstrate protein translation, and given the small number of samples available for study, protein quantification and formal comparisons were not undertaken.

Concordance between ISH and Other Methods of GHRH mRNA and Protein Detection

In 10 tumors and 1 autopsy pituitary gland, ISH, Northern analysis, RT-PCR, Western analysis, and GHRH immunohistochemistry were performed (Figures 2 and 3). In detecting GHRH message, ISH, Northern analysis, and

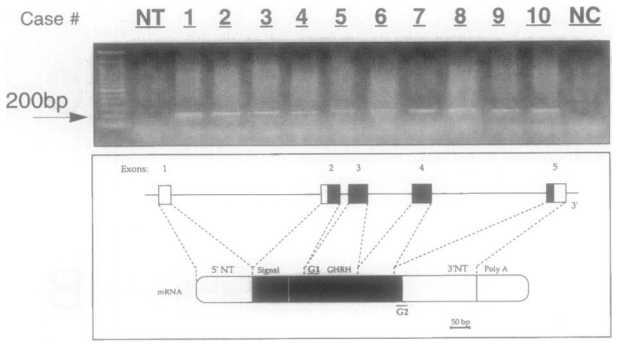

Figure 3. Detection of GHRH transcripts by RT-PCR in 10 somatotroph adenomas, a nontumorous autopsy pituitary (NT), and in a negative control (NC, corticotroph adenoma). The somatotroph adenomas and autopsy pituitary are the same cases as studied in Figure 2. In all somatotroph adenomas, an amplified fragment of predicted size (235 bp) was seen, appearing just above the 200-bp marker in the reference (leftmost lane). No message was detected in either the nontumorous autopsy pituitary or the negative control. With the exception of a positive result in case 2, these results are entirely concordant with Northern and Western analysis (Figure 2). In the lower panel, the GHRH gene and transcript map is shown, including primer sites. The antisense primer (G2) was used for the RT reaction, and both G1 (coding) and G2 (antisense) were used for PCR (modified from Wakabayashi et al[16]).

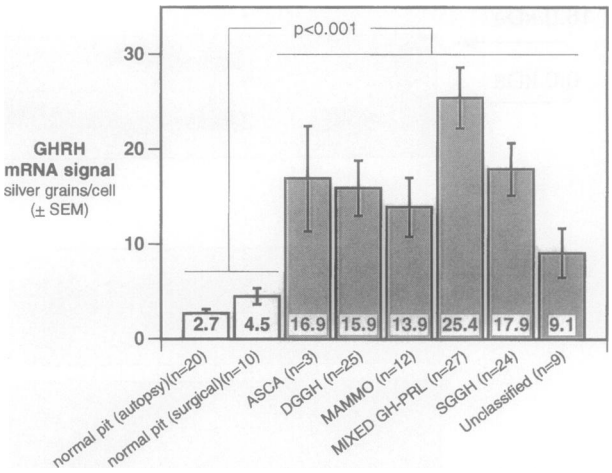

Figure 5. Mean GHRH mRNA signal intensities in 30 normal pituitaries (20 autopsy and 10 surgical) and in 100 somatotroph adenomas stratified by tumor ultrastructure: acidophil stem cell adenoma (ASCA), densely granulated GH cell adenoma (DGGH), mammosomatotroph adenoma (MAMMO), mixed somatotroph-lactotroph adenoma (MIXED GH-PRL), sparsely granulated GH cell adenoma (SGGH), and unclassifiable plurihormonal somatotroph adenomas. As shown, the mean GHRH signal among tumors was significantly higher than that observed in normal specimens (ANOVA, F ratio = 15.63, *post hoc* linear orthogonal contrast, $P < 0.001$).

RT-PCR were comparable, concordant results being observed in all but one instance (case 2) for which transcripts were demonstrable by RT-PCR but not by the other two methods. Furthermore, the GHRH mRNA signal intensities as determined by ISH and Northern analysis were quantitatively related, a significant linear relationship being present between the two ($r = 0.78$; 95% confidence interval, 0.33 to 0.94; $P < 0.01$; Figure 4). In all of nine samples for which GHRH message was detected by these methods, GHRH protein was also demonstrable by Western analysis, confirming translation of GHRH transcripts. Whereas GHRH could be readily detected on Western analysis, it could not be detected by immunohistochemistry on formalin-fixed sections in any of these cases, despite the use of vigorous antigen retrieval.

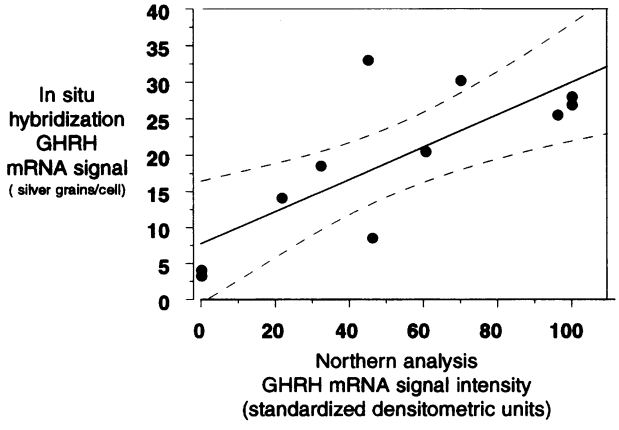

Figure 4. Scatterplot analysis revealing the relationship between GHRH mRNA signal intensities as determined by ISH and Northern analysis. For Northern analysis, densitometric values represent GHRH band densities internally standardized for loading variation by the amount of 18 S ribosomal RNA fraction in each lane. Note the positive linear correlation that exists between these two forms of transcript detection ($r = 0.78$; 95% confidence interval, 0.33 to 0.94; $R^2 = 0.60$; $P = 0.0035$). Dotted lines represent the 95% confidence intervals around the expected mean GHRH mRNA signal for any given densitometric value on Northern analysis.

GHRH mRNA Signal Intensity and Tumor Pathology

Some variability in the mean GHRH mRNA signal intensities was noted between different somatotroph adenoma subtypes (one-way ANOVA, F ratio = 2.54; $P = 0.033$; Figure 5). The mixed somatotroph/lactotroph adenomas had the highest signal intensity (25.41 ± 3.20), differing significantly from the unclassified somatotroph adenomas, which had the lowest (9.10 ± 2.60; Bonferroni correction, $P < 0.05$).

GHRH mRNA Signal Intensity and Ki-67 Labeling Index (Tumor Growth Fraction)

The MIB-1 antibody, conclusively discriminating proliferating from quiescent cells on the basis of nuclear expression of the Ki-67 antigen, permitted reliable quantification of the proportion of cycling cells and the derivation of a Ki-67 labeling index, or tumor growth fraction (Figure 6A). In comparing GHRH mRNA signal intensity with Ki-67-derived tumor growth fractions, a highly significant and positive linear correlation was observed ($r = 0.71$; $P < 0.001$; Figure 6B).

GHRH mRNA Signal Intensity and Tumor Size/Invasion Status

All tumors were graded according to size, invasion status, and radiological appearance according to the Hardy classification.[30] Of grades 0 to I, II, III, and IV, the primary

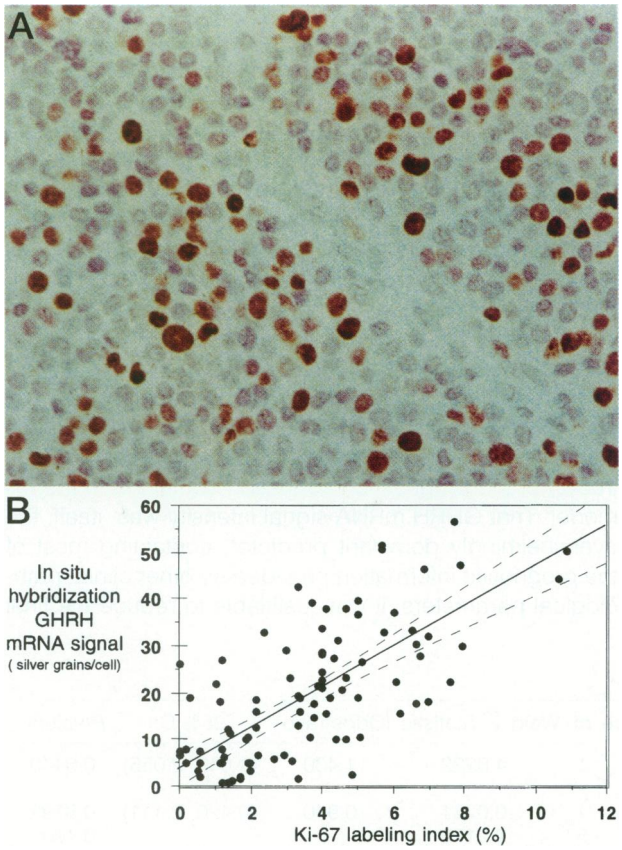

Figure 6. A: The Ki-67 nuclear antigen, present in G1, S, G2, and M phases of the cell cycle, as revealed by immunohistochemistry in an unusually aggressive somatotroph adenoma using the MIB-1 antibody. This method reliably distinguishes proliferating from noncycling cells. MIB-1 immunostaining, methyl green counterstain; magnification, ×200. **B:** Scatterplot analysis of GHRH mRNA signal intensity *versus* Ki-67 labeling index reveals the positive linear correlation that exists between these two variables ($r = 0.71$; $P < 0.001$). Dotted lines represent the 95% confidence intervals around the expected mean GHRH mRNA signal for any given Ki-67 labeling index.

study group was represented by 9, 42, 31, and 18 tumors, respectively. The mean GHRH signal intensity among invasive adenomas (grades III and IV) was significantly higher than that of noninvasive adenomas (grades 0 to II; 23.36 ± 2.0 *versus* 13.19 ± 1.84, ANOVA, F ratio = 5.02, *post hoc* linear orthogonal contrast, *t*-statistic = 3.56, $P = 0.001$; Figure 7).

The mean GHRH mRNA signal intensity of microadenomas (grades 0 and I) was lower than that of macroadenomas (grades II to IV; 11.05 *versus* 18.88 silver grains per cell). This difference, although of conceptual importance, fell short of statistical significance (*post hoc* linear orthogonal contrast, *t*-statistic = 1.97, $P = 0.08$). Given that there were only nine microadenomas in this series, there was insufficient statistical power to ascribe significance to this trend.

GHRH mRNA Signal Intensity and Preoperative Serum GH Levels

In all patients, multiple basal determinations of the preoperative serum GH level had been made, the mean of

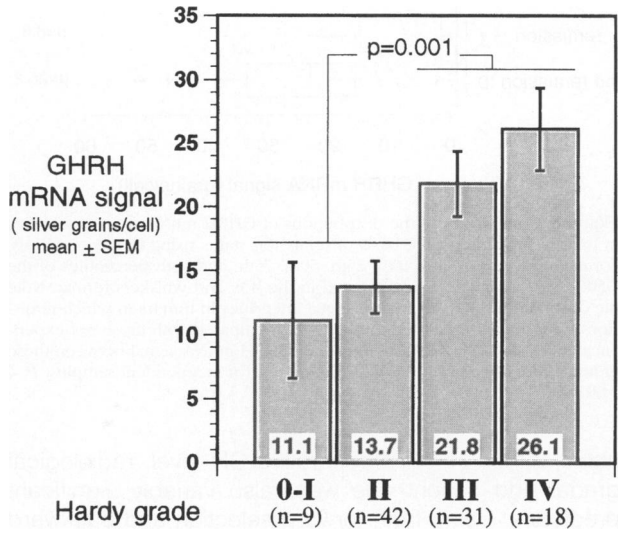

Figure 7. Mean GHRH mRNA signal intensity in tumors stratified on the basis of size and invasiveness according to the radiological classification of Hardy. The mean GHRH mRNA signal of invasive tumors (grades III and IV) was significantly higher than that of noninvasive tumors (grades 0, I, and II; ANOVA, F ratio = 5.02; *post hoc* linear orthogonal contrast, $P < 0.001$). The mean GHRH signal was higher in macroadenomas (II, III, and IV) as compared with microadenomas (0 and I); however, this difference did not reach statistical significance ($P = 0.08$.)

which was used for comparison. A significant positive linear correlation was noted between the mean preoperative GH level and tumor GHRH mRNA signal intensity ($r = 0.56$, $P < 0.01$).

GHRH mRNA Signal Intensity and Surgical Outcome

Response to surgical therapy was considered a dichotomous outcome variable defined solely on the basis of postoperative suppressibility of the serum GH level to less than 2 ng/ml during an oral glucose tolerance test. Based on this criterion, remission was achieved in 43 of 100 patients. Although nearly all of the remaining 57 patients experienced substantial declines in basal GH levels, their failure to suppress below the established threshold placed them in the no remission category. The mean GHRH mRNA signal intensity observed in tumors in which remission was achieved was markedly lower than in those in which it was not (8.79 ± 1.4 *versus* 25.2 ± 1.8; two-sample *t*-test for independent samples, *t*-statistic = 7.13; $P < 0.001$; Figure 8).

To delineate more precisely the relationship between GHRH mRNA transcript accumulation and remission likelihood, particularly as compared with other predictors currently used in clinical practice, an outcome model was developed by means of logistic regression. First, univariate analysis was performed to assess the prognostic relevance of the following predictors: patient age, sex, tumor pathology, tumor size, and invasion status (Hardy grade), mean preoperative GH level, Ki-67 labeling index, and GHRH mRNA signal intensity. As shown in Table 1, GHRH mRNA signal intensity and the Ki-67 labeling index were the most significant prognostic vari-

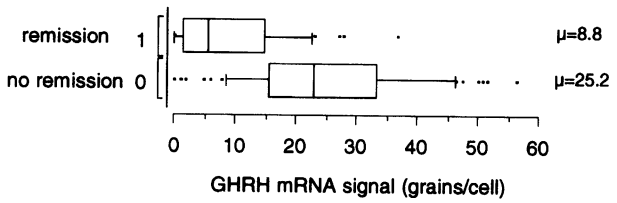

Figure 8. Comparison of the distributions of GHRH mRNA signal intensities in tumors stratified on the basis of remission status using boxplot analysis. For each population, the 10th, 25th, 50th, 75th, and 90th percentiles of the GHRH mRNA signal are represented in the box and whiskers format. Note the distribution of GHRH mRNA signal intensities in tumors in which remission was achieved is shifted to the left, as compared with those not experiencing remission. In addition, the mean GHRH mRNA signal between these groups differs significantly (two-sample *t*-test for independent samples, $P < 0.001$).

ables, although the preoperative GH level, radiological grade, and patient age were also variably significant predictors. Using both forward selection and backward elimination in delineating the most stable model possible, the significance of these predictors was evaluated. Regardless of the modeling strategy used, only GHRH mRNA signal intensity ($P < 0.005$) proved to be a consistently significant predictor in every model having satisfactory goodness of fit. Conventional clinicopathological predictors such as Ki-67 labeling index, the mean preoperative GH level, and the tumor size/invasion status, although each being of variable significance as univariate predictors, lost their explanatory contribution once GHRH mRNA signal intensity was entered into the model. In the saturated multivariate model, in which all relevant predictors from univariate analysis were present, only GHRH mRNA signal intensity retained predictive significance (Table 2). The likelihood ratio χ^2 statistic for the saturated model was 51.27, whereas that of a univariate model containing only GHRH mRNA signal intensity was 39.31. This implies that the GHRH mRNA signal intensity alone represented 77% (ie, 39.31/51.27 × 100%) of the prognostic information contained in the full model. That GHRH mRNA signal intensity was, itself, the overwhelmingly dominant predictor, containing most of the prognostic information provided by other clinicopathological parameters, it was justifiable to reduce the final

Table 1. Univariate Analysis: Postoperative Remission Likelihood

Variable	Category	Remission prevalence	df	Wald χ^2 Statistic	Odds ratio*	95% CI	*P* value
Age			1	4.0222	1.430	(1.016, 2.055)	0.0449
Sex	female†	43.9%					
	male	42.4%	1	0.0231	0.940	(0.420, 2.111)	0.8792
Pathological subtype			5	9.2179			0.1007
	Unclassified GH cell†	44.4%	1				
	Acidophil stem cell	33.3%	1	0.1133	0.625	(0.024, 9.157)	0.7364
	Densely granulated	60.0%	1	0.6408	1.875	(0.402, 9.300)	0.4234
	Mammosomatotroph	66.7%	1	1.0177	2.500	(0.431, 16.158)	0.3131
	Mixed GH-PRL	29.6%	1	0.6564	0.526	(0.109, 2.603)	0.4178
	Sparsely granulated	29.2%	1	0.6769	0.515	(0.103, 2.609)	0.4107
Radiological grade			3	10.9898			0.0118
	Hardy grade 0–I†	66.6%					
	Hardy grade II	57.1%	1	0.2753	0.667	(0.127, 2.895)	0.5998
	Hardy grade III	32.3%	1	3.1801	0.238	(0.043, 1.094)	0.0745
	Hardy grade IV	16.7%	1	5.8912	0.100	(0.013, 0.582)	0.0152
Preoperative GH level			1	7.1317	0.793	(0.658, 0.925)	0.0076
Ki-67 labeling index			1	19.9018	0.546	(0.409, 0.698)	0.0001
GHRH mRNA signal			1	23.2279	0.296	(0.172, 0.464)	<0.0001

*The odds ratios for age, preoperative GH, and GHRH mRNA signal intensity are given for a 10-U increase in the variable.
†For categorical variables, the dagger marks the reference category to which other members of the group were compared.

Table 2. Analysis of Maximal Likelihood Estimates (Full Model)

Variable	df	Parameter estimate (β)	χ^2	*P* value	Odds ratio
Intercept	1	0.8167	0.2236	0.6363	
GHRH	1	−0.1054	7.9954	0.0047	0.900
Preoperative GH	1	−0.0022	0.0379	0.8456	0.998
Ki-67	1	−0.1838	0.9373	0.3330	0.832
Age	1	0.0189	0.5687	0.4508	1.019
Tumor pathology	5		5.8932	0.3168	
Acidophil stem cell adenoma	1	0.5258	0.1135	0.7362	1.692
Densely granulated	1	1.6216	2.6275	0.1050	5.061
Mammosomatotroph	1	1.6233	2.0842	0.1488	5.070
Mixed GH-PRL	1	0.6512	0.4213	0.5163	1.918
Sparsely granulated	1	−0.0359	0.0014	0.9699	0.965
Radiologic grade	3		0.8158	0.8457	
Hardy grade II	1	−0.3862	0.1360	0.7122	0.680
Hardy grade III	1	−0.3108	0.0771	0.7813	0.733
Hardy grade IV	1	−0.9925	0.6280	0.4281	0.371

Likelihood ratio χ^2 statistic = 51.266 (12 df).

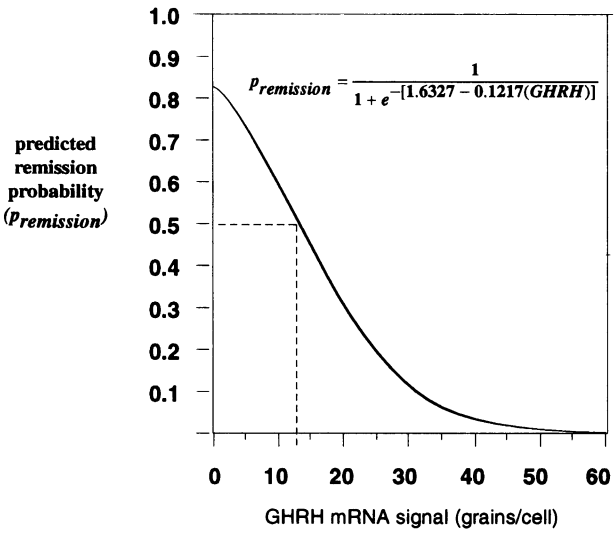

Figure 9. Plot of predicted postoperative remission probabilities *versus* the tumor GHRH mRNA signal intensity (n = 100). This plot, based on the logistic function constituting our outcome model, reveals that increases in GHRH mRNA signal intensity are associated with precipitous declines in postoperative remission likelihood. The GHRH signal corresponding to a 50% remission likelihood is noted (13.4 silver grains/cell).

fitted model to a univariate model containing GHRH mRNA signal intensity as the sole explanatory variable. In doing so, it also allowed the statistical effect of GHRH mRNA signal intensity on postoperative outcome to be clearly isolated. The final fitted model, where $P_{remission}$ is the probability of remission, is as follows:

$$\text{Logit}\left[\frac{p_{remission}}{(1 - p_{remission})}\right] = 1.6327 - 0.1217(\text{GHRH}),$$

$$p_{remission} = \frac{1}{1 + e^{-[1.6327 - 0.1217(GHRH)]}}. \quad (1)$$

As illustrated in Figure 9, the effect of GHRH transcript accumulation was strongly adverse; increases in GHRH mRNA signal intensity were associated with precipitous declines in remission likelihood. For example, an increment in GHRH mRNA signal intensity of 10 silver grains per cell was associated with more than a threefold reduction in the odds favoring remission (odds ratio = 0.30; 95% confidence interval, 0.18 to 0.49).

To validate the adequacy with which the derived logistic model represented our data, two standard goodness-of-fit criteria were evaluated.[31] First, a Hosmer-Lemeshow statistic was calculated (χ^2 = 2.89, 8 df, P = 0.94); its lack of significance legitimized our acceptance of adequate model fit. Second, the area under the receiver operator characteristic curve for representing this model was high (c = 0.84), indicating both good fit and high predictive accuracy for this model. As discussed below, a third validation of good model fit was provided by the model's satisfactory performance in a secondary patient population.

Given that all clinical data had been collected retrospectively and that patients were referred back to their primary physicians for ongoing care, the only endpoint consistently shared by all patients was the 1-month postoperative check, at which point a determination of remission status was made by one of us (D. W. Killinger). Whereas this fulfilled the immediate objectives of this study, it was not possible to determine from the available data the long-term prognostic relevance of GHRH mRNA transcript accumulation from such standpoints as tumor recurrence or relapse-free survival. Of patients in whom remission was achieved, all patients were referred back to their primary care physicians, and many were lost to follow-up. Of patients in whom surgical remission was not achieved, postoperative management was not uniform. Virtually all patients received one or more forms of adjuvant therapy (somatostatin analogues, radiation therapy, dopamine agonists, or repeat surgery). This lack of uniformity in postoperative management among the surgical failures made assessment of tumor regrowth and endocrine relapse problematic, particularly when attempting to isolate the effect of GHRH mRNA transcript accumulation from the effects of postoperative adjuvant therapies.

Outcome Prediction in a Secondary Test Population

In evaluating tumor samples from the secondary population, the investigators were blinded to all information except the pathological subtype of the tumor. In each case, ISH for GHRH mRNA was performed and the signal intensity quantified as described. Based only on the GHRH mRNA signal intensity, the derived outcome model (Equation 1) was applied and the probability of surgical remission was predicted for each case. A cutoff probability for remission of 0.5 was selected. Accordingly, when the model predicted a remission probability of greater than 0.5, the case was designated as a predicted remission; alternatively, values of 0.5 or less were designated as predicted failures. Predicted results were compared with actual results using a contingency table analysis (Table 3). The model correctly predicted the actual surgical outcome in 22 of 30 (73.3%) of cases (continuity adjusted χ^2 = 5.17, 1 df, P = 0.023). Correctly predicting 13 of 19 successes and 9 of 11 failures, the model had a sensitivity and specificity in predicting remission of 68 and 82%, respectively.

Table 3. Contingency Table Analysis of Predicted Remission Status (Toronto Model) and Actual Remission Status in the Secondary (Mayo Clinic) Patient Sample

Predicted remission status (Equation 1)	Actual remission status (Mayo Clinic patients)		
	Remission	No remission	Totals
Remission	13	2	15
No remission	6	9	15
Totals	19	11	30

Continuity corrected, χ^2 = 5.17, 1 df, P = 0.023.

Discussion

The biological behavior of pituitary adenomas is known to vary greatly. Some adenomas, such as those found in up to 20% of unselected autopsies, show neither a capacity for growth nor a capacity for hormone secretion and are thus relegated to a subclinical existence as incidental autopsy findings.[1] Others will manifest clinically; however, their noninvasive nature, limited growth capacity, and overall indolent character lend themselves to curative resection and a durable endocrinological remission. Finally, there are those adenomas that assume a more aggressive phenotype, being so prone to invasive, destructive growth and recurrence that they defy any and all therapeutic intervention. In terms of the multistep model of human tumorigenesis, the problem can be viewed as one of neoplastic progression; however, the actual events underlying the process are poorly understood.[32,33] Of the various pituitary adenoma subtypes, the problem of neoplastic progression appears especially relevant to GH-producing adenomas, of which a disproportionately large number are already invasive macroadenomas at the time of presentation[4].

Herein, we have identified accumulation of GHRH mRNA transcripts as a potentially important event in the progression of GH-producing pituitary tumors. In the normal nontumorous pituitary, GHRH mRNA transcripts were either absent (23/30) or present at very low levels (7/30) only. In contrast, transcript accumulation was evident in 91 of 100 consecutive GH-producing pituitary adenomas, the mean signal intensity being more than fivefold higher than that observed in normal pituitary specimens. Of greatest importance, however, was the significant relationship between transcript accumulation and tumor behavior. Our finding of a positive correlation between the GHRH mRNA signal intensity and Ki-67 labeling index, together with the observation that the mean GHRH mRNA signal among invasive adenomas was almost twice that of noninvasive adenomas, indicates preferential accumulation of GHRH transcripts in tumors capable of growth and invasion. That GHRH transcript accumulation is also associated with endocrine aggressiveness was evidenced by the positive linear correlation between tumor GHRH mRNA signal intensity and mean preoperative GH levels. When evaluated together with other conventional parameters of tumor aggressiveness, including tumor size and invasiveness, Ki-67 labeling index, tumor pathology, and preoperative GH levels in a multivariate model of surgical outcome, the degree of GHRH transcript accumulation eclipsed all other parameters as the single most significant explanatory variable. In fact, once GHRH mRNA signal intensity was entered into the logistic model, all remaining variables failed to make additional explanatory contribution. The statistical effect of GHRH mRNA transcript accumulation on outcome was strongly adverse, increments in GHRH mRNA signal intensity being associated with precipitous declines in remission likelihood. Finally, the reproducibility of the derived outcome model was validated by the model's significant performance in predicting the outcomes of a second population of patients with somatotroph adenomas treated at another institution, suggesting that these methods can be generalized to other populations of acromegalic patients.

As outlined previously, the only endpoint consistently shared by all patients was the 1-month postoperative check, at which point a determination of remission status was made on the basis of suppressibility of the GH level to <2.0 ng/ml during an oral glucose tolerance test. Although it is true that this outcome criterion is a short-term one, it is nonetheless a robust outcome criterion. Historically, when less stringent criteria were applied (ie, normalization of basal GH levels or suppressibility of GH levels to <5 ng/ml on oral glucose tolerance test), rates of recurrence were high, indicating that these patients did not in fact achieve a true remission; recurrence in this situation was the result of reconstitution/regrowth of residual tumor cells. In more recent surgical series, wherein suppressibility of GH to <2 ng/ml has been used as the remission criterion, tumor recurrence has been infrequent and patients typically experience a durable and disease-free remission over the long term.[6] Although this does not diminish the need for follow-up data concerning the relationship between GHRH mRNA transcript accumulation and long-term outcome, it does indicate that the outcome criterion employed in this study, despite its short-term and front-ended nature, does have some long-term prognostic relevance.

The culpability of GHRH as a potential contributor to neoplastic progression derives from several lines of physiological, pathological, and experimental evidence. First, GHRH is the principal positive regulator for pituitary somatotrophs, stimulating both their secretory function and proliferative activity.[11,12,34] Acting through specific membrane receptors, GHRH stimulates GH secretion and somatotroph proliferation through a variety of potent effector mechanisms that include 1) signal transduction by a classic stimulatory G-protein pathway, 2) activation of the protein kinase A/adenylate cyclase second messenger cascades, 2) recruitment of the inositol triphosphate/protein kinase C cascade, and 4) the induction of early response genes such as c-*fos*.[11,35–41] Given that GHRH is the most important physiological stimulator of pituitary somatotrophs, the potential oncological implications of pathological GHRH excess are readily apparent. In states of pathological GHRH excess, such as occurs in association with rare GHRH-producing tumors (eg, pancreatic endocrine tumors and carcinoid tumors), chronic GHRH stimulation leads to somatotroph hyperplasia, GH hypersecretion, and clinical acromegaly. In some instances, progression from somatotroph hyperplasia to adenomatous transformation has been well documented.[42,43] A parallel phenomenon has been demonstrated in transgenic mice bearing the human GHRH transgene. That these animals develop hypersomatotropism, elevated GH levels, somatotroph hyperplasia, and, eventually, GH-producing pituitary tumors provides compelling and conclusive evidence of the tumor-promoting potential of GHRH excess.[44–46] Finally, the discovery and characterization of the human oncogene *gsp* has further elucidated the tumor-promoting effect of a chronically activated GHRH stimulatory pathway.[36,40] Identified in up to 40% of somatotroph adenomas, mutations of this gene

involve the α-chain of the stimulatory GTP-binding protein that normally transduces the GHRH signal. The result is a constitutively activated G-protein that mimics a persistent GHRH stimulatory signal, culminating in adenomatous transformation of the affected cell. Activating mutations of *gsp* are regarded as transforming events only; for unknown reasons, their presence does not appear to confer aggressive behavior.[32,47]

Collectively, the above studies support the hypothesis that aberrant GHRH activity may play a role in the progression of somatotroph adenomas. Whereas recent demonstrations of GHRH gene and protein expression in somatotroph adenomas support this concept,[13–16,48] the clinical and biological implications of these findings had, until now, not been explored. The present study, in demonstrating accumulation of GHRH mRNA transcripts within a large series of somatotroph adenomas and systematically correlating their presence with clinically and biologically relevant differences in tumor behavior further strengthen this link. Furthermore, it strongly suggests that somatotroph adenomas are themselves both a local source of GHRH synthesis and a target for GHRH action. Our demonstration that both GHRH and GHRH-R mRNA transcripts are co-expressed in somatotroph adenomas points to GHRH-mediated autocrine and/or paracrine stimulation as one mechanism adversely associated with and/or contributing to their proliferative capacity, secretory activity, invasive potential, and surgical responsiveness. Crucial to the plausibility of such a mechanism is the fact that chronic exposure to GHRH appears unassociated with receptor desensitization in the neoplastic somatotroph. As reviewed by Spada and Lania,[49] patients with somatotroph adenomas continue to release GH after repeated GHRH injection, whereas normal subjects do not; among the latter, a marked refractoriness to repeated GHRH provocation is seen. The same lack of receptor desensitization has also been demonstrated by neoplastic somatotrophs *in vitro*.[50] In the present study, there was no GHRH-R down-regulation at the transcriptional level, as Northern analysis revealed a fairly constant and seemingly constitutive level of GHRH mRNA expression in all cases, including those in which the level of GHRH message was high. Whereas autocrine and/or paracrine stimulation are frequently invoked as somewhat generic tumor-promoting mechanisms in endocrine neoplasia, this is first comprehensive demonstration of the biological and clinical consequences that might accompany putative activity of this type in the pituitary. Implicit to this supposition of autocrine/paracrine stimulation are two fundamental requirements, both of which appear to be fulfilled in this tumor system: 1) increased local GHRH production and 2) a concomitant lack of GHRH-R down-regulation.

Our correlations have been based solely on levels of GHRH message as determined by ISH. The significant linear relationship demonstrated between ISH and Northern analysis does legitimize this approach as a valid means of quantifying the level of GHRH message. Of greater importance, however, is the confirmation that overexpressed GHRH transcripts are in fact translated into protein. That this does occur was evident from the observed concordance between the results of Western blotting and ISH. In addition to demonstration of the mature (~5-kd) immunoreactive form of GHRH, the frequent presence of a second larger (6- to 16-kd) immunoreactive species consistent with the expected size range of the pro-GHRH precursor molecule further confirms endogenous GHRH production by these tumors. The presence of the pro-GHRH precursor in these tumors was first demonstrated by Rauch et al,[13] who not only demonstrated a 10-kd pro-GHRH precursor in these tumors by Western analysis but also quantified pro-GHRH content using size-exclusion chromatography and also demonstrated GHRH immunoreactive cells within some pituitary adenomas. Our attempts at GHRH immunohistochemistry on formalin-fixed specimens, despite application of antigen retrieval, yielded negative results in all but two cases, including those in which the levels of GHRH mRNA was high by ISH. Even in the nine examples for which GHRH was demonstrable by Western blotting, immunohistochemistry was uniformly negative. This suggests that technical limitations rather than a failure of protein translation probably account for the observed negativity of GHRH immunohistochemistry in those tumors expressing high levels of GHRH message by ISH.

The significance of our observation that 7 of 30 nontumorous pituitaries expressed low levels of GHRH mRNA is uncertain. This finding is, however, consistent with previous demonstrations of pro-GHRH precursor within the normal pituitary as well as the release of mature GHRH peptide by normal somatotrophs *in vitro*.[13,48,51] The particular finding of Joubert et al[48] that normal pituitaries release less GHRH *in vitro* than do somatotroph adenomas is in keeping with the significantly higher levels of GHRH mRNA we observed in somatotroph adenomas as compared with nontumorous pituitaries. Still, the physiological significance of GHRH production by the normal pituitary is unclear. It has been proposed that GHRH may, in consort with the competing hypophysiotropic hormone somatostatin, exert some degree of local neuroendocrine control over normal pituitary function.[48,52]

In calling attention to a possible role for locally generated hypothalamic hormones in the progression of somatotroph adenomas, these data provide the basis of a new paradigm from which to view the biology and pathogenesis of these neoplasms. Historically, the development and progression of pituitary tumors have been the subject of two conceptually opposing theories. The hypothalamic hypothesis, a once dominant concept that engendered substantial experimental, clinical, and conceptual support, proposed that pituitary adenomas arose as the downstream consequence of a stimulatory imbalance of hypothalamic hormones emanating from a dysregulated hypothalamus.[32,33,53–55] With the demonstration that most human pituitary adenomas are monoclonal neoplasms, neither preceded nor accompanied by a phase of pituitary hyperplasia, this view has been subordinated in favor of the pituitary hypothesis, which suggests pituitary adenomas to be the result of subcellular alterations intrinsic to a single adenohypophyseal cell. Al-

though these theories have been considered mutually exclusive, the present data provide an important link between the two. In proposing that locally produced GHRH may modify the behavior of established adenomas, this model effectively merges pertinent components of the traditional hypothalamic hypothesis with the more contemporary pituitary hypothesis, and does so without violating the underlying essence of either. What emerges is a unified hypothesis that not only highlights the merits of existing theories but also addresses the problems of biological behavior and neoplastic progression, issues not readily reconciled by previous hypotheses.

Whereas our data suggest an unrecognized but biologically relevant level of regulatory control exercised by somatotroph adenomas and mediated via GHRH, it is equally probable that other locally generated hypophysiotropic hormones may exert comparable effects upon their respective tumor types. For example, mRNA transcripts for gonadotropin-hormone-releasing hormone,[56] thyrotropin-releasing hormone,[57] and corticotropin-releasing hormone[58] have been identified in various types of pituitary adenomas. Although the clinical effects of such expression are unknown, the fact that hypophysiotropic hormones are expressed in so broad a spectrum of pituitary tumor types suggests a more unifying and pervasive role of such hormones in pituitary tumor biology than is currently appreciated. Furthermore, as most normal adenohypophyseal cells are subject to dual coordinated regulation by both stimulatory and inhibitory hypophysiotropic hormones, aberrant activity of stimulatory hormones would appear to be only a part of the equation. Thus, it is equally plausible that locally generated inhibitory hypothalamic hormones may also modify the behavior of pituitary adenomas, perhaps in a clinically favorable way. In this regard, locally produced somatostatin may be of particular importance in modifying the behavior of somatotroph adenomas. Several investigators have demonstrated somatostatin gene and/or protein expression in somatotroph adenomas.[52,58,59] The pioneering studies of Peillon and colleagues are of special relevance in this context, as they have shown an inverse relationship between somatostatin mRNA levels and GH secretory activity in somatotroph adenomas[60] as well as a tendency for noninvasive adenomas to contain higher amounts of somatostatin precursor as compared with invasive adenomas.[52]

Having demonstrated that GHRH is overexpressed in aggressive pituitary tumors, the mechanisms responsible for this overexpression remain to be elucidated. The human GHRH gene has been localized to chromosome 20p12.1. The structure of its promoter region, including the transcriptional start site, have been characterized, but virtually nothing is known of the regulation of GHRH gene transcription in either health or disease.[12] The 5′ flanking region upstream of the transcription start site contains fairly typical TATA and CCAAT-like elements, ones devoid of any obvious vulnerability toward transcriptional activation, and thus provides few clues to the accumulation of GHRH transcripts described herein.

Conclusions

The present work comprehensively draws upon morphological, molecular, cell kinetic, and clinical data in an effort to evaluate the role of GHRH in the progression of GH-producing pituitary tumors. Using ISH, Northern analysis, and RT-PCR, overexpression of this gene was demonstrated within the majority of somatotroph adenomas tested, and protein translation was confirmed by Western blotting. In contrast, expression was absent or low in the nontumorous pituitary gland. As evidenced by the significant associations between GHRH mRNA signal intensity and proliferative activity, invasiveness, and preoperative GH levels, GHRH transcripts appear to preferentially accumulate among aggressive tumors not subject to surgical cure. That this is the case was further confirmed by our analysis of surgical outcome wherein not only was GHRH mRNA signal intensity a highly significant negative predictor of postoperative remission, but it also out-performed all currently known clinicopathological predictors of aggressive behavior. Finally, the generalizability of these conclusions were validated by the significant performance of our outcome model in a second population of somatotroph adenomas from another institution. Collectively, these results provide strong molecular, clinicopathological, and statistical evidence that overexpression of the GHRH gene is a prognostically relevant event associated with the neoplastic progression of GH-producing pituitary tumors. As such, it is among the first statistically validated demonstrations of a prognostically informative alteration in this tumor system. In light of the spectrum of other hypophysiotropic hormones known to be expressed by pituitary tumors, our findings suggest that pituitary adenomas can, in the course of their evolution, assume the capacity for local hypophysiotropic hormone production that may, in turn, serve to modify tumor behavior. Whereas the immediate objective of this report was to assess the mechanistic relevance of GHRH-mediated autocrine/paracrine stimulatory activity, and the data provided herein do support a relationship between the latter and aggressive behavior, there is the eventual possibility that determinations of GHRH gene and/or protein expression may also be of clinical utility as a prognostic marker, essentially gauging the inherent aggressiveness of somatotroph adenomas. At present, however, the diagnostic role of routine GHRH determinations in predicting the behavior of an individual somatotroph adenoma behavior is unclear. In that quantitative GHRH mRNA determinations by ISH or Northern analysis are both labor intensive and costly, routine clinical application of these techniques, as currently performed, would be difficult to justify, particularly in the absence of prospective data validating the long-term prognostic significance of such determinations. Certainly, the eventual availability of sensitive antisera that would permit immunohistochemical detection of GHRH protein should increase the feasibility of such determinations as routine diagnostic procedures, although the need for prospective data affirming the long-term prognostic significance of such expression would still be required before its diagnostic potential could be endorsed as a routine prac-

tice. Still, the current retrospective data, in showing a definitive and adverse relationship between GHRH overexpression and tumor growth kinetics, invasiveness, secretory activity, and the likelihood of immediate postoperative remission, provide the necessary foundation for future studies designed to assess long-term prognostic correlates of local GHRH mRNA/protein expression and to clarify the potential diagnostic utility of GHRH determinations. Until that time, however, such determinations are best regarded as investigative tools only. Measurement of serum GH levels or serum insulin-like growth factor-1 levels remain the most sensitive means of detecting tumor recurrence, although future studies will be required to determine whether GHRH gene/protein expression could predict, *a priori*, patients destined for recurrence and/or regrowth in advance of elevations in convention biochemical indices.

An important corollary to the mechanistic relationship demonstrated herein between local GHRH expression and tumor behavior is the possibility that GHRH and/or its downstream effectors may come to represent cellular targets amenable to therapeutic manipulation. The recent design and synthesis of GHRH antagonists as potential pharmacological agents for the adjuvant treatment of acromegaly certainly support such a view, especially in the context of the findings reported here.[61] In analogy to analogues of the hypophysiotropic hormone somatostatin, which have emerged as successful therapeutic adjuvants for acromegaly-associated tumors, GHRH antagonists may harbor similar therapeutic potential, allowing for a more comprehensive approach to the postoperative management of these frequently aggressive neoplasms.

Acknowledgments

We acknowledge the excellent technical assistance of Mrs. Anca Popescu, Mr. Fabio Rotondo, Mrs. Sherrilyn Hubbard, and Dr. Z. Cheng. We are grateful to members of Dr. Thapar's Ph.D. thesis advisory committee from the Department of Cellular and Molecular Pathology, University of Toronto (Drs. J.O. Minta, D. S. R. Sarma, E. Cutz, and J. T. Rutka) for their assistance throughout this project. This work was made possible through the generous contributions of Mr. and Mrs. Stephen Jaroslowsky (Montreal) and the Lloyd Carr-Harris Foundation (Toronto).

References

1. Thapar K, Kovacs K, Muller P: Clinical-pathologic correlations of pituitary tumors. Baillière's Clin Endocrinol Metab 1995, 9:243–270
2. Nabarro JDN: Acromegaly. J Clin Endocrinol 1987, 26:481–512
3. Molitch ME: Clinical manifestations of acromegaly. Endocrinol Metab Clin North Am 1992, 21:597–614
4. Scheithauer B, Kovacs K, Laws ER Jr, Randall R: Pathology of invasive pituitary tumors with special reference to functional classification. J Neurosurg 1986, 65:733–744
5. Laws ER Jr: Neurosurgical management of acromegaly. Contemporary Diagnosis and Management of Pituitary Adenomas. Edited by PR Cooper. Park Ridge, IL, American Association of Neurological Surgeons, pp 53–59
6. Fahlbusch R, Honegger J, Buchfelder M: Surgical management of acromegaly. Endocrinol Metab Clin North Am 1992, 21:669–692
7. Ross DA, Wilson CB: Results of transsphenoidal microsurgery for growth hormone-secreting pituitary adenoma in a series of 214 patients. J Neurosurg 1988, 68:854–867
8. Thapar K, Kovacs K, Laws ER: Pituitary tumors. Tumors of the Central Nervous System. Edited by PM Black, JJ. Loeffler. Cambridge, MA, Blackwell Science, 1997, pp 363–422
9. Thapar K, Laws ER: Transsphenoidal surgery for recurrent pituitary tumors. Operative Neurosurgery. Edited by AH Kaye, PM Black. New York, Churchill Livingstone (in press)
10. Laws ER, Chenelle AG, Thapar K: Recurrence after transsphenoidal surgery for pituitary adenomas: clinical and basic science aspects. Pituitary Adenomas: From Basic Research to Diagnosis and Therapy. Edited by K von Werder, R Fahlbusch. Amsterdam, Elsevier, pp 3–9
11. Thorner MO, Vance ML, Horvath E, Kovacs K: The anterior pituitary. Williams Textbook of Endocrinology. Edited by JD Wilson, DW Foster. Philadelphia, WB Saunders, 1992, pp 221–310
12. Mayo K, Godfrey P, Suhr S, Kulik D, Rahal J: Growth hormone-releasing hormone: synthesis and signaling. Rec Prog Hormone Res 1995, 50:35–73
13. Rauch C, Li J, Croissandeau G, Berthet M, Peillon F, Pagesy P: Characterization and localization of an immunoreactive growth hormone-releasing hormone precursor form in normal and tumoral human anterior pituitaries. Endocrinology 1995, 136:2594–2601
14. Kawamoto K, Uozumi T, Arita K, Takechi A, Kawamoto H: Secretion of growth hormone (GH)- releasing hormone by GH-producing pituitary adenoma assessed by cell immunoblot analysis. Endocr J 1995, 42:89–93
15. Levy A, Lightman S: Growth hormone-releasing hormone transcripts in human pituitary adenomas. J Clin Endocrinol Metab 1992, 74: 1474–1476
16. Wakabayashi I, Inokuchi K, Hasegawa O, Sugihara H, Minami S: Expression of growth hormone (GH)-releasing factor gene in GH-producing pituitary adenomas. J Clin Endocrinol Metab 1992, 74: 357–361
17. Mouhieddine OE, Levy L, Benlot C, Peillon F, Joubert D: Growth hormone (GH)-releasing hormone tonically inhibits in vitro endogenous somatostatin in human GH-secreting tumors. J Clin Endocrinol Metab 1995, 80:1691–1695
18. Mayo K, Vale W, Rivier J, Rosenfeld M, Evans R: Expression-cloning and sequence of cDNA encoding human growth hormone-releasing hormone. Nature 1983, 306:86–88
19. Stefaneanu L, Kovacs K, Horvath E, Lloyd R: In situ hybridization of pro-opiomelanocortin (POMC) gene expression in human pituitary corticotrophs and their adenomas. Virchows Arch Pathol Anat 1991, 419:107–113
20. Thapar K, Kovacs K, Horvath E, Stefaneanu L, Chambers E, Mortimer A: Effects of spaceflight on morphology of the rat adenohypophysis. J Appl Physiol 1994, 77:1411–1420
21. Thapar K, Stefaneanu L, Kovacs K, Scheithauer BW, Lloyd RV, Laws ERJ: Estrogen receptor gene expression in craniopharyngioma: an in situ hybridization study. Neurosurgery 1994, 35:1012–1017
22. Chomczynski P, Sacchi N: Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-choroform extraction. Anal Biochem 1987, 162:156–159
23. Gaylinn BD, Harrison JK, Zysk JR, Lyons CE, Lynch KR, Thorner MO: Molecular cloning and expression of a human anterior pituitary receptor for growth hormone-releasing hormone. Mol Endocrinol 1993, 7:77–84
24. Hsu SM, Raine L, Fanger H: The use of antiavidin antibody and avidin-biotin peroxidase complex in immunoperoxidase technics. Am J Clin Pathol 1981, 75:816–821
25. Taylor CR, Shan-Rong S, Chaiwun B, Young L, Imam SA, Cote RJ: Strategies for improving the immunohistochemical staining of various intranuclear prognostic markers in formalin-paraffin sections: androgen receptor, estrogen receptor, progesterone receptor, p53 protein, proliferating cell nuclear antigen, and Ki-67 antigen revealed by antigen retrieval techniques. Hum Pathol 1994, 25:263–270
26. Harlow E, Land DP: Immunoblotting. Antibodies: A Laboratory Manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1988, pp 471–510
27. Thapar K, Kovacs K, Scheithauer B, Stefaneanu L, Horvath E, Pernicone P, Murray D, Laws ER Jr: Proliferative activity and invasiveness

among pituitary adenomas and carcinomas: an analysis using the MIB-1 antibody. Neurosurgery 1996, 38:99–107
28. Melmed S: Acromegaly. The Pituitary. Edited by S Melmed. Cambridge, MA, Blackwell Science, 1995, pp 413–442
29. Klibanski A, Zervas NT: Diagnosis and management of hormone-secreting pituitary adenomas. N Engl J Med 1991, 324:822–831
30. Hardy J: Transphenoidal surgery of the normal and pathological pituitary. Clin Neurosurg 1969, 16:185–217
31. Hosmer DW, Lemeshow S: Applied Logistic Regression. New York, John Wiley and Sons, 1989, p 307
32. Melmed S: Etiology of pituitary acromegaly. Endocrinol Metab Clin North Am 1992, 21:539–551
33. Thapar K, Kovacs K, Laws ER: The classification and molecular biology of pituitary adenomas. Adv Tech Stand Neurosurg 1995, 22:4–53
34. Frohman L: Growth hormone-releasing hormone. Endocr Rev 1986, 7:223–253
35. Spada A, Vallar L, Faglia G: Cellular alterations in pituitary tumors. Eur J Endocrinol 1994, 130:43–52
36. Vallar L, Spada A, Giannattasio G: Altered Gs and adenylate cyclase activity in human GH secreting pituitary tumors. Nature 1987, 330: 566–567
37. Lei T, Arams E, Buchfelder M, Fahlbusch R: Relationship between protein kinase C and adenylyl cyclase activity in the regulation of growth hormone secretion by human pituitary somatotrophinomas. Neurosurgery 1996, 39:569–576
38. Billestrup N, Mitchell RL, Vale W, Verma IL: Growth hormone-releasing factor induces c-fos expression in cultured primary pituitary cells. Mol Endocrinol 1987, 1:300–305
39. Billestrup N, Swanson LW, Vale W: Growth hormone-releasing factor stimulates proliferation of somatotrophs in vitro. Proc Natl Acad Sci USA 1986, 83:6854–6857
40. Landis CA, Masters SB, Spada A, Pace AM, Bourne HR, Vallar L: GTPase inhibiting mutations activate the a chain of GS and stimulate adenylyl cyclase in human pituitary tumors. Nature 1989, 340:692–696
41. Frohman LA, Downs TR, Chomczynski P: Regulation of growth hormone secretion. Front Neuroendocrinol 1992, 13:344–405
42. Faglia G, Arosio M, Bazzoni N: Ectopic acromegaly. Endocrinol Metab Clin North Am 1992, 21:575–595
43. Sano T, Asa SL, Kovacs K: Growth hormone-releasing hormone-producing tumors: clinical, biochemical, and morphological manifestations. Endocr Rev 1988, 9:357–373
44. Lloyd RV, Jin L, Chang A, Kulig E, Campler SA, Ross BD, Downs TR, Frohman LA: Morphologic effects of hGRH gene expression on the pituitary, liver, and pancreas of MT-hGHRH transgenic mice: an *in situ* hybridization analysis. Am J Pathol 1992, 141:895–906
45. Asa S, Kovacs K, Stefaneanu L, Horvath E, Billestrup N, Gonzalez-Manchon C, Vale W: Pituitary adenomas in mice transgenic for growth hormone-releasing hormone. Endocrinology 1992, 131:2083–2089
46. Stefaneanu L, Kovacs K, Horvath E, Asa SL, Losinski NE, Billestrup N, Price J, Vale W: Adenohypophysial changes in mice transgenic for human growth hormone-releasing factor (hGRF): a histological, immunocytochemical and electron microscopic investigation. Endocrinology 1989, 125:2710–2718
47. Landis CA, Harsh G, Lyons J, Davis RL, McCormick F, Bourne HR: Clinical characteristics of acromegalic patients whose pituitary tumors contain mutant Gs protein. J Clin Endocrinol Metab 1990, 71: 1416–1420
48. Joubert D, Benlot C, Lagoguey P, Garnier P, Brandi A, Gautron J, Legrand J, Peillon F: Normal and growth hormone (GH)-secreting adenomatous human pituitaries release somatostatin and GH-releasing hormone. J Clin Endocrinol Metab 1989, 68:572–577
49. Spada A, Lania A: Hormone receptors in pituitary adenomas. Pituitary Adenomas. Edited by AM Landolt, ML Vance, PL Reilly. New York, Churchill Livingstone, 1996 pp 59–71
50. Spada A, Elahi FR, Arosio M, Sartorio A, Guglielmino L, Vallar L, Faglia G: Lack of desensitization of adenomatous somatotrophs to growth-hormone releasing hormone in acromegaly. J Clin Endocrinol Metab 1987, 64:585–91
51. Benlot C, Pagesy P, Peillon F, Joubert-Bression D: Growth hormone-releasing hormone and somatostatin in normal and tumoral human pituitaries. Peptides 1991, 12:945–950
52. Levy L, Bourdais J, Mouhieddine B, Benlot C, Villares S, Cohen P, Peillon F, Joubert D: Presence and characterization of the somatostatin precursor in normal human pituitaries and in growth hormone secreting adenomas. J Clin Endocrinol Metab 1993, 76:85–90
53. Reichlin S: Pathogenesis of pituitary tumors. Pituitary Adenomas: New Trends in Basic and Clinical Research. Edited by G Faglia, P Beck-Peccoz, B Ambrosi, P Travaglini, A Spada. Amsterdam, Elsevier Science, 1991, pp 113–122
54. Melmed S: Acromegaly. N Engl J Med 1990, 322:966–977
55. Faglia G, Spada A: The role of the hypothalamus in pituitary neoplasia. Baillière's Clin Endocrinol Metab 1995, 9:225–242
56. Miller G, Alexander J, Klibanski A: Gonadotropin-releasing hormone messenger RNA expression in gonadotroph tumors and nomal human pituitary. J Clin Endocrinol Metab 1996, 81:80–83
57. Pagesy P, Croissandeau G, Le Dafniet M, Peillon F, Li JY: Detection of thyrotropin-releasing hormone (TRH) mRNA by the reverse transcription-polymerase chain reaction in the human normal and tumoral anterior pituitary. Biochem Biophys Res Commun 1992, 182:182–187
58. Levy A, Lightman S: The pathogenesis of pituitary adenomas. Clin Endocrinol 1993, 38:559–570
59. Li J, Pagesy P, Berthet M, Racadot O, Kujas M, Racadot J, Peillon F: Somatostatin cells in human somatotropic adenomas. Virchows Arch Pathol Anat 1992, 420:95–101
60. Pagesy P, Li JY, Rentier-Delrue F, Delalande O, Le Bouc Y, Kujas M, Joubert D, Martial J, Peillon F: Growth hormone and somatostatin gene expression in pituitary adenomas with active acromegaly and minimal plasma growth hormone elevation. Acta Endocrinol 1990, 122:745–752
61. Zarandi M, Horvath J, Halmos G, Pinski J, Nagy A, Groot K, Rekasi Z, Schally A: Synthesis and biological activities of highly potent antagonists of growth hormone-releasing hormone. Proc Natl Acad Sci USA 1994, 91:12298–12302