CLINICAL STUDY

Quantitative analysis of somatostatin receptor subtypes (1–5) gene expression levels in somatotropinomas and correlation to in vivo hormonal and tumor volume responses to treatment with octreotide LAR

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Abstract

Objective: To determine whether the somatostatin receptor subtype (SSTR) expression profile correlates with hormonal and tumor volume responses to postsurgical octreotide long acting repeatable (OCT LAR) treatment.

Design and methods: Quantitative real-time RT-PCR was used to evaluate the absolute mRNA copy numbers for all five SSTR subtypes in 22 somatotropinomas. Response to OCT LAR was studied by hormone levels (GH and IGF-I) and tumor volume (sella turcica magnetic resonance imaging).

Results: SSTR5 was present at the highest level followed by SSTR2, SSTR3, SSTR1, and SSTR4 (2327 (1046–5555), 2098 (194–23 954), 97 (0–460), 14 (0–29 480), and 0 (0–652) copies respectively). Positive correlations were found between SSTR2 levels and the percentage decrease of GH and IGF-I after 3 (r = 0.49, P < 0.027 and r = 0.49, P < 0.029 respectively) and 6 (r = 0.59, P < 0.006 and r = 0.58, P < 0.008 respectively) months of OCT LAR. A negative correlation was found between SSTR5 mRNA levels and the percentage decrease of GH after 3 months of OCT LAR (r = 0.52, P < 0.016, n = 21). A higher SSTR2/SSTR5 ratio was observed among patients who obtained hormonal control with OCT LAR, when compared with those uncontrolled (2.4 (0.7–10) vs 0.3 (0.1–7.7), P < 0.006 and 6 (r = 0.59, P < 0.006 and r = 0.58, P < 0.008 respectively) months of OCT LAR. A ROC curve analysis showed a SSTR2/SSTR5 ratio of 1.3 as the best predictor of disease control, with a sensitivity of 88% and a specificity of 92% – area under curve, 0.9. A positive correlation was also found between SSTR2 mRNA levels and the percentage decrease in tumor volume after 6 months of OCT LAR (r = 0.79, P = 0.002, n = 12).

Conclusions: Somatostatin receptor subtype 2 mRNA expression levels in somatotropinomas correlate positively with in vivo hormonal and tumor volume responses to OCT LAR.

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Introduction

The primary treatment of acromegaly is surgical excision of the somatotropinoma by a transsphenoidal approach. However, around 80% of those tumors are macroadenomas and hormone hypersecretion is controlled only in 46–52% of the patients with non-invasive macroadenomas after surgery (1–4). For this reason, adjuvant medical treatment is often necessary, preferably with long-acting somatostatin analogs (SA), such as octreotide and lanreotide, which normalize growth hormone (GH) in 48–57% of the patients and insulin-like growth factor-1 (IGF-I) in 47–67% (5). Additionally, tumor shrinkage of at least 10% of the initial tumor volume occurs in 21–47% of the patients under adjuvant therapy with SA and in 32–89% of the patients under primary SA therapy (5, 6).

Several predictors of SA therapy efficacy have been studied. Pre-treatment GH levels appear to be well-established as a negative predictor of achieving SA-mediated disease control (5). Also, attaining GH levels below 5 μg/l after 3 months and IGF-I levels below 550 μg/l after 6 months of SA treatment appears to be good positive predictors of long-term control (7).
GH suppression test, following a single s.c. injection of octreotide, has been extensively studied with variable results (8–15). Scintillography with \(^{111}\)In-pentetreotide (Octreoscan) has also been studied, with limited success at predicting SA-mediated disease control (14, 16, 17). When molecular analysis of the resected tumor is possible, the presence of the gsp oncogene is indicative of a good response to SA treatment (18). In addition, expression profiles of the somatostatin receptor subtypes (SSTR) in tumor fragments may serve as a predictive tool of response to SA therapy, although further study is required (19–24).

The presently available SA bind preferentially to SSTR2 and have a ten-fold lower affinity for SSTR5 (25). Consistent with this, several authors have described that patients bearing tumors with the highest SSTR2 expression respond better to SA treatment in terms of hormonal control (19–21, 23). Some groups have also studied the \textit{in vitro} anti-proliferative action of SA and its correlation with the tumor SSTR expression pattern (26–28). Somatostatin, lanreotide, SSTR1, SSTR2, and SSTR5 ligands (BIM-23926, BIM-23190, and BIM-23268) have been shown to effectively reduce cell viability (26, 27). However, the correlation between SSTR expression and \textit{in vivo} tumor volume reduction during treatment with SA has not been reported. Therefore, in the present study, we sought to determine whether SSTR1–5 mRNA levels of somatotropinomas are correlated with \textit{in vivo} hormonal and tumor volume responses to octreotide LAR (OCT LAR) therapy.

**Subjects and methods**

**Subjects**

Only patients who were not cured by surgery were included in this study. Tumor tissue samples were obtained from 22 somatotropinomas during transsphenoidal surgery. A portion of the tumor was retained for pathology and the remaining fragments were placed in a nucleic acid stabilizing solution (RNA Later, Ambion, Austin, TX, USA) overnight at 4°C, as specified in the manufacturer’s protocol, and then stored in liquid nitrogen until RNA extraction.

Diagnosis of acromegaly was done based on the presence of classic clinical features and the lack of GH suppression to <1 \(\mu\)g/l during an oral glucose tolerance test, elevated IGF-I for age and/or positive GH staining of the tumor specimen by immunocytochemistry. One acromegalic patient (no. 2) exhibited prolactin co-secretion as noted by elevated prolactin levels and confirmed by positive immunostaining for prolactin of the tumor specimen. Surgical failure was defined by the lack of GH suppression to <1 \(\mu\)g/l during an oral glucose tolerance test and IGF-I above the upper limit of reference values (ULRV) for age assessed at least 3 months after surgery. Medical treatment with OCT LAR was started with 20 mg at 28-day intervals. Treatment was preceded by an acute test with 100 \(\mu\)g s.c. octreotide with GH levels assessed before and 2 h after the administration of the drug. After 3 months of therapy, GH and IGF-I levels were assessed on the day of the fourth injection, just prior to the administration of OCT LAR, and the dose was increased to 30 mg in the next injection if biochemical parameters of acromegaly control were not achieved (GH < 2.5 \(\mu\)g/l and normal IGF-I for age).

This study was approved by the Ethics Committee of the Hospital Universitário Clementino Fraga Filho and the Institutional Review Board of the University of Illinois at Chicago and Jesse Brown Veterans Affairs Medical Center (JBVAMC), Chicago. Informed consent was obtained from each patient before the study.

**Methods**

**Quantitative real-time PCR (qrtPCR) of SSTR subtypes cDNA from tumor samples**

Details of RNA extraction, RNA quantification, and RT for SSTR1–5 have been previously reported in detail including primer selection, verification of primer specificity, and confirmation of efficiency as well as construction of standard curves (19). To control for variations in the amount of RNA used in the RT reaction and the efficiency of the RT reaction, the expression level (copy number) of three housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase – \(\beta\)-actin, and hypoxanthine ribosyltransferase) was determined for each sample as reported previously (19). The geometric means of the copy numbers for these three genes within each sample were used as a normalization factor (NF), as described by Vandesompele (29). The results were then reported as median (minimum–maximum) of SSTR copy number-background/NF.

It should be noted that SSTR1–5 expression profiles and partial hormone data were previously reported for 15 out of the 22 samples included in this report (19). In order to accurately compare the expression levels of previous samples, with that of the seven new samples, RNA from all samples was reverse-transcribed in parallel and all samples were run on the same plate for qrtPCR amplification for each receptor and housekeeping gene transcript. When comparisons were made between the SSTR subtype levels of those samples previously reported and rerun for this study the relative levels closely align (see Fig. 1 for examples), thus demonstrating the accuracy and repeatability of the qrtPCR assessment of gene expression.

**Hormone assays**

GH was assayed by chemoluminescence (Diagnostic Products Corporation, Los Angeles, CA, USA) with an IMMULITE 1000 (Deerfield, IL, USA) analyzer. The
The detection limit of the assay is 0.01 μg/l (0.026 mU/l). The inter- and intra-assay coefficients of variation were 6.2 and 6.5% respectively. The assay detects the 22 kDa isoform.

IGF-I was assayed by IRMA after ethanol extraction of binding proteins (Diagnostic Systems Laboratories). The detection limit of the assay is 0.80 μg/l. The inter-assay coefficients of variation were 8.2, 1.5, and 3.7% for the low, medium, and high levels of the standard curve respectively. The intra-assay coefficients of variation were 3.4, 3.0, and 1.55% for the low, medium, and high levels of the standard curve respectively. The results were expressed as %IGF-I ULRV.

**Tumor volume assessment**

Magnetic resonance imaging (MRI) of the sella turcica was performed before and after 6 months of treatment with OCT LAR on a clinical 1.5T scanner, using T1-weighted gradient recalled-echo, in the sagittal and coronal planes. When assessed post-operatively, it was performed at least 3 months after the procedure. The acquisitions were repeated before and after the administration of gadolinium. Tumor volume was calculated by the Di Chiro and Nelson formula:

\[
sagittal \times \text{coronal} \times \text{axial diameters} \times \pi/6
\]

The same experienced radiologist, blind for the molecular analysis of the tumors and for the biochemical response to OCT LAR treatment, analyzed all the MRI.

**Statistical analysis**

Analyses were performed by SPSS (version 11.0 for Windows; Chicago, IL, USA). Mann–Whitney’s non-parametric test was used to compare numeric variables between groups. Correlations between numeric variables were studied using the Spearman’s correlation test. A ROC curve analysis was used to evaluate SSTR2/SSTR5 ratio when compared with hormonal disease control. \( P < 0.05 \) was considered significant.

**Results**

**SSTR mRNA content in somatotropinomas**

Out of the total number of patients, 11 (50%) were females. The median age at diagnosis for the whole group was 40 years (24–62 years). All tumors included in the present study were macroadenomas and pathology studies revealed no normal pituitary tissue in the collected samples. A variable amount of mRNA for each of the five SSTR subtypes was found (Table 1), with no significant differences between age or sex (data not shown). Median mRNA content of SSTR1 was 14 (0–29 480), for SSTR2 2098 (194–23 954), for SSTR3 97 (0–460), for SSTR4 0 (0–652), and for SSTR5 2327 (1046–5555). Examination of the relative amount of SSTR mRNA subtypes within individual tumors showed SSTR5 was the dominant SSTR subtype in 50% (11/22) of the tumors analyzed (nos 2, 5, 6, 9, 10, 13, 15, 17, 18, 20, and 21), while SSTR2 mRNA was dominant in 45% (10 out of 22; nos 1, 3, 4, 7, 8, 11, 12, 16, 19, and 22). In one patient (no. 14), the predominant mRNA was SSTR1.

**Hormonal response to treatment with OCT LAR and correlations with SSTR1–5 mRNA copy numbers**

The median GH at diagnosis was 35.2 μg/l (6.8–133; \( n = 19 \); data not shown) and after surgery was 13.7 μg/l (2.3–197; \( n = 22 \); Table 2, basal). Median IGF-I at diagnosis was 227% of the ULRV (114–703; \( n = 13 \); data not shown) and after surgery was 195.2% (103–612; \( n = 22 \); Table 2, basal). Hormonal response to treatment with OCT LAR is also presented in Table 2. Median GH at 3 and 6 months was 3.3 μg/l (0.1–142; \( n = 21 \)) and 2.5 μg/l (0.1–72.3; \( n = 21 \)) respectively. Median IGF-I at 3 and 6 months was 146.1% ULRV (38.9–475.2; \( n = 22 \)) and 99% ULRV (22.1–692.4; \( n = 21 \)) respectively. The median percentage of GH reduction after 3 and 6 months of OCT LAR therapy was 68% (−16 to 97) and 73% (−57 to 99).
respectively. The median percentage of IGF-I reduction after 3 and 6 months of OCT LAR therapy was 27.2% ULRV (K 247.9 to 82.4) and 49% ULRV (K 407 to 81) respectively. Nine patients (41%; nos 2, 3, 4, 8, 11, 12, 16, 21, and 22) achieved hormonal control with OCT LAR (safe GH and normal IGF-I for age). Two patients (9%; nos 10 and 18) had increased IGF-I for age with GH >2.5 mg/l and one patient (5%; no 7) had GH <2.5 mg/l with normal IGF-I for age.

Positive correlations were found between SSTR2 mRNA levels and the percentage decrease of GH after 3 and 6 months of OCT LAR (r = 0.48 and 0.57; P = 0.027 and 0.007 respectively; n = 21; Fig. 2a and b respectively) and between SSTR2 mRNA levels and the percentage decrease of IGF-I after 3 and 6 months of OCT LAR (r = 0.45 and 0.55; P = 0.041 and 0.01 respectively; n = 22 and 21 respectively; Fig. 2c and d respectively). A negative correlation was found between SSTR5 mRNA levels and the percentage decrease of GH after 3 months of OCT LAR (r = -0.52; P = 0.016; n = 21). A tendency toward a negative correlation was found between SSTR5 mRNA levels and the percentage decrease of GH after 6 months and the percentage decrease of IGF-I after 3 months of OCT LAR therapy (r = -0.4 and P = 0.07 for both; n = 21 and 22 respectively). No correlation was found between SSTR5 mRNA levels and the percentage decrease of IGF-I after 6 months of OCT LAR therapy. A higher SSTR2 mRNA level was observed among patients who obtained hormonal control of disease with OCT LAR (GH < 2.5 μg/l and normal IGF-I for age) when compared with those uncontrolled (4725 (1662–23 954) vs 801 (194–13 995) respectively; P = 0.008). Also, a higher SSTR2/SSTR5 ratio was observed among patients who obtained hormonal control of disease with OCT LAR when compared with those uncontrolled (2.4 (0.7–10) vs 0.3 (0.1–7.7) respectively; P = 0.001; Fig. 3). A ROC curve analysis evidenced a SSTR2/SSTR5 ratio of 1.3 as the best predictor of disease control, with a sensitivity of 88% and a specificity of 92% (area under curve, 0.9).

No significant relationship was observed between the hormonal response to medical therapy and the expression levels of the other SSTR subtypes.

### Tumor volume response to treatment with OCT LAR and correlations with SSTR1–5 mRNA copy numbers

Magnetic resonance imaging was available for 13 patients. The remaining patients underwent computerized tomography, which was not used for the evaluation of tumor volume reduction because of lower definition of the images. Median tumor volume before treatment with OCT LAR was 3.02 cm³ (0.1–24.5; n = 13; Table 2, basal) and after 6 months of LAR 1.82 cm³ (0.04–17.0; n = 13; Table 2). The median tumor volume reduction was 32.4% (–18.6 to 67.5) after 6 months of therapy with OCT LAR. Eight patients (62%; nos 1, 4, 7, 8, 15, 16, 20, and 22) had significant (>25%) tumor volume reduction after OCT LAR therapy. In three patients (nos 4, 6, and 19), the tumor volume reduction was evaluated pre-operatively.
Separated analysis of these patients was not performed because of the impossibility to make any statistical statement in such a small sample of patients. A positive correlation was found between SSTR2 mRNA levels and the percentage of tumor volume reduction after 6 months of therapy with OCT LAR (0.81; $P < 0.001$; $n = 13$). However, a negative correlation was found between SSTR3 mRNA levels and the percentage of tumor volume reduction ($r = -0.6; P = 0.029; n = 13$; Fig. 4). No significant correlation was found between SSTR5 mRNA expression and tumor volume response to OCT LAR therapy.

**Discussion**

This is the first series, to date, evaluating the correlation between SSTR1–5 mRNA levels in somatotropinomas with both *in vivo* anti-secretory and anti-proliferative response to treatment with OCT LAR. We report that SSTR2 mRNA levels positively correlate with the percentage GH and IGF-I reduction, as well as the percentage of tumor volume reduction, up to 6 months of treatment with OCT LAR.

The finding that patients whose tumors express higher SSTR2 mRNA levels tend to respond better to OCT LAR therapy in terms of hormonal control is consistent with preliminary data reported by our group in a subset of these patients (19) and reports by others (20–23). On the other hand, it is important to note that other factors such as initial GH levels (5) may also influence tumor responsiveness to OCT LAR therapy. Additionally, the present work also confirms our previous findings that patients bearing tumors with high SSTR5 mRNA levels tend to do worse under LAR therapy (19). In fact, a significant negative correlation was found between SSTR5 mRNA levels and the percentage GH reduction after 3 months of therapy with OCT LAR and negative correlations of borderline significance were found between SSTR5 mRNA levels and the percentage GH reduction after 6 months and between SSTR5 mRNA levels and the percentage IGF-I reduction after 3 months of OCT LAR therapy. Also, a higher SSTR2/SSTR5 ratio was observed among patients who obtained hormonal control of disease with OCT LAR when compared with those uncontrolled (2.4 ± 0.3; $P = 0.001$). Exploring this finding, the ROC curve analysis showed a SSTR2/SSTR5 ratio of 1.3 was a good predictor of disease control, with a sensitivity of 88% and a specificity of 92%. It is worthy to note that in a previous study from our group (8), acute testing with s.c. OCT was investigated as a predictor of the response to treatment with OCT LAR and a 75% reduction of GH levels during the test proved to have a sensitivity of 90% and a specificity of 60% to predict biochemical control of acromegaly. The present approach of investigating the SSTR mRNA expression profile of the tumors provides a higher specificity in predicting disease control than acute testing with s.c. OCT.

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<th>Tumor volume</th>
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% IGF-I, % of the upper limit of the reference values of IGF-I.
The reason why higher SSTR5 mRNA levels in somatotropinomas is associated with a poorer prognosis, in terms of hormonal response to OCT LAR therapy, remains to be clarified. However, given the fact that the affinity of octreotide is more than tenfold higher for SSTR2 than for SSTR5 (31, 32), we might speculate that higher levels of SSTR5, when compared with SSTR2, may be required for OCT LAR to efficiently reduce GH levels. It is also possible that the relative levels of SSTR5 expression may dictate its association with other G-protein-coupled receptors, which may in turn modify the ultimate response to the ligand. This possibility is based on reports showing SSTR5 can form homodimers (33) or heterodimers with SSTRs (34), dopamine receptors, and ghrelin receptors (35) depending on the relative levels expressed (34, 36).

The finding of a positive correlation between SSTR2 mRNA levels and in vivo tumor volume reduction, as well as hormonal control, during treatment with OCT LAR is an exciting and novel result. In vitro studies of pure GH and GH/prolactin-secreting pituitary adenomas have previously shown that SSTR1 (27), SSTR2, and SSTR5 (26) activation can confer the anti-proliferative actions of SA. In studies conducted with non-pituitary tissues, all the SSTR subtypes have been shown to have anti-proliferative effects, while the SSTR2 and SSTR3 were specifically shown to initiate apoptosis in select cell types (25). Our present observations specifically support the role of SSTR2 in mediating the anti-proliferative and/or pro-apoptotic action of octreotide.

The main caveat of this finding refers to the interpretation of the mechanisms of tumor reduction after surgery, once such shrinkage can be due, at least partially, to progression of the surgical debulking effect on the tumor mass, including debris absorption even after 3 months of surgery, in addition to the role of the SA. In effect, tumor volume reduction with OCT LAR therapy was assessed pre-operatively in only three patients as commented previously. Additionally, the role of each SSTR subtype in tumor shrinkage is not fully understood. Therefore, although the finding of this correlation is very exciting, this should be confirmed in further studies.

The finding of a negative correlation between SSTR3 mRNA levels and tumor volume reduction during treatment with OCT LAR was unexpected, since studies conducted with non-pituitary tissues have shown SSTR3 was positively associated with OCT-induced apoptosis (25). Also, an interesting case report of a patient, which displayed tumor shrinkage, without hormonal control, in response to SA treatment, showed the tumor preferentially expressed SSTR3 (37). This isolated case suggests that SSTR3 can confer apoptotic
activity in pituitary tumor tissue. However, preferential expression of SSTR3 or SSTR2 may not be absolutely required for SA-mediated tumor shrinkage based on another recent report showing effective tumor shrinkage, but not hormonal control, in a patient with a tumor that expressed relatively low levels of SSTR2 when compared with the other SSTR subtypes (38). This latter finding, coupled with our current observations, indicates multiple factors are likely involved in SA-mediated tumor shrinkage. In addition, it is not clear if SSTR3 mRNA levels could really influence the response to OCT LAR that has a very low affinity for this receptor subtype. For these reasons, this finding should be further confirmed.

In summary, in this acromegalic patient population, tumor SSTR2 mRNA expression levels positively correlated with in vivo hormonal and tumor volume response to OCT LAR. Also, a SSTR2/SSTR5 ratio of 1.3 was a strong predictor of the hormonal control of the disease with OCT LAR treatment. These findings indicate that, although qRT-PCR of SSTR mRNA does not directly evaluate the final presence of functional SSTR proteins on the cell membrane, SSTR mRNA levels may still be useful in predicting responsiveness to OCT LAR therapy in acromegaly.

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