Somatostatin Receptors Subtypes 2 and 5, Dopamine Receptor Type 2 Expression and *gsp* Status as Predictors of Octreotide LAR® Responsiveness in Acromegaly

clinical case report

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ABSTRACT

We present two acromegalic patients in which clinical and molecular data are discussed in regard to their ability to predict long term octreotide LAR® therapy response. Case reports: Patient 1: female, 36 years old at diagnosis. Basal GH and IGF-I at diagnosis were 133 ng/mL and 181% above the upper limit of reference values (ULRV), respectively. Growth hormone during acute test with subcutaneous octreotide decreased from 133 to 13 ng/mL. Patient started on primary octreotide LAR® therapy (20mg q28 days) and achieved biochemical parameters of disease control after 6 months. Molecular analysis of tumor fragments: *gsp* +; quantitative analysis of *SSTR* (somatostatin receptor) and DR (dopamine receptor) mRNA – SSTR2 23954; SSTR5 2407; DR2 total 17016 copies. Patient 2: male, 38 years old at diagnosis. Basal GH and IGF-I at diagnosis were 120 ng/mL and 114% ULRV, respectively. Patient underwent non-curative trans-sphenoidal surgery. Post-operative GH and IGF-I were 112 ng/mL and 137% ULRV, respectively. Growth hormone during acute test with subcutaneous octreotide decreased from 112 to 7 ng/mL. Octreotide LAR® therapy (20 mg q28 days) was then initiated. After 6 months of treatment, patient did not attain biochemical control of disease and displayed increased tumor volume. Molecular analysis of tumor fragments: *gsp* not done; quantitative analysis of SSTR and DR mRNA – SSTR2 416; SSTR5 3767; DR2 total 3439 copies. In conclusion, these two cases illustrate how laboratory data can be conflicting as predictors of octreotide LAR® responsiveness and how molecular analysis of tumor fragments can help explain different behaviors in clinically similar patients. **(Arq Bras Endocrinol Metab 2008; 52/8:1288-1295)**

Keywords: Acromegaly; Somatostatin receptor; Dopamine receptor; Octreotide LAR®.

RESUMO

Expressão dos Receptores da Somatostatina Subtipos 2 e 5 e do Receptor da Dopamina tipo 2 e *gsp* Status como Preditores de Resposta ao Octreotide LAR® na Acromegalia.

Apresentamos dois pacientes acromegálicos nos quais dados clínicos e moleculares são discutidos quanto à sua capacidade de predizer a resposta a longo prazo ao tratamento com octreotide LAR®. Relato dos casos: Paciente 1: Feminina, 36 anos de idade ao diagnóstico. GH e IGF-I ao diagnóstico 133 ng/mL e 181% acima do limite superior do valor de referência (LSVR), respectivamente. GH durante o teste agudo com octreotide subcutâneo diminuiu de 133 para 13 ng/mL. Foi iniciado tratamento primário com octreotide LAR® (20 mg q28 dias) e a paciente alcançou os parâmetros bioquímicos de controle de doença depois de seis meses. Análise molecular do tumor: *gsp* +; análise quantitativa do mRNA de *SSTR* (receptores de somatostatina) e DR (receptor de dopamina) – SSTR2 23.954; SSTR5 2.407; DR2 total 17.016 cópias. Paciente 2: Masculino, 38 anos de idade ao diagnóstico. GH e IGF-I ao diagnóstico 120 ng/mL e 114% LSVR, respectivamente. Paciente foi sub-

metido à cirurgia trans-esfenoidal não-curativa. GH e IGF-I pós-operatórios 112 ng/mL e 137% LSVR, respectivamente. GH durante o teste agudo diminuiu de 112 para 7 ng/mL. Foi iniciado tratamento com octreotide LAR® (20 mg q28 dias). Após seis meses o paciente não alcançou controle bioquímico e apresentou aumento do volume tumoral. Análise molecular do tumor: *gsp* não estudado; análise quantitativa do mRNA de SSTR e DR – SSTR2 416; SSTR5 3.767; DR2 total 3.439 cópias. Em conclusão, estes dois casos ilustram como dados laboratoriais podem ser conflitantes enquanto preditores de resposta ao tratamento com octreotide LAR® e como a análise molecular de fragmentos do tumor pode ajudar a explicar comportamentos diferentes em pacientes clinicamente semelhantes. (**Arq Bras Endocrinol Metab 2008; 52/8:1288-1295**)

Descritores: Acromegalia; Receptor de somatostatina;Receptor de dopamina; Octreotide LAR®.

INTRODUCTION

Trans-sphenoidal surgery is the most common initial treatment approach of patients with somatotropinomas (1). However, somatotropinomas are more often expansive macroadenomas and surgery is usually not curative. Surgical "cure" is correlated to the magnitude of initial GH levels, tumor size and invasiveness. The best results are achieved in microadenomas and macroadenomas restricted to the sella turcica, in which cure rates are around 75%. Regarding the more invasive macroadenomas, the cure rates drop to 33 – 44% depending on the invasion magnitude (2).

Because surgery alone is frequently not curative, further treatment is often required. Somatostatin analogs (SA) are a cornerstone of medical therapy for acromegaly. Octreotide LAR® therapy achieve 'safe' growth hormone (GH) levels (<2.5 ng/mL) in 48-57% and insulin-like growth factor type I (IGF-I) normalization in 47-67% of the patients (3). Additionally, tumor shrinkage of at least 20% of the initial tumor volume occurs in 75% of the patients (4).

A main issue in medical therapy of acromegaly is the elevated cost. Considering that 'safe' GH levels and IGF-I normalization are not achieved in one third of the patients, it is useful to predict which patient is more likely to respond to SA. Several clinical, biochemical and molecular data have been evaluated for that purpose.

Several predictors of SA therapy efficacy have been studied. Pre-treatment GH level appear to be well-established as a negative predictor of achieving SA-mediated disease control (5). Also, attaining GH levels below 5 ng/mL after 3 months and IGF-I levels below 550 ng/mL after 6 months of SA treatment appear to be good positive predictors of long term control (6). GH suppression test, following a single subcutaneous injection of octreotide (acute test), has been extensively studied with variable results (7-14). Scintilography with 111In-pentetreotide (Octreoscan) has also been studied, with limited success at predicting SA-mediated disease control (8,15,16). Patients bearing densely granulated somatotropinomas on electron microscopy respond better to SA therapy than patients with sparsely granulated tumors (17). Immunohistochemical detection of somatostatin receptor subtype 2 (SSTR2) correlates positively with percent GH reduction on acute test and IGF-I after 6 months of treatment (18,19). When molecular analysis of the ressected tumor is possible, the presence of the *gsp* oncogene seems to be indicative of a good response to SA treatment (20-26). Despite the fact that most studies suggest that *gsp*-positive adenomas respond better to octreotide LAR® compared to *gsp*-negative tumors, a recent study failed to find differences in octreotide LAR® sensitivity according to *gsp* status (18). In addition, expression profiles of the mRNA somatostatin receptor subtypes (SSTR) in tumor fragments may serve as a predictive tool of biochemical and tumor volume response to SA therapy (24,27-32).

In this papers we present two patients in which clinical and molecular data are discussed in regard to their ability to predict long term SA therapy response.

SUBJECTS AND METHODS

Acute test with subcutaneous octreotide

Octreotide – 100 mcg – was administered subcutaneously and blood samples were drawn for GH measurements before and two hours after administration of the drug. A positive GH response in this test was defined as >75% decrease of the GH levels. Sensitivity, specificity, positive and negative predictive values of the acute test were respectively 0.90, 0.60, 0.69 and 0.86 for a reduction of 75% of the GH on the test (14).

Growth hormone and IGF-I assays

Growth hormone was assayed by chemoluminescence (Diagnostic Products Corporation, Los Angeles, CA) with an IMMULITE 1000 analyser. The detection limit of the assay is 0.01 µg/L (0.026 mU/L). Inter and intra-assay coefficients of variation were 6.2% and 6.5%, respectively. The assay detects the 22 KDa isoform. The IRP (International Reference Preparation) used is 98/574.

Insulin-like growth factor-I was assayed by IRMA after ethanol extraction of binding proteins (Diagnostic Systems Laboratories, Webster, TX). The detection limit of the assay is 0.80 µg/L. 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. Intra-assay coefficients of variation were 3.4%, 3.0% and 1.5% for the low, medium and high levels of the standard curve, respectively. Results were expressed as the percent above the upper limit of the reference value (%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 (patient #2) it was performed 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 x coronal x axial diameters x $\pi/6$ (33).

Quantitative real-time RT-PCR (qrtRT-PCR) of DR and SSTR subtypes mRNA from tumor samples

Total RNA was extracted from 30 mg of tissue sample obtained during trans-sphenoidal surgery using the RNeasy® Mini Kit (Qiagen, Valencia, CA). To avoid contamination by genomic DNA, the RNA samples were treated with RNase-free DNase Set (Qiagen), as specified in the manufacturer's protocol. Reversal transcription was done using 1 µg of total RNA in a 20 µL volume. Synthesis of first strand cDNA suitable for PCR amplification was done using Kit First Strand cDNA Synthesis® (Fermentas, Hanover, MD) with random hexamer primer (0.2 µg/µL) and the cDNA was treated with RNase H, as specified in the manufacturer's protocol.

Primers sets for DR2 total, DR2 long isoform, SSTR2 and SSTR5 were selected using Primer 3 software with human genomic sequences as templates. Primer pairs (sense / anti-sense and Genbank accession number) used in PCR reactions were: DR2 total: cgagcatcctgaacttgtgtg / gcgttattgagtccgaagagg (NM_016574); DR2 long isoform (DR2L): ctcctccatcgtctccttct / cggtgcagagtttcatgtcc (NM_000795); SSTR2: ggcatgtttgactttgtggtg / gtctcattcagccgggattt (NM001050); SSTR5: ctggtgtttgcgggatgtt / gaagctctggcggaagttgt (NM001053).

One microliter aliquots of the resulting cDNA were amplified by real time PCR using the primers (0.375 µL), mixed with iQ SYBR® Green Supermix 12.5 µL. The iQ SYBR® Green Supermix magnesium chloride (MgCl2) concentration is 6 mM and the final concentration in the reaction was 3 mM. Thermal cycling profile consisted of a pre-incubation step at 95 °C for 10 min, followed by 35 cycles of denaturation (95 °C, 30 s), annealing (61-64 °C, 1 min) and extension (72 °C, 30 s). Total RNA samples that were not reverse transcribed were run to control for genomic and/or technical DNA contamination (background). It should be noted that a standard curve was run with each set of samples to estimate copy number. At the end of the amplification the final product was subjected to graded temperature dependent dissociation, to verify that only one product was amplified. The detection limit of the method is 10 copies.

To control for variations in the amount of sample and the RNA quality used in the RT reaction and the efficiency of the RT reaction, the expression level (copy number) of three commonly used housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase – GAPDH, β-actin, hypoxanthine ribosyltransferase - HPRT) was determined for each sample. Primers sets were also selected using Primer 3 software with human genomic sequences as templates. Primer pairs (sense / anti-sense and Genbank accession number) used in PCR reactions were:

GAPDH: aatcccatcaccatcttcca / aaatgagccccagccttc (NM002046); β-actin: actcttccagccttccttcct / cagtgatctccttctgcatcct (NM001101); HPRT: ctgaggatttggaaagggtgt / taatccagcaggtcagcaaag (BT019350). To determine if these genes were appropriate to use as internal controls, the stability of expression was calculated using the GeNorm 3.3 visual basic application for Microsoft Excel (http://medgen.ugent.be/~jvdesomp/genorm/) as previously developed and validated by Vandesompele *et al.* (34). This program calculates the average pairwise variation of a particular gene with all other control genes (M), allowing elimination of the worst scoring control genes and recalculation of new M values for the remaining genes. M values < 1.5 are indicative of a "stable gene". The geometric means of copy numbers for the most stable genes are then used as a normalization factor (NF). In our study, these three house keeping genes showed to be "stable" (all values were < 1.5). Therefore the geometric means of the copy numbers for these three genes within each sample were used as a normalization factor (NF). Results were then reported as median (minimum-maximum) of receptor copy number minus background divided by NF.

Analysis of the presence of *gsp* oncogene

Deoxyribonucleic acid (DNA) was extracted using QIAamp DNA MiniKit (Qiagen, Valencia, CA) according to the protocol for isolation from fresh tissue. Exons 8 and 9 (including codons 201 and 227, respectively) of the *gsp* gene were amplified by polymerase chain reaction (PCR) using oligonucleotide primers as described previously (35). Each 50-μL PCR reaction mix contained 2 μL of DNA, 20 pmol of each primer and 5 units of DNA-Polymerase (TTH biotools; Biotools, Madrid, Spain). Thermal cycling profile consisted of a pre-incubation step at 95°C for 10 min, followed by 40 cycles of denaturation (95°C, 1 min), annealing [56°C (for codon 201) or 54°C (for codon 227), 1 min] and extension (72°C, 1 min). The PCR products were then purified using the MinElute PCR Purification kit (Qiagen) and sequenced using the same oligonucleotide primers with an ABI3730XL analyzer (Applied Biosystems, Foster City, CA).

CASE REPORTS

Patient 1: Female, 36 years old at diagnosis, presented with classical signs and symptoms of acromegaly that was confirmed by lack of GH suppression to less than 1 ng/mL on oral glucose tolerance test (OGTT) and elevated IGF-I for age. Basal GH and IGF-I at diagnosis were 133 ng/mL and 181% ULRV, respectively. Sella turcica MRI revealed a macroadenoma with 4.73 cm³ (Figure 1A). Growth hormone during acute test with 100 mcg subcutaneous octreotide decreased from 133 to 13 ng/mL. Patient started on primary octreotide LAR® therapy (20mg q28 days) as a part of a clinical research trial. After 3, 6 and 12 months of octreotide LAR® therapy (20mg q28 days), GH decreased to 5.2, 1.7 and 2.3 ng/mL, respectively and IGF-I to 79, 90 and 82% ULRV, respectively. Tumor volume decreased to 2.48 and 2.09 cm³ (Figure 1B) at 6 and 12 months (Table 1). Patient un-
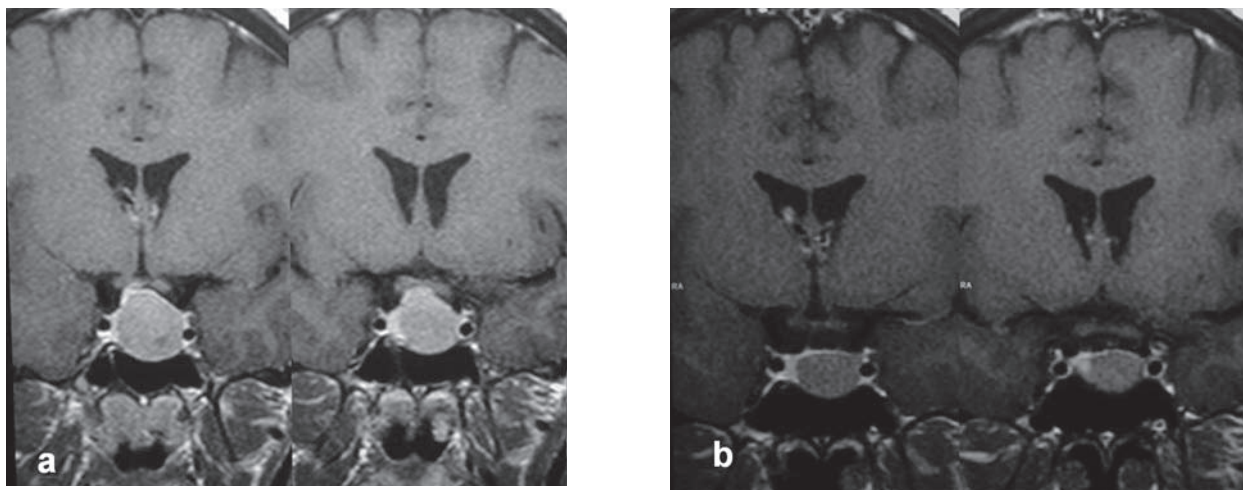

Figure 1. Sella turcica magnetic resonance, coronal view, T1 weighted , with contrast enhancement, before (a) (tumor volume 4.73 cm³) and 12 months after (b) (tumor volume 2.09 cm³) octreotide LAR® treatment.

derwent trans-sphenoidal surgery because of irregular supply of octreotide LAR® by the public health system. Molecular analysis of tumor fragments: *gsp* +; SSTR and DR mRNA expression – SSTR2 23954; SSTR5 2407; DR2 total 17016 copies (Table 2).

Patient 2: Male, 38 years old at diagnosis, was referred to our outpatient clinics for acromegaly treatment. Diagnosis was also supported by lack of GH suppression on OGTT and elevated IGF-I for his age. Basal GH and IGF-I at diagnosis were 120 ng/mL and 114% ULRV, respectively. Sella computed tomography showed a macroadenoma with 2.3 x 1.8 cm with supra and left para-sellar extension. Patient underwent non-curative trans-sphenoidal surgery. Post-operative GH and IGF-I were 112 ng/mL and 137% ULRV, respectively. Tumor volume on sella turcica MRI 3 months after surgery was 3.28 cm³ (Figure 2A). Growth hormone during acute test with subcutaneous octreotide decreased from 112 to 7 ng/mL. Octreotide LAR® therapy (20mg q28 days) was then initiated. After 3 months of therapy, GH decreased to 13 ng/mL, however after additional 3 months (30mg q28 days) it increased to 30 ng/mL. Insulin-like growth factor type I values were 293 and 290% ULRV, respectively. Tumor volume increased to 3.9 cm³ (Figure 2B) at 6 months of medical treatment (Table 1). Considering increased tumor volume and GH levels and unchanged IGF-I, a second surgical procedure was indicated. Post-operative GH and IGF-I were 19 ng/mL and 160% ULRV, respectively. Molecular analysis of tumor fragments: *gsp* not done; SSTR and DR mRNA expression – SSTR2 416; SSTR5 3767; DR2 total 3439 copies (Table 2).

Table 1. Laboratory and imaging data in both patients.

	Diagnosis			3 months		6 months			12 months			Acute test GH (ng/mL)	
	GH (ng/mL)	IGF-I (%ULRV)	Tumor volume (cm3)	GH (ng/mL)	IGF-I (%ULRV)	GH (ng/mL)	IGF-I (%ULRV)	Tumor volume (cm3)	GH (ng/mL)	IGF-I (%ULRV)	Tumor volume (cm3)	Basal	2 h
Patient 1	133	181	4.73	5.2	79	1.7	90	2.48	2.3	82	2.09	133	13
Patient 2	120 112†	114 137†	‡ 3.28†	13	293	30	290	3.9	ND	ND	ND	112	7

%ULRV: percent above the upper limit of the reference value; † Post-operative data (1st surgery); ‡ Tumor volume was not assessed at diagnosis because the patient presented with a sella computed tomography.
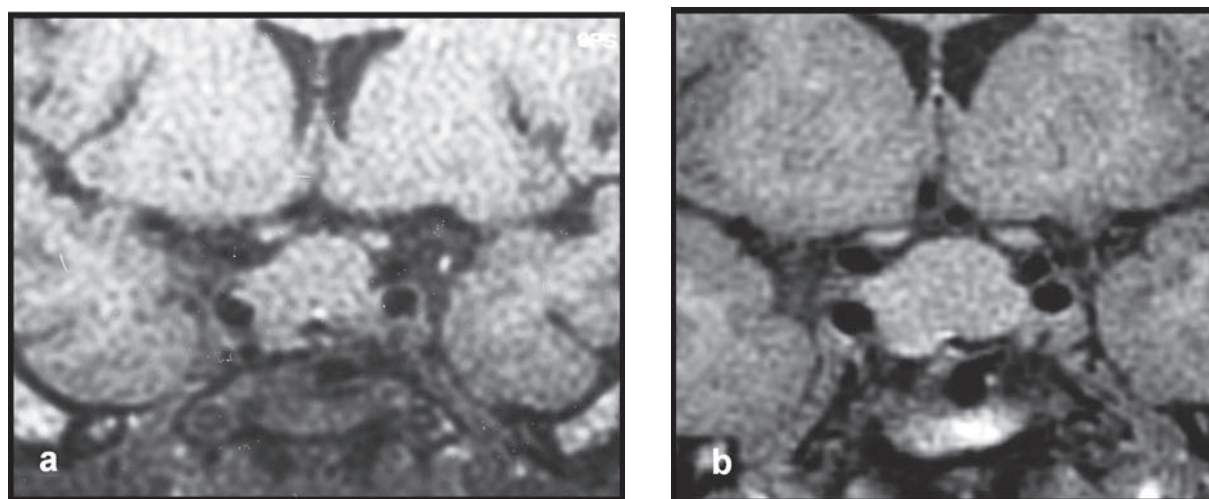

Figure 2. Sella turcica magnetic resonance, coronal view, T1 weighted, with contrast enhancement, before (A) (tumor volume 3.28 cm³) and 6 months after (B) (tumor volume 3.90 cm³) octreotide LAR® treatment.

Table 2. Dopamine and somatostatin receptor subtypes mRNA expression (copy number corrected for normalization factor) and *gsp* oncogene in both patients.

	Patient 1	Patient 2
DR2 total / NF	17016	3439
DR2 long isoform / NF	11260	1182
SSTR2 / NF	23954	416
SSTR5 / NF	2407	3767
gsp	+	ND

DR: dopamine receptor; SSTR: somatostatin receptor; NF: normalization factor; ND: not done.

DISCUSSION

These two case report illustrate how laboratory and molecular data can be used to predict responsiveness to SA therapy in acromegaly. Considering GH levels at diagnosis, the first patient data are not in accordance to the literature. It has already been demonstrated that patients with higher initial GH concentrations are less likely to normalize IGF-I concentrations during treatment (5). Despite very high initial GH levels, after only 3 and 6 months of octreotide LAR® therapy, normalization of IGF-I and near normal GH levels (<2.5 ng/mL) were observed. On the other hand, despite of initial impressive GH reduction under octreotide LAR®, the second patient did not achieve disease biochemical control. On the contrary, raising hormone levels associated with increased tumor volume, regardless of octreotide LAR® dose titration, determined a second surgical approach. Both patients displayed similar GH suppression during acute test with subcutaneous octreotide. An explanation for dissociation between response, in the acute test and during the long-term therapy with octreotide LAR®, might be that the percentage of GH decrease is used as parameter of responsiveness. On the other hand, the goal during the therapy with octreotide LAR® is to reach GH below 2.5 ng/mL. Therefore, the patient may have a significant percentage of GH decrease during the acute test and a reduction of GH levels during long-term therapy with octreotide LAR®. However, this decrease may not be sufficient to reach tolerable GH levels (< 2.5 ng/mL). In addition, data regarding acute test with octreotide as a predictor of responsiveness to long-term Octreotide LAR® is conflicting in the literature. It may be explained by methodological differences among

the studies such as acute test protocol and parameter used as positive response during the acute test. Regarding our patients, the distinct biochemical behavior in response to octreotide LAR® therapy, despite similar initial GH levels and response to acute test, raises the question regarding other factors determining tumor SA sensitivity.

One possible explanation to this discrepancy is the identification of *gsp* oncogene in patient #1. Although this analysis was not done in patient #2, the fact that patient #1 is *gsp* positive increases her chance to be successfully controlled with octreotide LAR®. In fact, several studies explored SA responsiveness in the light of *gsp* status and it seems that *gsp* positive patients are better responders (20-26), although there is some controversy in the literature (18).

Regarding DR and SSTR mRNA profile in both tumors, it is interesting to note that both patients displayed higher SSTR than DR mRNA levels, differently from the previously observed by our group in a larger series of patients. Median DR2 total, DR2 long isoform, SSTR2 and SSTR5 mRNA expression were 5276, 1709, 1316 and 2068 copies, respectively [unpublished data for DR and reference (32) for SSTR]. However, patient #1 had predominant SSTR2 mRNA levels over the other studied receptors and patient #2 had predominant SSTR5 mRNA levels (Table 2).

Patient #1 shows a very good response to octreotide LAR®, considering biochemical status and tumor shrinkage as well. It should be stressed that her SSTR2 mRNA levels are 18 times higher than median expression of acromegalic patients in our previous series, which may help to explain, along with *gsp* status, her impressive octreotide sensitivity. On the other hand, patient #2 shows a poor response to octreotide LAR® in terms of biochemical control as well as tumor shrinkage. His SSTR2 mRNA levels are 3 times lower and his SSTR5 mRNA levels are 1.8 times higher than median expression of acromegalic patients. Somatostatin receptor subtype 5 mRNA levels have already been shown to be predictors of unfavorable response to octreotide LAR® therapy (32). Also, SSTR2/SSTR5 ratio has been previously explored by our group as a predictor of biochemical disease control with octreotide LAR®. A SSTR2/SSTR5 ratio of 1.3 displayed the best profile with sensitivity 88% and specificity 92% (32). In the present cases reported, SSTR2/SSTR5 ratios were 9.95 and 0.11 for patients #1 and #2, respectively, justifying the octreotide LAR® responsiveness observed.

Internalization phenomenon due to somatostatin exposure was evaluated *in vitro*, but there is no data regarding that *in vivo* (36,37). Data from studies that evaluated the long-term efficacy of SA failed in demonstrating the tachyphylaxis effect in acomegaly. In fact, Cozzi and cols. (6) observed sustained suppression of GH/IGF-I hypersecretion throughout the study, without tachyphylaxis in any patient even after four years of continuous treatment. Even if the exposure to octreotide LAR® could affect the SSTR pattern expression, both patients in this study were submitted to octreotide LAR® before surgery (before collecting the tumor sample), allowing comparison of the receptor expression profile between them.

Finally, exploring the knowledge that DR2 forms heterodimers with SSTR2 and SSTR5 and that the composition of these receptor complexes can alter the response to SA (38, 39), it is important to stand out that patient #1 and #2 had DR2 mRNA levels 3.2 times higher and 1.5 times lower than median expression of acromegalic tumors, respectively. To support the present observations, a recent report revealed that *in vitro* response to octreotide was enhanced in adenomas which expressed higher levels of DR2 (40).

In conclusion, these two cases illustrate how molecular analysis of the tumor fragment can predict octreotide LAR® responsiveness, in contrary to biochemical data, which showed conflicting results. Therefore, we suggest the routine analysis of SSTR and DR expression in acromegalic patients who have been operated on.

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