Published in final edited form as:

Mol Cell Endocrinol. 2015 December 5; 417: 73–83. doi:10.1016/j.mce.2015.09.016.

Differential Somatostatin Receptor (SSTR) 1-5 Expression and Downstream Effectors in Histologic Subtypes of Growth Hormone Pituitary Tumors^{3,1}

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Abstract

Purpose—The aim of this study was to examine whether differential expression of somatostatin receptors (SSTR) 1-5 and downstream effectors are different in densely (DG) and sparsely (SG) granulated histological growth hormone (GH) pituitary tumor subtypes.

Methods—The study included 33 acromegalic patients with 23 DG and 10 SG tumors. SSTR1-5 were measured by qPCR and immunoblotting. Signaling candidates downstream of SSTR2 were also assessed.

Results—SSTR2 mRNA and protein levels were significantly higher in DG compared to SG tumors. Downstream of SSTR2, p27^{kip1} was decreased (2.6-fold) in SG compared to DG tumors, suggesting a potential mechanism of SSA resistance in SG tumors with intact SSTR2 expression. Re-expression of E-cadherin in GH pituitary cell increased p27^{kip1} levels.

Conclusions—Histological subtyping correlated with SSTR2, E cadherin and p27^{kip} protein levels and these may serve as useful biomarkers in GH tumors to predict behavior and response to therapy with SSA.

Keywords

Acromegaly; somatostatin receptor; dense GH tumors; sparse GH tumors

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1. Introduction

Somatostatin is an inhibitory peptide hormone found throughout the central nervous system and in many peripheral tissues (Reichlin, 1983, Patel, 1997). It has diverse biological actions that include inhibition of hormone secretion, neurotransmitter actions and suppression of proliferation in both normal and tumor cells (Lamberts, 1988, Schettini, 1991, Lamers, 1987, Bousquet, Puente, Buscail et al., 2001). Somatostatin activates five somatostatin receptors subtypes (SSTR1-SSTR5), which are variably expressed in both normal and tumor tissues (Patel, 1999, Schulz, Handel, Schreff et al., 2000). Somatostatin analogs (SSAs), were initially developed to target SSTRs in the treatment of patients with growth hormone (GH) pituitary tumors causing acromegaly and pancreatic neuroendocrine tumors to decrease excess hormone secretion associated with these conditions (Lamberts, van der Lely, de Herder et al., 1996).

The primary action of the currently available SSAs, octreotide and lanreotide, used in the treatment of patients with acromegaly due to GH pituitary tumors, is predominantly via activation of SSTR2 with additional binding to SSTR5 to decrease GH secretion and tumor growth (Weckbecker, Lewis, Albert et al., 2003). Although initial reports suggested high rates of response and remission in the majority of acromegalic patients treated with SSAs, recent literature suggests remission in less than 30% in unselected patients (Freda, 2002, Mercado, Borges, Bouterfa et al., 2007, Melmed, Cook, Schopohl et al., 2010). Differences in treatment outcome may be attributed to prior selection bias in patient recruitment in early studies and the use of SSAs as primary medical therapy versus secondary therapy after pituitary tumor surgical debulking. (Colao, Auriemma, Lombardi et al., 2011). The field needs a better understanding of the underlying mechanisms of resistance to SSAs and tools to predict responders versus nonresponders to SSAs as medical therapy. Previously clinical predictors of response to SSA included pre-treatment GH levels, GH suppression test after a single octreotide injection and the use of an radiolabeled octreotide imaging, although results using these tools have been with inconsistent (Freda, Katznelson, van der Lely et al., 2005, Lamberts, Uitterlinden, Schuijff et al., 1988, Plockinger, Bader, Hopfenmuller et al., 1997, Legovini, De Menis, Billeci et al., 1997).

Designation of subtypes of GH tumors based on specific histological markers including cytokeratin staining pattern, has also been associated with differential response to surgery (Bakhtiar, Hirano, Arita et al., 2010, Mazal, Czech, Sedivy et al., 2001, Kiseljak-Vassiliades, Carlson, Borges et al., 2014), as well as to SSAs as medical therapy (Bhayana, Booth, Asa et al., 2005, Ezzat, Kontogeorgos, Redelmeier et al., 1995, Fougner, Casar-Borota, Heck et al., 2012, Brzana, Yedinak, Gultekin et al., 2013, Bando, Sano, Ohshima et al., 1992). Densely granulated (DG) tumors, defined by perinuclear cytokeratin distribution, have shown a better response rate to medical therapy with SSAs compared to sparsely granulated (SG) tumors, characterized by dot-like keratin immunoreactivity commonly referred to as fibrous bodies (Bhayana et al., 2005, Ezzat et al., 1995, Fougner et al., 2012, Brzana et al., 2013, Bando et al., 1992). The mechanisms underlying the differences in histology and response to surgical and medical therapy are unknown. Initially, investigators suggested that an activating mutation in G protein subunit α (GSP) leading to constitutively active cAMP, which is an intracellular

target for somatostatin analog inhibition, was more commonly detected in DG tumors (Bakhtiar et al., 2010, Spada, Arosio, Boichicchio et al., 1990). However, less than half of patients harboring gsp mutation and 18% of patients without the mutation displayed a response to SSAs in subsequent studies (Bakhtiar et al., 2010). Recently, several groups have shown that increased mRNA or protein levels of SSTR2, in DG compared to SG tumors, may partially explain the differences in SSA response between these two GH tumor subtypes (Bakhtiar et al., 2010, Fougner et al., 2012, Brzana et al., 2013, Kato, Inoshita, Sugiyama et al., 2012). Many tumors, however, are resistant to SSAs (defined as inability to obtain IGF-1 normalization and/or continued tumor growth) despite high expression of SSTR2 (Colao et al., 2011, Wildemberg, Neto, Costa et al., 2013, Gadelha, Kasuki and Korbonits, 2013).

The evaluation of resistance to somatostatin analogues, as it relates to histological subtypes, has predominantly focused on differential expression of SSTR2 mRNA or protein (Fougner et al., 2012, Brzana et al., 2013, Kato et al., 2012, Mayr, Buslei, Theodoropoulou et al., 2013). Investigators also reported that SSTR5 mRNA or protein levels are higher in SG compared to DG tumors (Mayr et al., 2013), although others did not confirm a difference (Kato et al., 2012). An increase in the ratio of SSTR2-to-SSTR5 of 1.3 or higher was reported to be a good predictor of SSA response and disease control, but whether this correlates with different histologic subtype has not been reported (Taboada, Luque, Neto et al., 2008). Although SSTR1-5 mRNA expression levels in GH tumors have been reported, the levels of SSTR1, 3, 4 and 5 protein has been difficult to determine due to lack of available reagents (Kato et al., 2012, Reubi, Schaer, Waser et al., 1994, Reubi, Waser, Schaer et al., 2001, Casarini, Jallad, Pinto et al., 2009). Two recent studies explored differential expression of SSTR1-5 protein in GH histological subtypes with very different results. One group reported that SSTR2 was exclusively expressed in DG and SSTR5 in SG (Mayr et al., 2013), whereas others found no correlation between SSTR subtype expression and GH tumor subtype (Plockinger, Hoffmann, Geese et al., 2012).

The goal of our study was to determine SSTR1-5 mRNA and protein levels in DG compared to SG tumors, and to examine additional mechanisms to explain variable responsiveness to medical therapy with SSAs. To further elucidate potential additional mechanisms of SSTR resistance, we also determined the expression of effectors downstream of SSTR2 which might explain non-response in tumors with moderate to high SSTR2 expression and tested our results in a GH pituitary tumor cell model system.

2. Material and Methods

2.1 Patients

A retrospective chart review of 33 patients with acromegaly due to a GH pituitary tumor, who had undergone transsphenoidal pituitary surgery by a single neurosurgeon, was performed with Colorado Multiple Institutional Review Board (COMIRB) approval. The diagnosis of acromegaly was based on clinical and biochemical findings with elevated GH and IGF-1 levels, and confirmed on pathological examination by a single neuropathologist (BKD). Demographic patient information, including age and gender, was collected as well as tumor size at the time of diagnosis using standard magnetic resonance images (MRI) of

pituitary, with and without gadolinium contrast. Tumor dimensions were recorded and tumor volume was calculated using formula: $0.5 \times \text{width} \times \text{length} \times \text{height} \text{ (mm}^3\text{)}$ (Lundin and Pedersen, 1992). Post-operative IGF-1 level at 3-month and annual follow up were recorded. IGF-1 level was determined using Quantitative Chemiluminescent Immunoassay (Immunodiagnostic Systems; Isys technologies) with detection level of 10 ng/mL. IGF-1 is standardized to World Health Organization (WHO) international standard 02/254. IGF-1 levels were calculated and reported as fold-change from upper limit of normal (ULN), matched for age and gender. Patients with persistent disease at 3-months post-operatively, in conjunction with clinical evidence of GH excess, were placed on a SSA (octreotide LAR or lanreotide) with dose titration every 3-4 months until IGF-1 normalization or maximum dose (monthly octreotide LAR 30-40mg or lanreotide autogel 120mg) for 3 months. Data were collected with respect to SSA responsiveness to induce remission based on evidence of IGF-1 normalization corrected for age and gender.

2.2 Cell culture and reagents

GH3 cells were purchased from the American Type Cultures Collection (Manassas, VA). Cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan UT), 100 U/mL penicillin, and 100 ug/mL streptomycin at 37 C in humidified 5% CO₂. E-cadherin-LZRS-GFP retroviral construct was a generous gift from Dr. Al Reynolds (Vanderbilt University, Nashville, Tennessee). GH3 cells were transduced with E-cadherin or vector constructs as previously described (Ireton, Davis, van Hengel et al., 2002).

2.3 Quantitative Real-Time PCR

Total RNA was extracted from tissues using TRIzol reagent, according to the manufacturer's protocol (Invitrogen) and was purified with the RNeasy minikit (Qiagen, Valencia, CA) and 2.5µg was reverse transcribed with the iScript cDNA synthesis kit (Bio-rad, Hercules, CA). GH tumor cDNA was diluted 1:10 and SSTR1,2,3,4,5, Aip, Zac1, Shp1, p21, Cdkn1b/p27^{kip1} expression were determined using Taqman Gene Expression Assays (Life Technologies, Grand Island, NY) after normalization to 18S (Life Technologies, Grand Island, NY) RNA levels. Human SSTR4 amplicon (IDT, Coralville, IA) were used as a positive control. Real-time PCR was performed in duplicate on an ABI7300 system using Gene Expression Master Mix (Applied Biosystems). Real time PCR for human E-cadherin and rat Cdkn1b/p27^{kip1} from GH3 cells was performed using Power SYBR Green PCR master mix (Applied Biosystems, Foster city, CA) in an ABI PRISM 7300 real-time recycler (Applied Biosystems). Primers used were human E-cadherin Forward: GCTGGACCGAGAGAGTTTCC, and Reverse: CGACGTTAGCCTCGTTCTCA. Rat Cdkn1b/p27^{kip1} Forward:CGGCGCAAGAGAGGC, and Reverse:CAGTGCTTCTCCAAGTCCCG; GAPDH was used as control Forward:TGGAGTCTACTGGCGTCT, and Reverse: TGTCATATTTCTCGTGGT. The experiment was repeated in triplicates.

2.4 Histopathological evaluation

Tumor tissues were submitted and fixed overnight in 10% buffered formalin as previously described (Kiseljak-Vassiliades et al., 2014). Briefly, sections from the paraffin blocks were

cut at 4 microns and stained with hematoxylin and eosin, Gomori reticulin, and periodic acid Schiff-orange G histochemical stains. Immunohistochemical staining (IHC) staining for all anterior pituitary hormones was performed using the peroxidase-antiperoxidase complex technique, with light hematoxylin counterstaining. Growth hormone tumor subtyping using pattern of cytoplasmic immunostaining for CAM5.2 (monoclonal, pre-diluted; Becton Dickinson) was assessed. Densely-granulated (DG) growth hormone adenomas were defined as those with growth hormone immunostaining and CAM5.2 immunoreactivity throughout the cytoplasm, corresponding to distribution of cytokeratin filaments throughout the cytoplasm and an absence of rounded CAM5.2 IHC-positive fibrous bodies. Conversely, sparsely-granulated (SG) growth hormone adenomas manifested GH immunostaining (often weaker than in DG GH adenomas) associated with numerous, diffusely-distributed FBs on CAM5.2. Overly mixed GH-PRL adenomas or adenomas with weak IHC for GH and/or rare CAM5.2-immunopositive fibrous bodies, such as acidophil stem cell adenomas, were not included in the study. E-cadherin antibody (Ventana Medical Systems, Tuscon, AZ) immunohistochemistry detection was performed as per manufactures protocol using VENTANA detection kit. p27^{kip1} antibody (Sigma-Aldrich, St. Louis, MO) immunohistochemistry on GH tumors was performed by IHC techniques as described below.

The monoclonal mouse antibody for SSTR2 and monoclonal rabbit antibody SSTR3 were purchased from Abcam (Cambridge, MA). The mouse MABs for SSTR1, 4 and 5 were developed and provided by H Schmidt, Novartis, Their specificity has recently been described (Schmid, Lambertini, van Vugt et al., 2012). IHC for SSTR1-5 was performed on 4 microns thin sections prepared from formalin-fixed paraffin-embedded tissue. Sections were deparaffinized in xylene followed by hydration in a graded series of ethanol (100, 95 and 70% ethanol) and water. Sections underwent antigen retrieval (AR) pretreatment by incubating them in a 10 mM sodium citrate (pH 6.0) for 10 minutes at 110C in a pressure cooker followed by one rinse in 1× Reaction Buffer (Ventana Medical Systems). Endogenous peroxidase was blocked by incubating sections with 3% hydrogen peroxide solution for 10 min at room temperature. After washing twice for 5 min, 10% normal goat serum diluted in Reaction Buffer for 30 min at room temperature was added to the sections to reduce unspecific absorption. Sections were subsequently incubated in a humid chamber at 4 ° C overnight with the antibodies (at different concentrations: SSTR1 at 1:1000, SSTR2 at 1:200, SSTR3 at 1:1000, SSTR4 1:2000, SSTR5 at 1:500 and p27^{kip1} at 1:200) diluted in antibody diluent solution. After washing, to visualize immunoreactivity, universal polymer peroxidase (EnVision) was added to the slides for 30 min followed by a freshly made DAB solution for 5 min, both at room temperature. Sections were counterstained with Harris hematoxylin for 2 minutes at room temperature, dehydrated in a graded series of alcohol (70%, 95% and 100%), incubated in xylene and placed on coverslips. Negative controls to confirm the specificity of the immunostaining included omission of the primary antibody incubation step in the IHC protocol, substitution of the primary antibody with non-immune immunoglobulin of the same isotype at the same concentration as the primary antibodies.

Each SSTR1-5 and p27^{kip1} section was graded, in a blinded fashion to the DG or SG status, by the study neuropathologist (BKD) for the percentage of immunostained stained adenoma cells. For p27^{kip1} grading only nuclear, not cytoplasmic, immunoreactivity was assessed in a

subset of samples. Grading was conducted on a subjective scale, with 0 – no staining, 1 – mild to moderate staining with 10-50% of cell positive, 2 – strong staining with 50-100% of cell positivity. Intensity of immunostaining was graded in comparison to control samples from normal pituitary tissue and pancreas.

2.5 Immunoblotting

Primary antibodies used for immunoblotting were obtained as follows: SHP1 and SHP2 (Cell Signaling, Beverly, MA; 1:1000), Zac1 (Santa Cruz, Dallas, TX; 1:350), AIP (Abcam, Cambridge, MA; 1:1000), p27^{kip1} (BD Biosciences, Franklin Lakes, NJ 1:1000).

Immunoblotting for normal human and pituitary tumors was performed as described previously (Xu, Knox, Michaelis et al., 2012). Briefly, the tissue was homogenized and protein lysates were quantified by bicinchoninic assay (Pierce, Rockford, IL) and electrophoresed through sodium dodecyl sulfate polyacrylamide gels and onto polyvinyl difluoride membranes. Primary antibodies were diluted in TBST/0.5% BSA/0.1% NaN₃, and the membranes were incubated in primary antibodies at 4 C overnight and then conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ) was diluted 1:3000 in TBST and incubated with the membranes for 1 h. The membranes were washed and visualized using enhanced chemiluminescence protocol (Pierce).

2.6 Immunofluorescence

For detection of E-cadherin and p27^{kip1} in GH3 E-cadherin stable overexpression experiments, GH3 cells were plated (50,000/well on poly-D-Lysine treated coverslips) and allowed to grow for 72 hours and then fixed with 4% PFA for 15 minutes at room temperature. The coverslips were then washed in PBS 3 times and covered with blocking solution (5% BSA, 0.2% Tritonx-100) for 1 hour at room temperature. The cells were then incubated overnight with primary antibody: E-cadherin dilution 1:200 (Cell Signaling 3195S, Rabbit), p27^{kip1} 1:1000 (Sigma Aldrich p2092, Mouse). The next day coverslips were washed with PBS 3 times and placed in secondary antibody (1:400 Life technologies AF647 [A21236], AF546 [A11035]) for 2 hours at room temperature. Cells were washed with PBS 3 times and were then mounted with prolonged gold containing DAPI (Invitrogen) and observed under confocal microscope (Olympus FV1000 FCS/RCIS, Tokyo, Japan). All images of the same channel used the same acquisition parameters. To quantify the intensity of p27^{kip1} NIH Image J software was used. All regions of interest (ROI) used the same area and were chosen based on having >80% of the area containing cells. Thirty six ROI were used to quantify intensity for each sample using the measure function. Background was subtracted from each ROI intensity measurement and then the data was plotted in Graphpad-Prism5. Student t-test was used to determine statistical significance.

2.7 Statistical analysis

Patient and tumor characteristics were compared between the two tumor subtypes SG and DG using two-sample t-tests or Chi-square tests as appropriate. Differences in response to surgical treatment by tumor subtype were assessed using Fisher's exact test given the small samples. Given the small numbers of patient samples (n=8) in the cohort who were not in remission after surgery, statistical analysis on rates of remission after medical therapy with SSAs could not be performed. The association between tumor subtype and SSTR1-5 mRNA

and protein expression was assessed using permutations tests. Comparisons between patients' characteristics and SSTR1-5 status were measured using one-way ANOVA or Spearman test as appropriate. All analyses were performed using SAS v9.3 (Cary, NC) or R v3.01 (The R Foundation for statistical computing). P-values < 0.05 were considered statistically significant.

3. Results

3.1 Patient characteristics

Thirty three GH tumor samples were divided by histologic subtype into 22 DG and 11 SG tumors. All patients, importantly, were naïve to medical treatment prior to the tumor resection. Baseline patient characteristics are outlined in Table 1. There was no significant difference in age, gender, volume of tumor or baseline IGF-1 level (assessed by fold change from upper limit of normal) in DG versus SG patients. Post-operatively, rates of remission, however, were more often achieved in patients with DG tumors (80.1%; N=17/21) compared to SG tumors (22.2%; N=2/9), $p=0.004$, as we have previously reported in a larger cohort (Kiseljak-Vassiliades et al., 2014).

3.2 Measurement of SSTR1-5 mRNA levels

SSTR1-5 mRNA levels were measured using qPCR (33 GH tumors: 22 DG and 11 SG) (Figure 1). DG tumors showed higher SSTR2 mRNA levels than SG tumors (median difference of 5.5-fold, $p=0.02$). The distribution range of SSTR2 mRNA levels was larger in DG tumors compared to SG tumors, where tumors showed consistently low mRNA levels. SSTR2 mRNA levels in normal pituitary samples were low, and similar to that of SG tumors ($p=0.58$). SSTR1, 3 and 5 mRNA expression levels, by qPCR, did not differ between GH tumor subtypes or from normal pituitary. Of interest however, both SSTR1 and SSTR3 mRNAs displayed a tri-modal distribution in DG tumors, while their expression was consistently lower in SG tumors (geometric mean difference 1.7, $p=0.25$ and geometric mean difference of 0.89 $p=0.22$). SSTR5 mRNA levels were higher (1.4 fold) in SG compared to DG tumors and normal pituitary samples, but not statistically different ($p=0.62$). SSTR4 mRNA was not detected in GH tumors or normal pituitary (data not shown).

SSTR2 mRNA levels were also significantly higher in male than female patients ($p=0.04$) and there was a trend of higher SSTR2 levels in patients who were in remission after surgery ($p=0.06$). There was no correlation between SSTR1-5 mRNAs and age or tumor volume. The ratio of SSTR2-to-SSTR5 was calculated and a ratio of >1.3 was observed in 48.3 % more patients with DG tumors than SG tumors ($p=0.01$) (supplementary Figure 1).

3.3 SSTR1-5 protein levels by immunohistochemistry

Immunohistochemistry was performed on 32 (22 DG and 10 SG) GH tumor samples to evaluate SSTR 1, 2, 3 and 5 subtype protein levels. We used a classification system where the level of staining for each individual SSTR was characterized as strong (+2), weak (+1) or no expression (0) in tumor and normal pituitary samples, as detailed in Methods (Figure 2). As shown on immunohistochemistry (Figure 2) SSTR were localized predominately at

the cell membrane. DG tumors (80%; N= 18/22) were more likely to have strong SSTR2 immunostaining compared to SG (30%; N=3/10), $p=0.0095$ (Figure 3). SSTR2 expression in SG tumors was usually, but not consistently weak (+1), with 60% (N=6/10) of SG tumors having weak, and 10% (N=1/10) no SSTR2 staining. SSTR1 and SSTR3 protein levels were comparable across the different GH tumor subtypes. SSTR5 had slightly higher, but not significantly different level of expression in SG tumors (80.0%; N=8/10) compared to DG (64%, N=14/22), $p=0.42$.

3.4 SSA responsiveness and SSTR expression

Since a majority of patients with DG tumors were in remission after surgery by an experienced neurosurgeon (80.1%; N=17/21), analysis of response to medical therapy with SSA in relation to SSTR expression in this tumor subtype was limited to 8 samples. The IHC expression of SSTRs and response to surgery is outlined in Supplementary Table 1. In the DG group, one patient with strong expression of SSTR 1, 2, 5 responded to SSA. On the other hand, one SSA non-responder displayed, strong expression of SSTR2, weak staining of SSTR5 and loss of SSTR1. In the patients with SG tumors who received SSA (N=6), there were only two responders, one patient with a tumor expressing weak SSTR1 and 2 and strong SSTR5; the other with high levels of SSTR1, 2, and 5. Four SG tumors did not respond to SSA (one with negative SSTR2 negative, two weak SSTR2 and one strong SSTR2 on immunohistochemistry). In summary these data show that SSTR expression did not fully explain or predict response to medical therapy with SSAs.

3.5 Low p27^{kip1} levels may be implicated in SSA resistance

To elucidate potential dysregulation of components downstream of SSTRs that may contribute to SSA resistance in GH tumor subtypes, we examined a panel of molecules shown to be downstream of SSTR2 (Lopez, Esteve, Buscail et al., 1997, Ben-Shlomo and Melmed, 2010, Theodoropoulou, Stalla and Spengler, 2010, Theodoropoulou and Stalla, 2013, Ferjoux, Lopez, Esteve et al., 2003, Luttrell and Lefkowitz, 2002, Lopez, Ferjoux, Cordelier et al., 2001, Pages, Benali, Saint-Laurent et al., 1999, Sellers, Alderton, Carruthers et al., 2000), as outlined in Figure 4, including: SHP1, SHP2, p27^{kip1}, p21, AIP and ZAC1. Levels of mRNA measured by qPCR and immunoblot detection of protein levels of these candidates in normal pituitary and GH tumor histological subtype samples are shown in Figure 5. Contrary to expectations, the expression of the docking proteins SHP1 and SHP2 were similar between SG and DG tumors. In contrast to suggestion by others (Leontiou, Gueorguiev, van der Spuy et al., 2008), ZAC1 and AIP levels did not differ between DG and SG tumor samples in our cohort. The cyclin-dependent kinase inhibitor, p27^{kip1} however, was downregulated in SG compared to DG tumors at the mRNA level (2.4-fold, $p=0.02$) (Figure 5A). Similarly, at the protein level assessed by immunoblotting p27^{kip1} was 2.6-fold lower in SG compared to DG tumors (Figure 5B), which was also confirmed by immunohistochemistry, where on average nuclear p27^{kip1} was consistently weaker in SG tumors (representative samples are shown in Figure 6B). We have also examined other cell cycle components including Cyclin E, Cyclin D1, Cyclin D3, Cdk2, Cdk4 as well as Akt and ERK signaling pathways none of which differ between DG and SG tumors (data not shown).

3.6 Correlation of p27^{kip1} with changes in E cadherin with GH histologic subtypes

As others have previously reported (Bakhtiar et al., 2010, Fougner et al., 2012, Obari, Sano, Ohyama et al., 2008, Sano, Rong, Kagawa et al., 2004, Xu, Sano, Yoshimoto et al., 2002, Fougner, Lekva, Borota et al., 2010), E-cadherin is one of the most dysregulated proteins in correlation to GH tumor subtypes. As shown in Fig 6A. E-cadherin is highly expressed in DG tumors, whereas it is low or absent in SG tumors. Besides playing a role in cell-cell adhesion, E-cadherin has been reported to be a major growth suppressor through upregulation of p27^{kip1} in human carcinoma cell lines (St Croix, Sheehan, Rak et al., 1998). To examine whether levels of E-cadherin might play a role in modulating the levels of p27^{kip1} expression in DG tumors, we overexpressed E-cadherin in GH3 pituitary cells and assessed changes in levels of p27^{kip1}. Overexpression of E-cadherin, to levels comparable in human DG tumors, was associated with a 2.1-fold increase in Cdkn1b/p27^{kip1} compared to vector on mRNA level (p=0.005, Figure 7A). Immunocytochemical analysis revealed a 1.7-fold increase of p27^{kip1} in E-cadherin expressing GH3 pituitary cells compared to vector controls (Figure 7B-C) (p=0.0009). Together, these data suggest that in addition to SSTR2 downregulation, SG tumors have dysregulated key components downstream of the receptor that may contribute to a tumor phenotype that is less responsive to surgery or medical therapy.

4. Discussion

Our study confirms the suggestion by others (Fougner et al., 2012, Brzana et al., 2013, Kato et al., 2012), that DG tumors have higher expression of SSTR2 at the mRNA and protein levels compared to SG tumors. Contrary to our predictions, SSTR1, 3 and 5 mRNAs, did not differ between GH tumor subtypes. In a limited cohort of GH tumors we correlated SSTR1-5 protein expression to clinical response to SSA therapy and observed that strong SSTR2 staining was not always a predictor of response to SSAs. We document alterations in the expression of the cell cycle kinase inhibitor p27^{Kip1} which was lower in SG compared to DG tumors and demonstrated that E-cadherin upregulation is associated with increased p27^{kip1} levels in GH3 pituitary cells. Together, these alterations may contribute to SSA resistance in SG tumors with intact SSTR2 expression.

Compared to previously published literature, in our cohort there was no difference in age, gender or tumor volume between two different GH tumors subtype patients likely due to our smaller sample size (Bakhtiar et al., 2010, Mazal et al., 2001, Kiseljak-Vassiliades et al., 2014, Bando et al., 1992, Obari et al., 2008, Mori, Inoshita, Takahashi-Fujigasaki et al., 2013). The response to surgery was dramatically different in patients with DG compared to SG growth hormone tumors similar to prior reports (Bakhtiar et al., 2010, Mazal et al., 2001, Kiseljak-Vassiliades et al., 2014).

SSTR subtype mRNA expression in relation to GH tumor subtype has been examined recently by several groups (Kato et al., 2012, Mayr et al., 2013). While Mayr and coworkers found positive correlation of SSTR2 in DG, and SSTR5 in SG tumors (Mayr et al., 2013), Kato et al. observed that DG tumors showed higher SSTR2 mRNA expression, and SSTR5 did not differ between the two groups (Kato et al., 2012). Our findings concur with those of Kato et al. and showed higher SSTR2 mRNA and protein expression in DG vs SG tumors,

and similar expression levels for other SSTRs between two subtypes. Similar to the studies by Kato et al (Kato et al., 2012), DG tumors in our cohort displayed widely variable SSTR2 mRNA expression levels, some with very low levels comparable to SG tumors levels, and some with moderate and high expression. These data suggest that SSTR2 expression alone does not predict SSA responsiveness or resistance. This hypothesis is also supported by recent study of Brzana et al. which reported that approximately 15% of DG tumors with positive SSTR2 staining did not responded to treatment with SSA (Brzana et al., 2013).

Although we did not document significant differential expression of SSTR1,3 and 5 between GH tumor subtypes, dimerization of SSTRs have been previous explored with potential therapeutic implications. SSTRs are capable of forming homodimers and heterodimers at the cell membrane, although the underlying molecular mechanism and modulation of intracellular signaling cascades have only been partially elucidated (Rocheville, Lange, Kumar et al., 2000, Sharif, Gendron, Wowchuk et al., 2007, War and Kumar, 2012, Grant, Alturaihi, Jaquet et al., 2008). SSTR2 is localized to the cell membrane where it can forms homodimers that upon agonist treatment dissociate into monomers and become internalized (Grant, Collier and Kumar, 2004). SSTR2 heterodimerization with SSTR3 and SSTR5 has been shown to modulate its downstream signaling to affect proliferation and trafficking, respectively (War and Kumar, 2012, Grant et al., 2008). When compared to SSTR2 transfectants alone, SSTR2/SSTR5 heterodimer showed reduced receptor internalization, increased receptor recycling and decreased desensitization when treated when a selective SSTR2 agonist (L-779,976). Based on observations by Taboada and coworkers (Taboada et al., 2008), we evaluated the ratio of SSTR2-to SSTR5 mRNA in DG vs SG tumors, and confirmed that a ratio >1.3 predicted SSA response in our cohort and was more frequently detected in DG compared to SG tumors. These data suggest the need for further investigation into the role of SSTR subtype interactions to modulate intracellular signaling and response to SSA.

Signaling pathways downstream of SSTR2, as outlined in Figure 4, have been extensively characterized (Lopez et al., 1997, Ben-Shlomo and Melmed, 2010, Theodoropoulou et al., 2010, Theodoropoulou and Stalla, 2013, Ferjoux et al., 2003, Luttrell and Lefkowitz, 2002, Lopez et al., 2001, Pages et al., 1999, Sellers et al., 2000). However, a differential expression of the key downstream components had not been previously explored based upon histologic subtyping. The anti-proliferative action of SSTR2 is initiated through its association with the docking proteins, SHP-1 and SHP-2 (Lopez et al., 1997) which in turn induce MAPK phosphorylation and activation and decrease cell proliferation (Sellers et al., 2000). Fougner and colleagues also reported that attenuation of MAPK signaling downstream of SSTR2 by Raf kinase inhibitory protein (RKIP) might also play a role in SSA resistance. We hypothesized that SHP-1 and -2 levels would be decreased in SG vs DG tumors, but our results showed the opposite. These results suggest that these proteins are likely not implicated in differential response of patients with different GH subtypes to SSA therapy. We, also examined candidates downstream of MAPK including ZAC1 (Theodoropoulou et al., 2010) and upstream by AIP (Chahal, Trivellin, Leontiou et al., 2012), whose activation leads to cell cycle arrest through upregulation of key cell-cycle inhibitor, p27^{Kip1}. While AIP and ZAC1 were not differentially expressed on protein level, p27^{Kip1} was significantly more often downregulated in SG compared to DG tumors. In

animal studies, germline deletion of p27^{Kip1} has been associated with development of pituitary hyperplasia and tumors (Fero, Rivkin, Tasch et al., 1996, Kiyokawa, Kineman, Manova-Todorova et al., 1996, Nakayama, Ishida, Shirane et al., 1996), but in human pituitary tumors p27^{Kip1} mutations are infrequent (Takeuchi, Koeffler, Hinton et al., 1998). Although our patient cohort was too small to draw definitive conclusions on whether low p27^{Kip1} correlates with SSA resistance, these data are suggestive that decreased expression of this cycle inhibitor in SG tumors, which are frequently not responsive to treatment with SSA, as previously reported in our larger cohort of patients (Kiseljak-Vassiliades et al., 2014), may contribute to differential response to SSA. To further assess the role of p27^{Kip1} in GH tumor subtypes we asked whether E-cadherin, known to also be downregulated or absent in SG tumors (Bakhtiar et al., 2010, Fougner et al., 2012, Obari et al., 2008, Sano et al., 2004, Xu et al., 2002), might modulate p27^{Kip1} levels in pituitary tumor cells. In the GH3 pituitary cell model we documented that increases in E-cadherin are associated with increased p27^{Kip1} expression. While Qian and colleagues previously suggested that p27^{Kip1} is methylated and undetectable in GH3 cells (Qian, Jin, Kulig et al., 1998), an earlier manuscript by the same group (Qian, Jin and Lloyd, 1997) demonstrated low but detectable p27^{Kip1} mRNA and protein which is comparable to our present findings. While both E-cadherin and p27^{Kip1} downregulation have been associated with invasive growth patterns and metastasis in human carcinoma (van Roy, 2014, Borriello, Bencivenga, Criscuolo et al., 2011), their alteration and prognostic value has also been reported in preneoplastic head and neck lesions (Massarelli, Brown, Tran et al., 2005), suggesting they may be useful biomarkers in GH pituitary tumors.

In summary, in contrast to our hypothesis that all of the SSTR subtypes would be differentially expressed at the mRNA or protein level in the different GH histologic subtypes, we confirmed, as others previously reported, that only SSTR2 showed differential expression (Brzana et al., 2013, Kato et al., 2012). While SSTR5 expression was not different between DG and SG tumors, SSTR2-to-SSTR5 mRNA ratio >1.3 was more often seen in DG tumors, implicating that interaction between SSTR subtypes might play a role in response to SSA therapy. Although the analysis of response to SSA in our study was limited due to an excellent surgical response and remission in patient with DG tumors, our data are supportive of the observations by other groups that low SSTR2 alone cannot explain SSA resistance. (Colao et al., 2011, Brzana et al., 2013, Wildemberg et al., 2013). The variability in mRNA and protein levels suggests that SSTR subtyping will not be clinically useful as a single biomarker. Here, for the first time, we report that p27^{Kip1} is differentially expressed between two GH tumor histological subtypes and based on *in vitro* data its expression might be regulated by E-cadherin. It would be of interest to examine if SG tumors with a low ratio of SSTR2-to-SSTR5 have a different response to Pasireotide, which more specifically targets SSTR5. Taken together our data suggest that further investigation is needed to derive a combination of protein or mRNA biomarkers to identify SSA responders and nonresponders for medical treatment of patients with GH pituitary tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by Investigator initiated grant from Novartis and VA Merit Review to MEW, N.I.H. K12CA086913-12 to KKV

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Highlights

Somatostatin analogues (SSA) are the treatment for growth hormone (GH) tumors.

Histological subtyping of GH tumors (DG and SG) may predict response to SSA.

We examined the expression of somatostatin receptors in GH tumor subtypes.

SSTR2 levels were significantly higher in DG compared to SG tumors.

Downstream of SSTR2 p27^{kip1} was decreased in SG compared to DG tumors.

Figure 1. SSTR1, 2, 3 and 5 mRNA level by qPCR

SSTR2 is significantly higher in DG than to SG tumors (*p=0.02). SSTR2 expression in normal pituitaries is similar to those of SG tumors. SSTR1, 3 and 5 expression did not differ between GH tumor subtypes and/or normal pituitary.

Figure 2. Immunohistochemistry scoring (IHC) method

Normal pituitary staining for SSTR1,2,3,5 is shown in left column. Examples of SSTRs IHC are shown for tumors, +2 (strong), +1 (weak) and 0 (negative) (40×). No tumor had negative IHC for SSTR5.

Figure 3. SSTR1, 2, 3 and 5 protein expression by immunohistochemistry
SSTR2 expression is significantly more often stronger in DG compared to SG tumors (* $p=0.0095$). SSTR1,3 and 5 protein levels did not differ between GH tumor subtypes

Figure 4. SSTR2 pathway downstream signaling

((Lopez et al., 1997, Ben-Shlomo and Melmed, 2010, Theodoropoulou et al., 2010, Theodoropoulou and Stalla, 2013, Ferjoux et al., 2003, Luttrell and Lefkowitz, 2002, Lopez et al., 2001, Pages et al., 1999, Sellers et al., 2000))

Figure 5. A. Candidates downstream of SSTR2 mRNA by qPCR

On mRNA level p27^{kip1} is downregulated in SG compared to DG tumors (*p=0.02) **B. protein expression by immunoblot.** p27^{kip1} is 2.6 fold higher in DG compared to SG tumors. SHP1, SHP2, ZAC1 and AIP were unchanged.

Figure 6. A. Immunohistochemistry of human GH tumors for E-cadherin

DG tumors have strong and diffuse E-cadherin staining, while in SG tumors E-cadherin is

low or absent (40×) **B. Immunohistochemistry of human GH tumors for p27^{kip}** DG

tumors on average show stronger staining, while in SG tumors p27^{kip} is low or absent (40×)

Figure 7. A. mRNA by qPCR

Overexpression of E-cadherin increases p27^{kip1} levels in GH3 by 2.1 fold cells (*p=0.005)

B. Immunofluorescence graph E-cadherin overexpression is associated with increases in p27^{kip1} protein levels (**p=0.0009) **C. Immunofluorescence slide example** of E-cadherin compared to vector shows nuclear DAPI, E-cadherin and p27^{kip1} stain

Table 1
Patients' clinical baseline characteristics

Characteristic	DG tumors N=22	SG tumors N=11	P value
Age (Mean $\pm$ SE)	49.0 $\pm$ 2.6	43.8 $\pm$ 3.9	0.28
Gender (N:% Females)	56.5%	60.0%	0.85
Tumor Volume (mm ³)	3264 $\pm$ 3361	5077 $\pm$ 4129	0.27
Baseline IGF-1 $\times$ FC ULN ($\pm$ SD)	3.3 $\pm$ 1.5	2.7 $\pm$ 1.6	0.29
Curative surgery (% of patients)	80.1%	22.2 %	0.004