Ki-67 is a predictor of acromegaly control with octreotide LAR independent of SSTR2 status and relates to cytokeratin pattern

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Abstract

Introduction: Only one study has evaluated Ki-67 as a predictor of the response to somatostatin analog therapy in acromegaly; however, other predictors like somatostatin receptor type 2 (SSTR2) and cytokeratin pattern expressions were not considered.

Objective: To evaluate whether Ki-67 is a predictor of octreotide LAR (OCT-LAR) response in somatotropinomas independent of SSTR2 and cytokeratin expression patterns.

Methods: Protein expression was analyzed by immunohistochemistry. The percentage of cell nuclei that were immunolabeled for Ki-67 and the percentage of cells with positive SSTR2 staining were calculated. SSTR2 expression was considered high when \( R > 25\% \), and a cutoff of 2.3\% was designated for Ki-67. Tumors were classified as densely or sparsely granulated according to the cytokeratin pattern.

Results: Thirty-one somatotropinomas were studied. Fourteen patients (45.2\%) were controlled with OCT-LAR therapy. The median Ki-67 labeling index (LI) was higher in patients not controlled with OCT-LAR than in those controlled (1.63 and 0.15 respectively, \( P = 0.002 \)). Higher SSTR2 expression and densely granulated tumors were correlated with control as well (\( P = 0.04 \) and 0.038 respectively). There was no difference in Ki-67 levels between patients with high and low SSTR2 expression (\( P = 0.651 \)). After multivariate analysis, both Ki-67 and SSTR2 remained statistically significant as predictors of OCT-LAR response (\( P = 0.017 \) and 0.012 respectively). The Ki-67 LI was higher in sparsely than in densely granulated tumors (\( P = 0.047 \)).

Conclusions: Ki-67 is a predictor of response to OCT-LAR in acromegaly, independent of SSTR2 expression and relates to cytokeratin patterns.

Introduction

Acromegaly is a chronic endocrine disease with high morbidity and mortality (1). Surgery is the treatment of choice for patients with microadenomas and macroadenomas with a high chance of surgical cure (2). However, \( \sim 40-60\% \) of patients with macroadenomas are unlikely to be cured by surgery alone (3). Somatostatin analogs (SSAs) are the first treatment option in acromegalic patients for whom surgical cure is improbable and for those not cured by surgery (2). The currently available SSAs can result in disease control in \( \sim 30\% \) of acromegalic patients based on prospective clinical trials (4, 5). Thus, considering the present accessibility of the diverse classes of drugs and their elevated costs, it would be valuable to identify predictive markers for the patients who are more likely to respond to SSA therapy.

Several predictors of SSA therapy efficacy have been evaluated, with the granulation pattern and expression of the somatostatin receptor subtype 2 (SSTR2) being the most established (6, 7, 8, 9, 10, 11). Patients harboring densely granulated tumors respond better to SSA treatment than those with sparsely granulated ones (7, 12). SSTR2 expression in somatotropinomas seems to represent the best predictor of the response to SSAs (9). Indeed, some studies have shown a positive correlation between the expression of SSTR2 mRNA and protein and the clinical response to SSAs (13, 14, 15, 16, 17, 18).
Ki-67 is a nuclear antigen expressed in all non-G0 phases of the cell cycle. Thus, in some studies, it has been shown to be a proliferative marker associated with tumor invasiveness or recurrence (19). Fusco et al. (20) found that a group of GH-secreting adenomas with low Ki-67 labeling index (LI) values had a better response to SSAs than those with higher Ki-67 LI values. However, they did not evaluate the SSTR2 expression levels in the two groups of tumors, which might explain the difference in treatment response. No other study has evaluated Ki-67 as a predictor of the response of acromegaly to SSA therapy.

In the current study, we aimed to evaluate whether Ki-67 LI is a predictor of octreotide LAR (OCT-LAR) response in somatotropinomas independent of SSTR2 and cytokeratin expression pattern.

Subjects and methods

This study was approved by the Ethics Committee of the Clementino Fraga Filho University Hospital/Federal University of Rio de Janeiro. All the patients signed informed consent forms before study entry.

Patients and tumors

Thirty-one consecutive acromegalic patients who were treated with surgery but not cured were included in the study. Patients underwent operations between 2006 and 2010. The biochemical diagnosis of acromegaly was based on the current criteria (21). The exclusion criteria consisted of a history of medical treatment with SSAs, dopamine agonists, or GH antagonist before surgery as well as previous radiotherapy for pituitary adenoma. Magnetic resonance imaging (MRI) of the sellar region before the surgery was analyzed and the Di Chiro and Nelson formula was used to calculate tumor volume (22). Tumor invasiveness was defined according to the Knosp–Steiner criteria (23).

Postsurgical evaluation

Biochemical evaluation was performed 12 weeks after surgery by evaluation of oral glucose tolerance test (OGTT) and serum insulin-like growth factor 1 (IGF1) levels in all subjects. Pituitary MRI was performed 3 months after the surgical procedure. Patients were considered as non-cured on the basis of the clinical picture, nadir GH levels after OGTT higher than 0.4 ng/ml, and plasma IGF1 levels higher than age-matched normal subjects. Medical therapy with OCT-LAR was started at a dose of 20 mg every 4 weeks, and the dose was increased to 30 mg every 4 weeks in uncontrolled patients after 3 months of therapy. Efficacy of medical therapy was evaluated at the last patient visit, and patients were considered uncontrolled if they had a basal GH value higher than 1.0 ng/ml and/or a plasma IGF1 level higher than age-matched normal subjects with at least 6 months of treatment with OCT-LAR at a dosage of 30 mg. Postsurgical follow-up ranged from 12 to 60 months (median 32 months). Tumor volume was not considered an endpoint in this series because the study included only postsurgical patients, which could lead to mistakes in the volume measurements due to confounding variables such as postsurgical changes.

Methods

Hormonal assessment Plasma GH levels were measured by a chemiluminescence assay kit (IMMULITE; Diagnostic Products Corp., Inc., Los Angeles, CA, USA). The inter- and intra-assay coefficients of variation (CV) were 6.0 and 5.8% respectively. The International Reference Preparation of GH was 98/574. Plasma IGF1 levels were measured by an immunoradiometric assay kit (DSL, Webster, TX, USA). The inter- and intra-assay CV were 2.6 and 4.5% respectively. The International Reference Preparation of IGF1 was 80/185.

Immunohistochemistry The SSTR2 expression and Ki-67 LI were analyzed using immunohistochemistry on paraffin-embedded tissue sections as described previously (17, 24, 25). A mouse MAB directed against the Ki-67 antigen (1:100, Dako, Carpinteria, CA, USA; MIB-1 clone, cat number M-7240) and a rabbit polyclonal antibody against SSTR2 (1:500, Gramsch Laboratories (Schwabhausen, Germany), cat number SS-800) were used. The cytokeratin expression pattern was analyzed as previously published (26) with a mouse MAB CAM5.2 (1:100, BD Biosciences (San Jose, CA, USA), cat number 349205).

For Ki-67, human breast carcinoma was used as a positive control. For CAM 5.2, normal kidney glomerular tissue was used as a positive control, and for SSTR2, normal human pituitary tissue was used as a positive control. Negative controls included omission of the primary antibody (for all) and pre-absorption of the anti-SSTR2 antibody with its immunizing receptor peptide.

Histomorphometry was performed using Image-Pro Plus 4.5.1 (Media Cybernetics, Silver Spring, MD, USA) coupled to a digital camera (Evolution, Media Cybernetics) and a light microscope (Eclipse 400, NIKON, Japan). For Ki-67, high-quality images (2048×1536 pixels) of immunostained tumor cells from ten representative areas with at least 1000 cells were captured using a 40× objective lens. Both immunolabeled and unlabeled nuclei were evaluated, and the percentage of positive cells (LI) was calculated. A cutoff point of 2.3% was designated for Ki-67 LI as previously published by our group (24). The statistical analyses were also performed with a 3.0% cutoff, as suggested by the WHO classification of pituitary adenomas (27). For SSTR2,
high-quality images of immunostained tumor cells were randomly captured using a 40× objective lens (ten microscopic fields). Both membrane-bound and intracytoplasmic stainings were considered. Tumors were staged according to the percentage of stained cells within the following score categories: 0 (<25% stained cells – low expression) or 1 (≥25% – high expression). Scale bar = 500 μm. Full colour version of this figure available via http://dx.doi.org/10.1530/EJE-13-0349.

### Statistical analysis

The statistical analyses were performed using SPSS version 16.0 for Windows (SPSS, Inc.). The results are reported as median values (minimum–maximum). The Mann–Whitney U non-parametric test was used to compare numeric variables between the groups. Fisher’s exact test or the χ² test was used to compare frequencies between the groups according to the sample size. For multivariate analysis, a binary logistic regression was performed. A P value < 0.05 was considered significant.

### Results

#### Patient/sample characteristics

Thirty-one consecutive postsurgical acromegalic patients (19 women) were included in the study. The median age at diagnosis was 42 (23–60) years. The median baseline GH level was 10.3 (1.1–255.0) ng/ml and the median baseline IGF1 level was 621 (264–1717) ng/ml. Twenty-eight of 31 tumors were macroadenomas (90.3%) and 19 (61.2%) were invasive. Median tumor volume at diagnosis was 4.7 cm³ (0.2–31.2 cm³). Fourteen (45.2%) tumors exhibited co-expression of GH and prolactin; others were pure somatotropinomas. Detailed clinical, laboratory, and radiological information about each patient is provided in Supplementary Table 1, see section on supplementary data given at the end of this article.

#### Ki-67 LI, SSTR2 expression, and cytokeratin pattern in the somatotropinomas

Twenty-five somatotropinomas (80.6%) expressed Ki-67, of which six (19.5%) demonstrated an LI higher than 3% and eight presented an LI higher than 2.3% (25.8%). Examples of Ki-67 nuclear immunostaining are shown in Fig. 1. There was no difference in age, gender, hormonal staining (mixed tumor or pure GH), baseline GH, or IGF1 levels between patients harboring somatotropinomas with low or high Ki-67 LI (Table 1).

Membrane and cytoplasmic staining for SSTR2 was observed (Fig. 1). All the somatotropinomas expressed SSTR2, with high levels of expression observed in 26 tumors (83.8%). There were no differences in age, gender, hormonal staining (mixed tumor or pure GH), baseline GH, or IGF1 levels between patients harboring somatotropinomas with low or high SSTR2A expression levels (Table 2). There was no difference in the median Ki-67 LI values between patients with high and low SSTR2 expression levels (0.81, range 0.0–4.5 and 1.25, range 0.0–20.6 respectively, P = 0.651; Fig. 2A).

The cytokeratin pattern was analyzed in 24 tumors. Thirteen tumors (54.2%) were classified as sparsely granulated, sparsely granulated, or mixed forms according to previously published classification (28). Mixed tumors were considered as densely granulated for analysis, as previously suggested (28).

### Table 1 Patient characteristics according to Ki-67 labeling index (LI). Results are presented as median (min–max).

<table>
<thead>
<tr>
<th></th>
<th>Ki-67 LI &gt; 2.3%</th>
<th>Ki-67 LI &lt; 2.3%</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>42 (23–60)</td>
<td>38.5 (24–48)</td>
<td>0.110</td>
</tr>
<tr>
<td>Female</td>
<td>50.0%</td>
<td>65.2%</td>
<td>0.362</td>
</tr>
<tr>
<td>Pure GH tumor</td>
<td>75.0%</td>
<td>47.8%</td>
<td>0.240</td>
</tr>
<tr>
<td>Treatment duration (months)</td>
<td>29 (9–57)</td>
<td>25 (12–35)</td>
<td>0.848</td>
</tr>
<tr>
<td>Baseline GH (ng/ml)</td>
<td>10.3 (1.1–133)</td>
<td>10.8 (4.9–255)</td>
<td>0.611</td>
</tr>
<tr>
<td>Baseline IGF1 (ng/ml)</td>
<td>618 (264–1717)</td>
<td>778 (345–1470)</td>
<td>0.096</td>
</tr>
</tbody>
</table>

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Table 2 Patient characteristics according to somatostatin receptor subtype 2 (SSTR2) expression. Results are presented as median (min–max).

<table>
<thead>
<tr>
<th></th>
<th>High SSTR2</th>
<th>Low SSTR2</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>43 (23–60)</td>
<td>40 (24–47)</td>
<td>0.514</td>
</tr>
<tr>
<td>Female</td>
<td>60.0%</td>
<td>61.5%</td>
<td>0.658</td>
</tr>
<tr>
<td>Pure GH tumor</td>
<td>40.0%</td>
<td>57.6%</td>
<td>0.403</td>
</tr>
<tr>
<td>Treatment duration</td>
<td>29 (9–57)</td>
<td>25 (12–35)</td>
<td>0.848</td>
</tr>
<tr>
<td>Baseline GH</td>
<td>10.6 (1.1–255)</td>
<td>8.2 (5.4–252)</td>
<td>0.979</td>
</tr>
<tr>
<td>Baseline IGF1</td>
<td>622 (264–1717)</td>
<td>621 (414–972)</td>
<td>0.829</td>
</tr>
</tbody>
</table>

Acromegaly was not controlled with OCT-LAR therapy in any of the patients (n=5) with SSTR2 expression levels lower than 25%. Of the 26 patients with SSTR2 expression levels higher than 25%, acromegaly was controlled with OCT-LAR in 14 (53.8%) cases (P=0.04). In addition, we performed a multivariate analysis through a binary logistic regression including both the SSTR2 and the Ki-67 LI as independent variables, with the acromegaly disease control with OCT-LAR therapy being the dependent variable. In this multivariate analysis, both Ki-67 and SSTR2 remained statistically significant as predictors of disease control with OCT-LAR (P=0.017 and 0.012 respectively).

There was a positive correlation between densely granulated tumors and response to SSA therapy. Disease control was achieved in 72.7% (8 of 11) of the patients harboring densely granulated tumors, while only 23.1% (3 of 13) of the patients harboring sparsely granulated tumors were controlled with SSA treatment (P=0.038).

There was a lower chance of disease control with OCT-LAR therapy in those patients harboring invasive tumors at diagnosis. Ten of 12 (83.3%) patients whose tumors were not invasive were controlled with OCT-LAR treatment, while only 4 of 19 (21.1%) patients harboring invasive tumors were controlled with medical treatment (P=0.001). Tumor volume was also greater in tumors from patients resistant to OCT-LAR treatment than in those whose disease was controlled by the treatment (7.6 cm³ (0.6–31.2 cm³) vs 1.35 cm³ (0.2–12.5 cm³); P=0.008).

Figure 2 Boxplot showing the Ki-67 labeling index (LI) in: (A) tumors with high or low somatostatin receptor type 2 (SSTR2); (B) sparsely or densely granulated tumors; (C) Tumoral volume in patients with high (≥2.3%) or low (<2.3%) Ki-67 LI, *P<0.05. Full colour version of this figure available via http://dx.doi.org/10.1530/EJE-13-0349.
Discussion

SSAs are currently the mainstay for the treatment of acromegaly (2). Although some studies, including a meta-analysis, indicate control rates of ~60% with SSA therapy (29, 30, 31), prospective clinical trials showed lower control rates with this class of drugs (~30–40%) (4, 5, 32). As there is a considerable percentage of patients whose acromegaly will not be controlled by SSAs, predictors of the response to this treatment are useful in clinical practice. Many possible predictors have been studied (8, 9).

We did not find any difference in the baseline GH levels between patients whose disease was controlled or not controlled with OCT-LAR therapy. Although some studies report that GH levels are predictors of meeting biochemical criteria for control with SSA therapy, in a meta-analysis, this was found to be valid only for primary therapy (29). In the same study, there was no relationship between baseline GH levels and response to OCT-LAR in the secondary therapy group, as in our present study.

SSAs act by binding to SSTR. There are five known SSTR types, and all but type 4 are present in the normal pituitary and in somatotropinomas (14). The currently available SSAs (lanreotide and OCT) bind preferentially to SSTR2. Therefore, as expected, the expression of SSTR2 is one of the few predictors of patient response to SSA therapy (8, 9). Indeed, some studies have found a positive correlation between SSTR2 mRNA expression and GH and IGF1 suppression during SSA therapy (13, 15). SSTR2 protein expression has also been found to be positively correlated with the clinical response to SSAs (16, 18). SSTR2 expression has a high negative predictive value, as patients with low SSTR2 expression do not respond to SSA therapy; however, the presence of high SSTR2 expression levels is not always associated with a good response to treatment, as post-signaling regulation could be involved (10, 18). In agreement with the literature, we found that no patient with low SSTR2 expression was controlled with OCT-LAR therapy, but some patients with high SSTR2 expression were also not controlled.

As mentioned earlier, SSTR expression has been evaluated in several studies, mainly based on detection of mRNA expression (13, 14). SSTR expression was also evaluated by immunohistochemistry (IHC) (17, 18), which, although semi-quantitative, can be routinely used in the evaluation of pituitary adenomas because it is a widely available technique. To validate the results of IHC, we previously compared the SSTR2 mRNA and protein expression levels and found a positive correlation between them (17).

Ki-67 is a marker of aggressiveness in pituitary tumors. It is related to invasiveness, a low chance of surgical cure, and higher rates of recurrence (19). In the current study, we also found a higher Ki-67 LI in the invasive tumors. Additionally, the tumor volume at diagnosis was higher in those somatotropinomas with a higher Ki-67 LI.

Fusco et al. (20) evaluated the response to OCT-LAR treatment in 40 acromegalic patients with different Ki-67 LI values. They observed for the first time that patients with higher Ki-67 LI values were less likely to respond to SSA treatment. However, SSTR2 expression was not evaluated and the response to OCT-LAR is mainly influenced by SSTR2 expression, as emphasized by the authors. Therefore, the relationship between the low expression of Ki-67 and control of the disease through SSA treatment could have been related to a different profile of SSTR expression in the tumors with low Ki-67 LI values.

Previously, no study has evaluated whether Ki-67 and SSTR2 are independent predictors of acromegaly control with SSAs. There is only one study that analyzed the acute effect of subcutaneous OCT in acromegalic patients (33). Through univariate analysis, the authors found that SSTR2 expression and Ki-67 were related to the acute effect of OCT in lowering GH levels. However, after a multivariate analysis, only SSTR2 expression remained a predictor of drug response. But, this study considered only the acute OCT test, and no study has considered the effects of long-term treatment with SSAs.

Figure 3 Examples of cytokeratin pattern expression: (A) densely granulated tumor and (B) sparsely granulated tumor. Scale bar = 500 μm. Full colour version of this figure available via http://dx.doi.org/10.1530/EJE-13-0349.
In this study, we confirmed that the Ki-67 LI is a predictor of acromegaly control with SSA therapy. We have also found that both SSTR2 and Ki-67 are independent predictors of disease control with SSA therapy, as we showed that there was no difference in Ki-67 LI between patients with low or high SSTR2 expression using univariate analysis. In addition, both markers remained statistically significant after multivariate analysis. Therefore, our study is the first to report that Ki-67 and SSTR2 are independent predictors of disease control with OCT-LAR treatment in acromegaly.

Previous studies have demonstrated that the cytokeratin pattern of the somatotropinomas is associated with clinical and therapeutic characteristics of these tumors (7, 28). The sparsely granulated somatotropinomas are associated with a poor response to treatment with SSAs (7). In this study, we found the same association. We also observed that the sparsely granulated tumors presented a higher Ki-67 LI than the densely granulated tumors, which is expected due to the known aggressiveness of these tumors.

As previously mentioned, Ki-67 is a marker of cellular proliferation. Thus, it is not directly involved in the mechanism of resistance to OCT-LAR in acromegalic patients. In this study, we demonstrated that the amount of SSTR2 is not responsible for the lower control rates that are observed in patients harboring tumors with higher Ki-67 LI values. One of the possible explanations is the higher frequency of sparsely granulated adenomas between those somatotropinomas with a high Ki-67 LI, although the mechanism involved in the resistance to SSA therapy in sparsely granulated adenomas has also not been fully described yet. Probably, the presence of higher Ki-67 LI values and of the dot-like cytokeratin pattern (sparsely granulated adenomas) represents a phenotype of tumors that have alterations in the post-receptor signaling pathways involved in the response to SSA therapy. Interestingly, a low AIP expression has been observed in tumors with the same phenotype (more invasive and resistant to SSA therapy) (24, 34). Therefore, it is possible that tumors with a high Ki-67 LI present a low expression of AIP or other proteins involved in the post-receptor signaling cascade, and this could be the mechanism behind the worse response to therapy in this group of tumors, as for example, AIP has already been proved to be important in the mechanism of action of SSAs (8, 35). Further studies are necessary to explain the inferior response to therapy in this subset of more aggressive tumors.

In conclusion, this study indicates that Ki-67 and SSTR2 are independent predictors of the response to OCT-LAR in acromegalic patients and that the use of both markers can identify the patients who are more likely to respond to medical therapy with OCT-LAR. Additionally, Ki-67 correlates well with the cytokeratin pattern and both can predict the therapeutic response to OCT-LAR.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-13-0349.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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