EBioMedicine 43 (2019) 537–552

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

EBioMedicine
Published by THE LANCET

Unmasking a new prognostic marker and therapeutic target from the GDNF-RET/PIT1/p14ARF/p53 pathway in acromegaly

Miguel Chenlo [a,j,1], Iria A. Rodriguez-Gomez [b,c,j,1], Ramon Serramito [d,j], Angela R. Garcia-Rendueles [a,j], Rocío Villar-Taibo [b,j], Eva Fernandez-Rodriguez [f], Sihara Perez-Romero [a,j], Maria Suarez-Fariña [a,j], Alfredo Garcia-Allut [d,j], Jose M. Cabezas-Agricola [b,j], Javier Rodriguez-Garcia [e,j], Pamela V. Lear [g], Rosa M. Alvarez-San Martin [h], Cristina Alvarez-Escola [i], Ignacio Bernabeu [b,j,*], Clara V. Alvarez [a,j,**]

[a] Neoplasia & Endocrine Differentiation P0L5, Centro de Investigación en Medicina Molecular y Enfermedades Crónicas (CIMUS), University of Santiago de Compostela (USC), Spain
[b] Servicio de Endocrinología y Nutrición, Complejo Hospitalario Universitario de Santiago de Compostela (CHUS)-SERGAS, Santiago de Compostela, Spain
[c] Servicio de Endocrinología y Nutrición, Hospital HM Modelo, A Coruña, Spain
[d] Servicio de Neurocirugía, Complejo Hospitalario Universitario de Santiago de Compostela (CHUS)-SERGAS, Santiago de Compostela, Spain
[e] Servicio de Análisis Clínicos, Complejo Hospitalario Universitario de Santiago de Compostela (CHUS)-SERGAS, Santiago de Compostela, Spain
[f] Servicio de Endocrinología y Nutrición, Complejo Hospitalario Universitario de Ourense, Spain
[g] Department of Physiology, Anatomy and Genetics, Oxford University, Oxford, United Kingdom
[h] Servicio de Endocrinología y Nutrición, Complejo Asistencial Universitario de Leon, Spain
[i] Servicio de Endocrinología y Nutrición, Hospital Universitario La Paz, Madrid, Spain
[j] Instituto de Investigación Sanitaria (IDIS), Santiago de Compostela, Spain

ARTICLE INFO

Article history:
Received 28 January 2019
Received in revised form 2 April 2019
Accepted 2 April 2019
Available online 8 April 2019

Keywords:
Pituitary tumors
Somatotropinomas
Endoscopy surgery
Acromegaly
ARF
GFRA4
SSA-resistance
Human pituitary cultures
Sorafenib

ABSTRACT

Background: Acromegaly is produced by excess growth hormone secreted by a pituitary adenoma of somatotroph cells (ACRO). First-line therapy, surgery and adjuvant therapy with somatostatin analogs, fails in 25% of patients. There is no predictive factor of resistance to therapy. New therapies are investigated using few dispersed tumor cells in acute primary cultures in standard conditions where the cells do not grow, or using rat pituitary cell lines that do not maintain the full somatotroph phenotype. The RET/PIT1/p14ARF/p53 pathway regulates apoptosis in normal pituitary somatotrophs whereas the RET/GDNF pathway regulates survival, controlling PIT1 levels and blocking p14ARF (ARF) and p53 expression.
Methods: We investigated these two RET pathways in a prospective series of 32 ACRO and 63 non-functioning pituitary adenomas (NFPA), studying quantitative RNA and protein gene expression for molecular-clinical correlations and how the RET pathway might be implicated in therapeutic success. Clinical data was collected during post-surgical follow-up. We also established new'humanized' pituitary cultures, allowing 20 repeated passages and maintaining the pituitary secretory phenotype, and tested five multikinase inhibitors (TKI: Vandetanib, Lenvatinib, Sunitinib, Cabozantinib and Sorafenib) potentially able to act on the GDNF-induced RET dimerization/survival pathway. Antibody arrays investigated intracellular molecular pathways.
Findings: In ACRO, there was specific enrichment of all genes in both RET pathways, especially GDNF. ARF and GFRA4 gene expression were found to be opposing predictors of response to first-line therapy. ARF cut-off levels, calculated categorizing by GNAS mutation, were predictive of good response (above) or resistance (below) to therapy months later. Sorafenib, through AMPK, blocked the GDNF/AKT survival action without altering the RET apoptotic pathway.
Interpretation: Tumor ARF mRNA expression measured at the time of the surgery is a prognosis factor in acromegaly. The RET inhibitor, Sorafenib, is proposed as a potential treatment for resistant ACRO.
Fund: This project was supported by national grants from Agencia Estatal de Investigación (AEI) and Instituto Investigación Carlos III, with participation of European FEDER funds, to IB (PI150056) and CVA (BFU2016-76973-R). It was also supported initially by a grant from the Investigator Initiated Research (IIR) Program (WI177773) and by a non-restricted Research Grant from Pfizer Foundation to IB. Some of the pituitary

* Correspondence to: Ignacio Bernabeu, Servicio de Endocrinologia y Nutrición, Complejo Hospitalario Universitario de Santiago de Compostela (CHUS)-SERGAS, 15706 Santiago de Compostela, Spain.
** Correspondence to: Clara V Alvarez, Neoplasia & Endocrine Differentiation P0L5, Centro de Investigación en Medicina Molecular y Enfermedades Crónicas (CIMUS), Av Barcelona s/n, 15782 Santiago de Compostela, Spain.
E-mail addresses: ignacio.bernabeu.moron@sergas.es (I. Bernabeu), clara.alvarez@usc.es (C.V. Alvarez).
[1] Equal co-first authors.

https://doi.org/10.1016/j.ebiom.2019.04.007

acromegaly samples were collected in the framework of the Spanish National Registry of Acromegaly (REMAH), partially supported by an unrestricted grant from Novartis to the Spanish Endocrine Association (SEEN).
CVA is also supported from a grant of Medical Research Council UK MR/M018539/1.

Research in context

Evidence before this study

25% of patients with a head pituitary tumor secreting growth hormone (GH), acromegaly, will be resistant to first-line therapy, surgery + fgSSA. Months later those patients will suffer radiotherapy or second/third surgery in the head while presenting multiple metabolic, cardiovascular, joint and oncological body comorbidities. There is no predictive factor of resistance to therapy. In the last years we have had a scarcity of new therapies for pituitary acromegaly addressing the tumors. One of the reasons is the difficulty to establish acromegaly primary cultures maintaining the somatotroph secretory phenotype and growing in vitro. To express and secrete GH a cell needs to express the transcription factor PIT1. The few rat pituitary cell lines secreting GH do not maintain the full panel of receptors characteristic of somatotrophs.
In normal pituitary somatotrophs the RET receptor located at the membrane is implicated into two opposite pathways. In one hand, when there is no GDNF ligand reaching the cell surface, RET behaves as a "dependence receptor" being intracellularly processed by caspase and inducing overexpression of PIT1, which activates the CDKN2A/p14ARF promoter leading to p53 accumulation and cell death. That is the reason why the somatotrophs are "dependent" on the GDNF ligand expression inducing RET receptor dimerization at the membrane, activating its tyrosine-kinase activity and leading to AKT phosphorylation, controlling PIT1 levels, enough for GH secretion but maintaining survival.

Added value of this study

We carried out a prospective study of more than seven years to evaluate the importance of the RET receptor pathways in somatotroph tumors analyzing and culturing fresh tissue obtained at initial surgery in correlation with clinical data during follow-up. GDNF, the RET ligand, is heavily overexpressed. Moreover, p14ARF-the p53 activator- expression measured at the time of the surgery is a prognosis factor of posterior resistance to therapy. We established a new "humanized" protocol for culturing human somatotroph adenoma cells, where all components are adjusted to concentrations found in human serum. Patients' cells are able to grow maintaining the somatotroph phenotype. Following testing of five multikinase inhibitors, we found out that a new pharmacotherapy with Sorafenib could be effective for resistant acromegaly.

Implications of all the available evidence

Mechanistic studies in human tumor samples in parallel with primary cultures from the same patients have been very relevant for medicine. Unfortunately, when tumor cells are cultured under non ideal conditions, the relevance of the results is lost.

We have been able to obtain a marker in the tumor of later resistance to therapy, p14ARF. Ultimately, the ability to identify patients who are resistant to first-line therapy and the trial of new pharmaceuticals should improve outcomes in acromegaly. Our in vitro evidence discards some kinase inhibitors but proposes Sorafenib as a putative treatment for those resistant patients. Using this new culture system other therapies, even more selective for RET, could be identified to later be tested in clinical trials.

1. Introduction

Pituitary tumors are the 16% (11–22%) of intracranial neoplasms [1]. Acromegaly is a rare disease caused by a benign pituitary adenoma of somatotroph cells (ACRO) secreting growth hormone (GH) (Orphanet: 963; GARD-5725). The majority of cases are sporadic, although familial cases have been described [2]. Acromegaly has symptoms derived from expansion of the tumor compressing the pituitary or the surrounding structures such as the cavernous sinus, optic chiasma or hypothalamus. Additionally, excess GH and consequently elevated IGF1 levels, produce peripheral signs with growth of soft tissues and multiple comorbidities (metabolic, cardiovascular, oncological, etc.; (reviewed elsewhere [3–5]). First-line therapy initially comprises endoscopic surgery aiming to cure the disease without damaging the remaining pituitary gland. However, the surgical cure rate is only around 50% (40–69%), being inversely related to tumor size and invasiveness outside the sella turcica [6]. Patients not cured by surgery undergo adjuvant therapy with first-generation analogs of somatostatin (fgSSA), the main hypothalamic neuropeptide that inhibits GH secretion [7]. However, only half of patients not cured by surgery respond to fgSSA [8]. In fgSSA-resistant cases second-line therapies include combined treatment with dopamine analogs, pegvisomant or pasireotide, and even re-intervention or radiotherapy. These procedures have a range of side effects without necessarily controlling the apparently benign tumor.

RET is a tyrosine kinase receptor activated by a ligand in the presence of a membrane co-receptor [9]. In mammals there are four different ligands for RET (Glial cell line-Derived Neurotrophic Factor (GDNF), Neurturin (NRTN), Artemin (ARTM), Persephin (PSPN)) and four respective co-receptors (GFRA1–4) [10,11]. In addition to the respective ligand-co-receptor interaction, some cross-interactions between ligands and co-receptors able to activate RET have been described. Normal human and rodent somatotrophs express RET, GDNF and GFRA1 [12,13], with RET working as a dependent receptor, a mechanism shared by a few other receptors [14]. Thus, in the absence of GDNF its ligand RET is processed at the membrane by Caspase-3 and induces overexpression of PIT1, leading to induction of the CDKN2A/ARF promoter and ARF mRNA expression; p14ARF protein then binds to and inhibits MDM2, leading to p53 accumulation and apoptotic cell death [15,16]. When GDNF is present, RET dimerizes, activating its tyrosine kinase which induces AKT and cell survival. Thus, somatotrophs rely on the presence of the RET ligand GDNF for survival.

Immunohistochemical studies have shown that ACRO are the only pituitary adenomas expressing all three proteins, RET, GDNF and GFRA1 [13]. More recently, in vitro experiments in nine acutely dispersed ACRO have suggested that ACRO maintain the RET/PIT1/ARF/p53 apoptotic pathway and need GDNF expression to survive [17]. In a

small group of ACRO tissues, GDNF expression was inversely correlated to PIT1 expression [17]. To date, no studies have shown whether other ligands or GFRA receptors are expressed in ACRO, nor are there any data relating to the clinical relevance of the RET/PIT1/ARF/p53 apoptotic or the RET/GDNF survival pathways in acromegaly.

In this prospective study our goal was to investigate the role of each of these RET pathways in acromegaly, specifically in relation to clinical tumor characteristics, response to treatment and prognosis. Additionally, we carried out in vitro tests on five multikinase inhibitors able to act on the GDNF-induced RET dimerization/survival pathway. Our main finding was that Sorafenib, a RET inhibitor, is a potential therapeutic agent in resistant ACRO.

2. Materials and methods

2.1. Study design and clinical data

This study followed the European Convention on Human Rights and Biomedicine (ETS n°164) reflected in the Spanish Law of Research in Biomedicine (14/2007). This was a prospective study of acromegaly (ACRO) patients recruited from October 2010 till February 2017 from three hospitals in Spain: Hospital Clinico Universitario de Santiago de Compostela (CHUS, $N = 20$), Hospital Universitario La Paz de Madrid ($N = 6$) and Hospital Universitario de León ($N = 4$). Two patients underwent reoperation. The study was approved by both a national ethics committee based in Hospital Virgen del Rocio (Sevilla) (Act 1/2010), as part of a national registry of Acromegaly (REMAH node 6) [18], and an institutional committee at CHUS, where the molecular analyses were performed. Approved written consent was obtained from each patient participating in the study. Patients underwent endoscopic endonasal transphenoidal surgery. Non-functioning pituitary adenomas (NFPA) were also prospectively collected in CHUS ($N = 50$) and Leon ($N = 13$) for comparison with ACRO at the molecular level.

The study was designed following REMARK guidelines [19] (http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumour-marker-prognostic-studies-remark/) (adapted to endocrine tumors). $N \geq 5$ was calculated as the sample size in a group to obtain significant differences. Sample size calculations were based on the known failure of first-line therapy [6–8]: surgery failure has been reported to vary from 31% to 60% in previous series; of remaining non-surgically cured patients, half will not respond to therapy with fgSSA, meaning that 15.5% to 30% of patients can be expected to be Resistant. So to have $N \geq 5$ in the Resistant group the current study needed to have a total size of 17–32 patients, and we therefore included 32.

Clinical data from ACRO were collected at the end of follow-up by one Clinical Endocrinologist in a pre-designed Excel file. Variables included were based on current guidelines [6,7,20] and are shown in Supplementary Table 1: gender; age; GH at diagnosis and after surgery; IGF1 index (defined as serum levels respective to the upper level of normality for the corresponding life decade) at diagnosis and after surgery (the most relevant within 4–24 weeks); maximal diameter of the adenoma in the magnetic resonance imaging (MRI), volume calculated from three diameters of the tumor, Knosp (grades of invasion of cavernous sinus), invasiveness (at MRI, extension of the tumor in any structure outside the sella turcica); cured at surgery (when IGF1 index 4–24 weeks after surgery was normal ≤1; if IGF1 index was repeatedly found to be 1–1·5, an Oral Glucose Tolerance Test was performed to assess curing); adjuvant therapy with SSA analogs (fgSSA, treatment with first-generation analogs, Octeotride-LAR or Lanreotide autogel); residual tumor (presence of tumor rest at control MRI 4–24 weeks after surgery); response to SSA analogs (Complete: normalization of IGF1 index after 6 months of treatment; Partial: when not normalized but there was a reduction of 50%); response to first-line therapy (The patient was either cured by surgery or responded to adjuvant SSA analogs); other treatments for those who did not respond to first-line treatment (pegvisomant, radiotherapy). Molecular data were analysed in pituitary tumor surgical surpluses by a single laboratory in Santiago de Compostela (CIMUS).

2.2. Tissue sample management

Adenoma tissue surplus was immersed directly in RNA Later (AM7021, Ambion, Netherlands), kept at 4 °C for 24 h, during which it was couriered to Santiago de Compostela, where it was kept at −20 °C for another 24 h and stored at −80 °C until further use. From 2014, for surplus tissue samples >10 mg at CIMUS, a small fresh piece was transferred to the culture room for h7H primary culture (see below).

2.3. RNA and DNA extraction

Nucleic acids were extracted using an AllPrep DNA/RNA Mini Kit (80284, Qiagen, Germany), in which DNA is first purified in an affinity column, and RNA is then purified from the eluted fraction in a second affinity column which includes treatment with an RNasa-Free DNase Set (79254, Qiagen, Germany). DNA was QC and quantified using a Quantifiler Human DNA kit (4343895, Applied Biosystems, UK). RNA was QC and quantified using a Nanodrop 2000 spectrophotometer (Thermo).

2.4. qRT-PCR assay

500 ng of total RNA were incubated with 0.5 IU RNAse free DNAse I (EN0521, Thermo), 1 microL 10× buffer and water as a total volume of 10 microL, at 37 °C for 30 min. The reaction was termnated by adding 1 microL 50 mM EDTA and heating at 65 °C for 10 min. cDNA was obtained after adding 1.5 microL with 300 IU MMLV (28025–013, Invitrogen, USA) and 6 microL 5× First-Strand Buffer and, following the supplier's protocol, 1·5 microL 10 mM dNTPs, 0·1 microL Random Primers, 3 microL 0·1 M DTT, 1microL RNaseOUT™ Recombinant Ribonuclease Inhibitor (40 units/microL) and H2O in a 30 microL reaction. 50 ng, 25 ng and 12·5 ng of a commercial human Pituitary Gland Poly A+ mRNA pool (1305204A, Clontech, USA) was similarly reverse-transcribed in three independent reactions to use as a technical control. Expression was detected by qPCR using 1 microL of the cDNA reaction plus 6 microL 2× TaqMan Gene Expression MasterMix (4369016 Applied Biosystems) and 6 microL diluted primers in 96 well plates in a 7500 Real-Time PCR System (4351105, Applied Biosystems, USA). For primers, when possible we used commercial Taqman Gene expression primer sets (Applied Biosystems) (see Supplementary Table 5). When isoforms or highly homologous genes were involved, (FSHB, LHB, SSTR2 and SSTR5), we used previously validated primers (See Supplementary Table 5) [1] with 6 microL Brilliant III Ultra-Fast SYBR MasterMix (600,882, Agilent Technologies, USA). GH isoform mRNA expression was detected by both assays. Each 96 well plate was used for a single gene reaction including duplicate samples of a group of 10 ACRO, 20 NFPA together with technical positive controls, negative control (ACRO9 reverse-transcribed in the absence of MMLV) and blank (all reagents without cDNA).

As control for general gene expression we used TBP based in published works [21,22] (http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_042279.pdf) and our previous experience [7]. ACTB is not considered a good control because it shows higher variability between samples from the same and from different tissues. TBP is considered as a gold standard control for quantitative mRNA assays as it shows a more stable expression pattern in different human tissues and cell types. The ΔCt method was used to express the results for each gene ($\Delta Ct_{gene} = Ct_{gene} - Ct_{TBP}$). We calculated some of the data using both TBP and ACTB and obtained similar results and statistical significance.

Genes that were undetectable in a sample ($Ct_{gene} > 40$) were classified according to the Ct_{TBP}. If other samples with a similar Ct_{TBP} had a

detectable Ct_{gene} (Ct gene <40), the particular sample was considered to have a very low expression for that gene and its Ct_{gene} transformed to 42 (two cycles over the limit). If a sample had a Ct_{TBP} that was higher than any other sample, it was considered to be technically undetectable for that gene (a lost sample for statistical comparisons). The mean of the three concentrations of the technical control (commercial human pituitary gland pool) was considered as 1. The ΔΔCt method, $\Delta Ct_{gene\text{-}sample} - \Delta Ct_{gene\text{-}technical\ control}$, was used to calculate the end value of the qPCR in each sample. Potency of $2^{-\Delta\Delta Ct}$ was the end result used for statistical comparisons.

2.5. GNAS mutational profile and STR analysis

The GNAS gene was amplified from 50 ng DNA by PCR (primers in Supplementary Table 5) using 0·5 IU DreamTaq DNA Polymerase (EP0701 Thermo Scientific), 2·5 microL DreamTaq Buffer, 0·5 microL 10 mM dNTPs, 0·5 microL 5microM Forward Primer and 0·5 microL 5 microM Reverse Primer and dH20 to a final volume of 25 microL. The PCR Product was Sanger-sequenced to check for mutations in nucleotide C601/codon R201 or nucleotide A680/codon Q227 (Supplementary Fig. 1a, Supplementary Table 3).

For assessment of similarity between pituitary tissue and primary culture DNA we used a combined panel of 16 STR AmpFiSTR NGM Select kit (4457889, Applied Biosystems, Germany) including Identifiler Plus Panel V1 (Applied Biosystems) as the read-out pattern. The 16 STR profiles obtained were compared to the 8 STR American Type Culture Collection (ATCC) database, using the ATCC Match algorithm according to ICLAC standards (https://www.lgcstandards-atcc.org/en/STR_Database.aspx).

2.6. Long-term primary culture of human pituitary adenomas under humanized (h7H) conditions

Previously [23,24], we designed a cell culture medium for human follicular thyroid cells, h7H, in which all components and additives were at concentrations within the human physiological ranges (based on reference values for human serum from the Mayo Clinic wherever possible (http://www.mayomedicallaboratories.com/test-catalog/)). In current study, based in our experience in short-term rodent and human primary pituitary cultures [16,17,25,26], the h7H medium was slightly modified from the follicular thyroid medium: while insulin, somatostatin and cortisol were maintained; bTSH and hGH were removed, and GHRH, Ghrelin, EGF, bFGF, T3 and Glucagon were added. (Supplementary Table 6: h7H components are listed in seven groups as osmolality and ions, hormones, growth factors, oligo elements, antioxidants and vitamins, metabolites and ethanol. The medium also included 10% serum (5% FBS and 5% NBCS)).

The excised fragment of the adenoma surplus was digested in 1 mg/mL collagenase IV (C9891-AG, Sigma, Israel) and 1× trypsin (T4674, Sigma, USA) in PBS for 45 min, and then filtered into complete h7H medium (including serum, Supplementary Table 6) through a 41-micron filter (NY4100010, Merck Millipore, Ireland) to remove undigested tissue fragments. Cells were washed several times with complete h7H medium and then seeded in one well of a 12-well TC-treated polystyrene plate (Costar, Thermo Scientific, USA). Every two weeks, cells were trypsinized and diluted 1:2. We performed h7H culture in 11 NFPA y 4 ACRO, of which 8 NFPA and 3 ACRO grew and were maintained for at least 21–33 passages. At the last passage, cells stop growing and were able to remain passively attached to the plate for weeks (looking senescent). Passages attained by cultures presented in current work were: ACRO28 reached p21; ACRO30 reached p23; ACRO32 reached p24; NFPA41 reached p33.

2.7. Analysis of secreted hGH and GDNF

For hGH secretion culture medium from plates was collected before passaging, after three-four days with the cells, and stored at −80 °C. Immediately after, cells were trypsinized and counted for the next passage, so the precise number of cells in the dish, and the precise time of incubation was known.

Clinical kits to determine hGH in human serum can have negative interferences in samples of culture medium containing bovine serum with heterophylic antibodies and higher biotin concentrations. This happened to us when we used the Immulite 2000 (Siemens Healthineers, Erlanger, Germany) an IRMA type assay using a rabbit anti-hGH antibody conjugated to bovine calf alkaline phosphatase and beads coated with murine monoclonal anti-hGH antibody; signal is detected through chemiluminiscence. This kit have been previously used to measure hGH regulation in acute cultures of primary ACRO adenomas [27]. In our hands, measurements using 25 microL of whole medium (incuding 10% bovine sera) and this kit gave undetectable secretion in the Immulite Siemens analyzer.

We decided to detect secreted hGH by loading 15 microL of collected medium onto a western blot in comparison with medium that was not put into the cells. After a positive result in quantity, specificity and molecular weight (Fig. 3), a second clinically validated assay was performed in the culture samples after revision of the literature. The COBAS hGH assay (Roche Diagnostics, Mannheim, Germany) had also been previously used to measure hGH in culture medium from pituitary adenomas [28]. COBAS is also an IRMA type assay but without components of bovine origin. 40 microL of sample medium is incubated with a biotin labelled monoclonal murine anti-hGH antibody and a rabbit polyclonal anti-hGH antibody conjugated to ruthenium chelate; streptavidin-magnetic beads on a magnetic electrode purify the sandwiches, followed by an electromagnetic impulse to initiate the light emission by the ruthenium that is detected in the Elecsys E170 automatic analyzer.

For GDNF secretion, 10,000/well h7H-primary pituitary acromegaly (or NFPA) cells were seeded in a 96-well dish, collected for different time-lengths and kept at −80 °C. Secreted GDNF levels were measured with a Human GDNF DuoSet ELISA (DY212, R&D Systems, USA) according to the manufacturer's protocol. Briefly, a 98-well microplate was coated with the capture antibody and incubated overnight at room temperature. Wells were blocked for 1 h at room temperature and standards and culture medium samples added and incubated for 2 h at room temperature. Detection antibody was added during for 2 h at room temperature. Avoiding exposure to light, streptavidin-HRP was added for 2 min, followed by the substrate solution for a further 20 min. After termination of the enzyme reaction with the stop solution, the plate was read at A450/A550nm in a microplate reader (Mithras LB 940, Berthold, Germany).

2.8. Immunocytochemistry

Procedures were performed as described [24]. Briefly, h7H-primary pituitary acromegaly cells were seeded and grown on glass coverslips in a 48-well dish. Wells were washed and fixed with 10% neutral formalin for 30 min, washed once and then incubated at room temperature for 20 min in methanol added at −20 °C. After further washing, permeabilization was carried out using Triton 0.2% in H_2O on ice for one hour. Primary and Secondary Antibodies are listed in Supplementary Table 8.

2.9. Rat pituitary cell line GH4C1

The rat GH4C1 somatothroph cell line was cultured in DMEM (D6046, Sigma, UK) containing 10% FBS [17]. Since no STR profile has been standardized for such rat cell lines, expression profiling was used to assess phenotype (see Supplementary Fig. 3 and previous ref. [29] to see constancy of phenotype). Transfection of RETL or RETS (Supplementary Table 7) was performed using Nucleofector II (AAD-1001S, Amaxa, Germany) with the A-20 program and L-kit (VCA-1005, Lonza, Germany) as described [16].

2.10. Evaluation of anti-survival action of tyrosine kinase inhibitors (TKIs) activity

TKIs (TINIB tools, Czeck Rep) stocks were prepared in DMSO and kept at −20 °C. An essential step in our assay was deciding which TKI concentrations tested in vitro were relevant for their in vivo action. TKI concentrations were based on the United States Food and Drug Administration (FDA) approved pharmacological review of each drug from data collected in Phase II-III clinical trials. We selected the mean concentration at steady-state (Cm_{SS}) detected in treated patients' serum (Supplementary Table 4).

h7H-primary pituitary acromegaly cells were seeded (10,000/well in mw48 for apoptosis; 50,000/mw12 for RNA or protein extracts) in full h7H medium with 10% serum. Two days later, cells were washed and the medium changed to a deprived medium (0·5% FBS without hormones or additives). Human GDNF (100 or 500 ng/mL, stock: 10 microg/mL in PBS containing 0·1%BSA; 345,872, Calbiochem, Israel), NRTN (100 ng/mL; stock: 10 microg/mL in PBS containing 0.1%BSA; 1297-NE-025, R&D Systems, USA) or vehicle (PBS containing 0·1% BSA) was added for 24 h. A full h7H medium (with serum and additives) condition was included as a control of basal apoptosis. Appropriate TKI dilutions or DMSO were added for the corresponding time with/without GDNF/NRTN.

For GH4C1 pituitary cells, 25,000 transfected cells/well were seeded in a 48-well dish in full medium. The following day, cells were washed and cultured in DMEM+0·1% BSA for 48 h with/without rat GDNF (50 ng/mL PF039-10ugmicrog, Calbiochem, USA) and TKIs.

2.11. Apoptosis assay

Medium containing GDNF, TKIs or vehicles was added for 24 h followed by addition of 5 microM Hoescht-33,258 (B1155, Sigma, USA) for 1 h. Apoptosis was measured by live microscopy in an IX51 equipped with CellSens software (Olympus). At least six fields including 75–100 cells were registered per well detecting condensed brilliant blue (apoptotic cells) and homogeneous soft blue (live cells).

2.12. 16-pad slide array

h7H-primary pituitary acromegaly cells were seeded and treated as described above for one hour. Cells were washed with ice-cold PBS and lysed with 30 microL of ice-cold Cell Lysis buffer (#7018, Cell Signaling Technology, USA). MicroBCA Protein Assay (23,235 Thermo IL USA) was used to measure protein concentration. The 16-pad PathScan Intracellular Signaling array kit (#7323, Cell Signaling Technology, USA) was used according to the manufacturer's instructions. In brief, the array was blocked using the array blocking buffer for 15 min at room temperature. 60 microg cell lysates/100 microL Array Diluent Buffer was then added and incubated for 2 h at room temperature on an orbital shaker. After washing, Detection Antibody cocktail was added and incubated for 1 h at room temperature. After washing, HRP-linked streptavidin was added and incubated for 30 min at room temperature. After washing, the Array glass slide was finally covered with 1× LumiGLO©/Peroxide solution and exposed to films in a linear time-course. The average pixel density of each marker was scanned and quantified by Quantity One (Bio-Rad) and normalized to pixel densities of the positive control spots.

2.13. Cell extracts and Western blot

A small fragment of frozen acromegaly tissue was mixed with 100 microL lysis buffer (0.05 M Tris-HCl, 0.01 M EGTA, 1 mM EDTA, 16 mM Triton X-100, 1 mM sodium orthovanadate, 0.05 M sodium fluoride, 0.01 M sodium pyrophosphate and 0.25 M sucrose, adjusted to 7·5 pH (all reagents from Sigma, USA), with freshly added Complete EDTA-free protease inhibitor cocktail tablets (11,836,170,001 Roche Diagnostics, USA). A Pit-Mill tissue lyser (85,300 Qiagen, Germany) was used for three minutes at 20 Hz. Lysates were centrifuged at 12000 rpm for 30 min and supernatants stored at −80 °C.

Cell lysates obtained for the above 16-path slide array were considered cytoplasmic. For extractions that included membrane proteins, wells were incubated for 20 min on ice with 30 microL of ice-cold Triton lysis buffer (50 mM Hepes pH 7·5, 1% Triton X-100, 150 mM NaCl, 5 mM EGTA, 1·5 mM MgCL, 10 mM Na pyrofosfate, 92 microg/mL Na_3VO_4, 2 microg/mL aprotinin and 4 mM PMSF, all reagents from Sigma, USA). For total cell extracts 15 microL of boiling 1% SDS/well were added to each well, which was then scraped and boiled at 95 °C for 5 min in a thermoblock, and finally diluted 1:3 in Triton lysis buffer [17]. All lysates were centrifuged at 12000 rpm for 10 min and supernatants stored at −80 °C.

MicroBCA Protein Assay (23,235 Thermo IL USA) was used to measure protein concentration. 15–20 microg of protein extracts run in 10–15% SDS-PAGE were electrotransferred to a PVDF membrane (Immun-Blot PVDF 162–0177; Bio-Rad, USA) with a semidry blotter. Membranes were blocked with 0·2% Tropix i-block (T2015, Applied Biosystems, USA) in 1× TBS for 2 h. Antibodies and dilutions are shown in Supplementary Table 8. Enhanced chemiluminescence assay (Pierce ECL Western Blotting Substrate, 32,106, Thermo Scientific, USA) was used for signal detection. Membranes were exposed to X-ray film (4741 19,289, Fujifilm, Japan) and developed (Developer G150, AGFA HealthCare, Belgium) and fixed (Manual Fixing G354, AGFA HealthCare, Belgium). *Statistical analysis* Statistical analysis and plotting of graphs were performed using GraphPad Prism 6 (GraphPad Software, USA), SPSS Statistics 20 (IBM, USA) and Corel Draw Graphic Suite 2017. Analysis were performed in an independent way by CVA's group and the Mathematics Medical Department at CHUS. Graphs show mean±SEM. Normality for each group of quantitative data was assessed using the Kolmogorov-Sminorv test with Dallal-Wilkinson-Lillie for 'p' value. The majority of the groups were non-parametric. For statistical comparisons we performed detailed bi-variant analysis. As the initial step in analysis of the data, correlations were assessed with Spearman's Rho (rs) and significance. Quantitative variables were compared by Mann-Whitney tests when non-parametric or unpaired t-tests when parametric. Qualitative variables were compared with a Chi-square independence test, using the Pearson 'p' value when $n>5$ or the Fisher exact 'p' value when $n<5$. ANOVA was used for comparisons of more than two groups; for culture experiments, where there was one control with no treatment, Dunnet's correction was applied. For the multivariate analysis (response to first-line treatment and ARF expression cut-off depending on GNAS mutation) we used Chi-squared with multivariate contingency table analysis. To calculate the discriminating threshold for ARF (and GFRA4) expression, a ROC curve was used.

3. Results

We analysed quantitative RET pathway gene expression in a prospective series of 32 sporadic ACRO surgical samples collected in 2010–2017, in relation to clinical characteristics, and expression of known ACRO genes and the G-protein subunit α (GNAS) DNA mutation, a recurrent mutation found in sporadic ACRO (Supplementary Table 1 and Supplementary Fig. 1a). In summary, 30 new patients and two undergoing reoperations were recruited from three hospitals (Santiago de Compostela, Madrid and León), comprising 66·6% females, median age 45·6 years old and median follow-up 4·2 years (Supplementary Table 2). 25% of samples contained mutated GNAS (Supplementary Table 2 and 3). The clinical characteristics of our series do not differ from other published series [30]. The majority of patients had a macroadenoma, with tumor volume and diameter being inversely related to age but positively related to GH and IGF1 levels, Knosp grade and invasion (Supplementary Table 2). Half of the patients ($N = 16$) have received pre-surgery therapy with fgSSA, although no correlation

of this with other clinical variables was found (Supplementary Table 2). Presence of GNAS mutation was not related to any other variable. Success of surgery was correlated with smaller, less invasive tumors that secreted less GH/IGF1. Resistance to adjuvant therapy with fgSSA was just significantly greater in males than females ($p = 0{\cdot}049$) but had no other significant correlation. An improved response to combined first-line treatment was observed in older patients ($p = 0{\cdot}049$) and smaller non-invasive tumors but had no other significant correlation.

3.1. Molecular taxonomy of the RET pathway in ACRO

Quantitative RNA expression analysis, using TaqMan qRT-PCR, was performed for the following gene groups: pituitary hormones (GH isoforms; PRL; POMC/ACTH; CG-alpha, the common alpha subunit for FSH, LH and TSH; and the specific beta subunits of the two gonadotropins, FSHB and LHB), our pathways of interest (RET receptor as total expression RETN, long RETL and short RETS isoforms; its four co-receptors GFRA1-2-3-4 and its four ligands GDNF, NRTN, ARTM and PSPN; PIT1 transcription factor; p14ARF isoform from the CDKN2A gene (here called ARF); and p53), hypothalamic neuropeptide receptors (GHRHR and somatostatin receptors SSTR2, SSTR5), other pituitary transcription factors (PROP1, SF1, TPIT), AIP as a gene implicated in familial pituitary adenomas (FIPA) [2], and two cell cycle genes previously studied in models of acromegaly (CDKN1A/p21 and CDKN1B/p27) (see Supplementary Fig. 1 for a comprehensive scheme of the genes studied). We compared all genes with a commercial pool of pituitary poly-A mRNA as a technical control for each gene, to equalize measurement efficiency from different plates during the study.

First, we identified a common profile of genes enriched in ACRO, to allow us subsequently to evaluate individual samples. To assess enrichment, we compared the expression of each ACRO gene with a similar prospective series of Non-Functioning Pituitary Adenomas (NFPA, $n = 63$). As expected, GH (22KDa (GHTaq) and 20 KDa (GHSyb) isoforms), GHRHR, SSTR2 and SSTR5 were very abundant in ACRO in comparison with NFPA (Fig. 1a). There was no difference between ACRO and NFPA in expression levels of other non-somatotroph hormones, such as POMC (corticotroph hormone), CG-alpha or LHB (the beta subunit of the LH, one of the two gonadotroph hormones) (Supplementary Fig. 1b). However, FSHB (the beta subunit of FSH, the other gonadotroph hormone) was significantly enriched in NFPA (Supplementary Fig. 1b).

From the RET receptor downstream, all genes in the RET pathway were significantly more highly expressed in ACRO than in NFPA (Fig. 1a). Especially abundant were the ligand GDNF, the somatotroph transcription factor PIT1 and the tumor suppressor ARF, while a smaller difference was found in p53, attributable to its regulation being at the protein level. Of the three non-somatotroph transcription factors studied, PROP1 presented low expression in all tumors, although it was significantly higher in ACRO than in NFPA; as expected, SF1 was significantly higher in NFPA, while TPIT was poorly expressed in both ACRO and NFPA, attributable to the fact that it is characteristic of corticotroph tumors (ACTH series) (Fig. 1a). AIP, a protein related to familial but not sporadic acromegaly [1], did not show differences in expression between ACRO and NFPA (Fig. 1A). p21, a gene activated by p53, was more abundant in ACRO (Fig. 1a), contrary to p27 that did not present differences (Supplementary Fig. 1b).

After this initial characterization of the ACRO series, each sample was individually evaluated for its level of enrichment of the most abundant somatotroph genes (GH, GHRH, PIT1) and depletion of non-somatotroph genes (TPIT, POMC, SF1, CG-alpha, FSHB, LHB) (Supplementary Fig. 1c). PRL was considered a 'neutral' gene as it can be co-secreted with GH. All samples passed our technical control, although two samples, ACRO23 - a re-operation following radiotherapy - and ACRO39, expressed some non-ACRO genes (Supplementary Fig. 1c). However, the level of GH expression in these two samples was still much higher than that in NFPA and thus they were included. PRL was co-expressed with GH in three samples: ACRO19, 25 and 32. We proceeded with our study in the confidence that the tissue from which we had extracted RNA was mainly ACRO.

Fig. 1b shows the RET pathway in normal somatotroph cells [15,16]. In the absence of the ligand GDNF, RET is a dependence receptor: the intracellular region is processed by Caspase-3, generating an intracellular fragment IC-RET and activation of PKC delta (PKCd); this induces PIT1 overexpression, exceeding permissive levels of an already abundant gene and inducing ARF expression. ARF binds to and blocks MDM2, the protein that ubiquitinates p53 prior to its destruction, leading to p53 accumulation and apoptosis. If the ligand is present, GDNF induces RET dimerization which inhibits the apoptotic pathway, activating AKT and maintaining regulated PIT1 expression, which is sufficient to produce GH but not to induce apoptosis [15,16]. This pathway explains the abundance of GDNF both in the normal pituitary (containing 50–60% of somatotroph cells) and to a greater extent in ACRO (Fig. 1a).

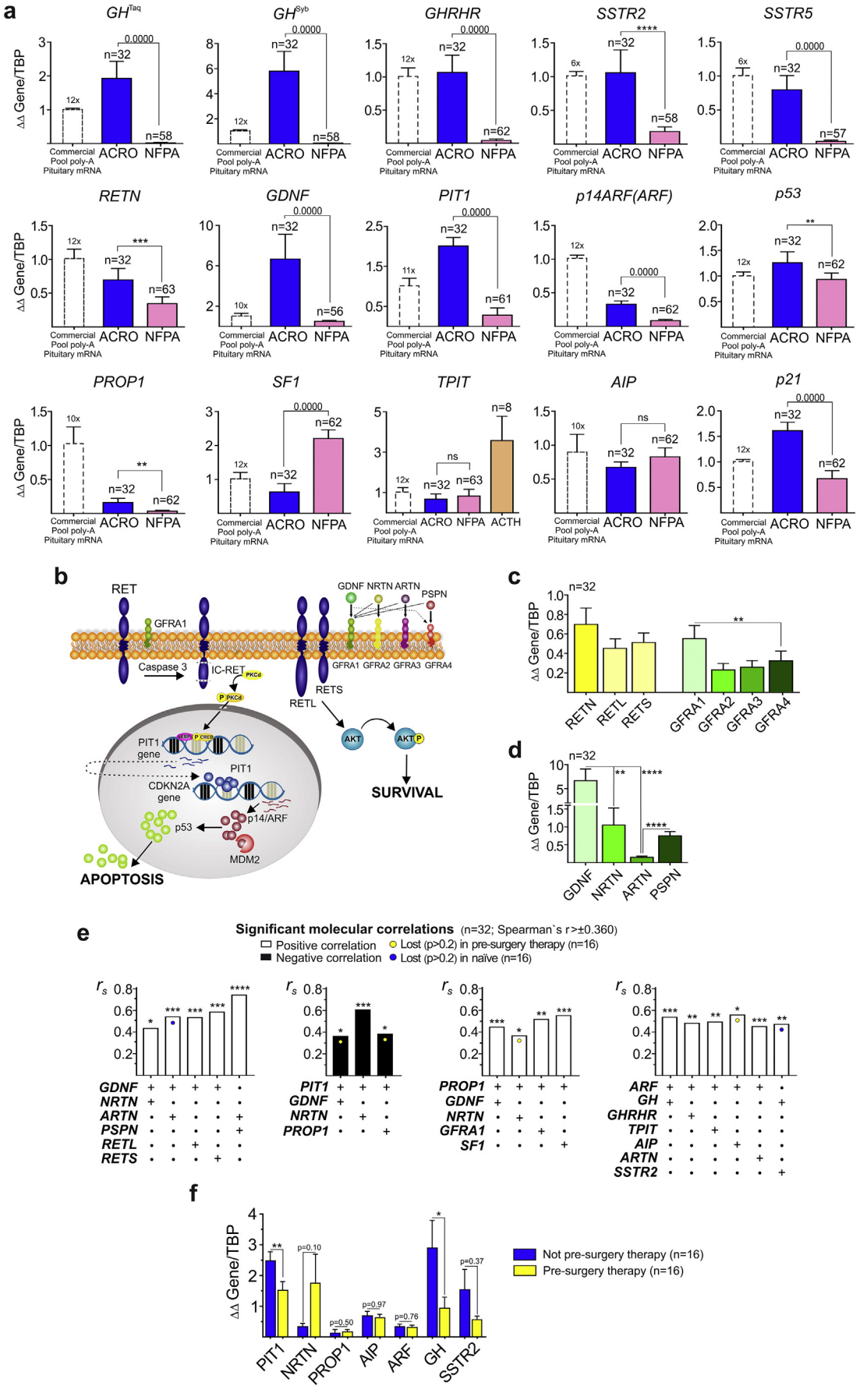
RET has a long (RETL) and a short (RETS) isoform varying in the length of its cytoplasmatic C-terminal tail, leading to different number of tyrosines candidates for phosphorylation and hence interaction with different substrates (Fig. 1b, right). As stated above, each RET co-receptor binds a characteristic ligand (GDNF-GFRA1, NRTN-GFRA2, ARTM-GFRA3, PSPN-GFRA4). (see STRING, https://string-db.org/cgi/network.pl?taskId=Unm9pNIMfJex). It is well established that GDNF activates RET through GFRA1 [31,32]. It has also been demonstrated that NRTN is able to activate RET through GFRA2 more potently than GDNF [33]. However, there is also cross-activation between ligands and co-receptors, as shown by transfection experiments and in primary culture. Thus NRTN is able to fully activate RET with equal potency to GDNF through GFRA1 in transfection studies although it does so slightly less potently than GDNF in primary cells expressing GFRA1 receptor (Fig. 1b) [33–36]. ARTM and PSP are less potent than GDNF or NRTN but still able to activate RET through GFRA1 in cells expressing the receptor [35–37].

In the ACRO series studied, expression of RETL and RETS isoforms were similar as approximate halves of total RET expression (RETN) (Fig. 1c). The co-receptor most abundantly expressed was GFRA1 followed by GFRA4 (Fig. 1c). This fitted with the major ligand expressed being GDNF, followed by NRTN and PSPN (Fig. 1d).

We calculated correlations between molecular variables. Spearman correlations were used to investigate gene expression and GNAS mutation. Significant but relevant correlations (Rho, $rs > 0{\cdot}36$) are shown in Fig. 1e. Expression of GDNF was positively correlated with NRTN and ARTM ligands, and both RET isoforms. Both GDNF and NRTN were negatively correlated with PIT1 but positively correlated with PROP1. PIT1 and PROP1 were negatively correlated with each other. PROP1 directly induces PIT1 expression during embryonic development, and once PIT1 is induced PROP1 is repressed through a mechanism that is known not to be directly repressed by PIT1 [38,39]. GFRA1 and SF1 were also positively correlated with PROP1. ARF expression was positively correlated with GH, GHRHR, AIP, ARTN and TPIT. SSTR2 was only correlated with GH expression. No correlations were found for p53, which was not unexpected as p53 is regulated mainly at the protein level. All other genes studied (including p21 and p27) and GNAS mutations were found not to be relevantly correlated.

Molecular correlations were somewhat reduced in significance when the group of naïve samples ($N = 16$) was analysed separately from the group with pre-surgery treatment ($N = 16$) (Supplementary Fig. 1d). This was expected as a consequence of halving the samples. However, the weakest correlations were lost including PIT1-GDNF, PIT1-PROP1, PROP1-NRTN, ARF-AIP and GH-SSTR2 (Fig. 1e, yellow or blue dots). When genes implicated in these lost correlations were analysed for differences between both groups we found significant differences exclusively in PIT1 and GH expression that is reduced, particularly GH, in pre-surgery treated samples (Fig. 1f).

The most relevant finding from these data was that the GDNF-RET survival pathway is strongly expressed in acromegaly and reinforced by redundancy in expression of RET ligands and RET receptor isoforms,


a
GH^Taq
GH^Syb
GHRHR
SSTR2
SSTR5
RETN
GDNF
PIT1
p14ARF(ARF)
p53
PROP1
SF1
TPIT
AIP
p21
ΔΔ Gene/TBP
Commercial Pool poly-A Pituitary mRNA
ACRO
NFPA
ACTH
b
RET
GFRA1
GDNF NRTN ARTN PSPN
GFRA1 GFRA2 GFRA3 GFRA4
Caspase 3
IC-RET
PKCδ
RETS
RETL
AKT
SURVIVAL
PIT1 gene
CDKN2A gene
p14/ARF
p53
MDM2
APOPTOSIS
c
d
e
Significant molecular correlations (n=32; Spearman`s r>±0.360)
Positive correlation
Negative correlation
Lost (p>0.2) in pre-surgery therapy (n=16)
Lost (p>0.2) in naïve (n=16)
f
Not pre-surgery therapy (n=16)
Pre-surgery therapy (n=16)

controlling PIT1 and inhibiting the RET apoptotic pathway. This prompted us to further explore the RET pathway in relation to clinical follow-up characteristics of the patients.

3.2. ARF is a prognostic factor of response to first-line combined treatment (surgery +fgSSA) in acromegaly

We investigated correlations between molecular and clinical variables, which were divided into three groups: diagnostic, pathologic and prognostic/follow-up. In Fig. 2A significant and relevant (rs ≥ 0·5) correlations are shown.

Of the analytical diagnostic variables, only basal serum GH at diagnosis was significantly correlated with GH, SSTR2, AIP and p53 RNA tumor expression, all of which were abundantly expressed in ACRO (Fig. 2a left). Pre-surgery therapy was negatively correlated with PIT1 and GH (Fig. 2a). This was expected since expression of those two genes were reduced with the treatment (Fig. 1e).

Regarding pathologic variables, two genes showed important correlations, ARF and TPIT. ARF expression was negatively correlated with invasiveness, Knosp grade and, importantly, with post-surgical residual tumor (Fig. 2a center). TPIT expression was negatively correlated with tumor diameter/volume, invasiveness and Knosp grade, but positively correlated with surgical cure (Fig. 2a right).

In the group of prognostic variables, response to fgSSA during follow-up was strongly and positively correlated with ARF expression (rs > 0·8), while GFRA4 was negatively correlated (rs > 0·7). Other genes less strongly correlated ith response to fgSSA were GH, TPIT and SSTR2 (rs 0·5–0·6). Since expression of the gene with the two most important correlations, ARF, was also related to a favorable course of disease (negatively correlated with residual tumor and positively correlated with response to fgSSA), we combined the two prognostic variables into a single 'Good Prognosis' Group 1, defined as responsive to combined first-line therapy (surgery + fgSSA). When this group was analysed for correlations, ARF expression showed the highest positivity (rs > 0·7) and significance, followed by TPIT (rs = 0·6), while GFRA4 was negatively correlated (rs = 0·5).

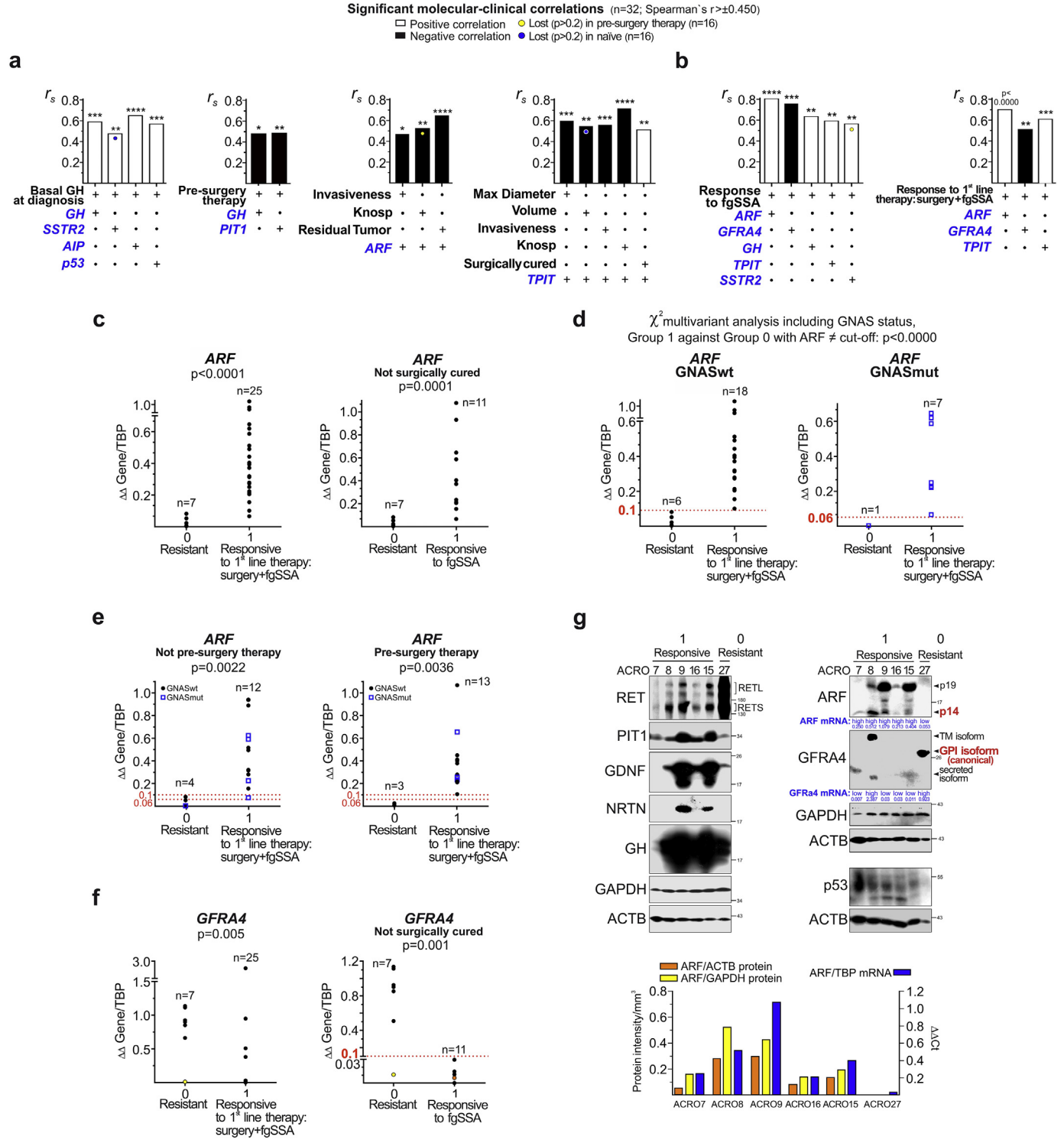
Molecular-clinical correlations were maintained, although with reduced p values, when analyzing separately naïve and pre-surgery therapy groups (N = 16 each) (Supplementary Fig. 2a), with some exceptions (Fig. 2a: yellow dots, lost in pre-surgery therapy; blue dots lost in naïve). Correlation between SSTR2 and basal GH at diagnosis, and TPIT and volume did not exist in naïve samples. Correlations between ARF and invasiveness, and SSTR2 and Response to fgSSA were lost in the group with pre-surgery therapy. All other correlations, especially those implicating ARF and GFRA4 with response to 1st line therapy were strongly maintained in both groups (Compare Fig. 2a and Supplementary Fig. 2a).

We then compared Group 1 with 'Resistant' Group 0, defined as not cured by surgery and resistant to fgSSA during follow-up. ARF mRNA expression values were much lower in Group 0 than Group 1 (Mann Whitney test, $p < 0·0001$, Fig. 2c left). Similar results were obtained when tumors not cured by surgery were analysed separately. ARF was again more highly expressed in samples responding to fgSSA than resistant samples (Fig. 2c right). Differences between follow-up groups for the other genes tested (SSTR2, SSTR5, TPIT, SF1) either did not reach the ARF levels of significance or were not significant (Supplementary Fig. 2c-d). Moreover, results did not change for ARF when ACTB was used as the control gene, instead of TBP to normalize RNA expression (Supplementary Fig. 2e). We then aimed to find a cut-off value for ARF expression which would classify an acromegaly as either Responsive Group 1 or Resistant Group 0 at the time of surgery, by constructing a ROC curve of ARF levels (Supplementary Fig. 2f). We found that placing the cutoff at ≥0·06 separated the groups with 100% sensitivity but 85·7% specificity. Placing the cutoff at ≥0·1, however, separated groups with 96% of sensitivity and 100% specificity. Although these values were very good as a clinical predictor we wanted to understand if there were other factors that might explain the blurred separation of three samples located at cut-off levels. In Group 1 the only value in the range 0·06–0·1 was in fact GNAS-mutated, while in Group 0 values in the same range were non-GNAS-mutated. We did not find any correlations between GNAS mutation and any molecular or clinical variables studied (Supplementary Table 2). Moreover, while the median values were no different between the GNAS-mutated and non-mutated groups, the range of ARF expression in the mutated group was much smaller (range 0·042–0·64, $n = 8$) than that in the non-mutated group (range 0·003–1·08, $n = 24$) (Supplementary Fig. 2 g). Therefore, a lower cut-off value would be expected for the mutated GNAS samples. In accordance with this, when we separated Group 0 and Group 1 using a cutoff of 0·1 for ARF expression in non-mutated GNAS ACRO and 0·06 in mutated GNAS ACRO, and performed a multivariant analysis using Chi-square test, we found maximal significance ($p < 0·0000$) (Fig. 2d top), with all samples showing 100% sensitivity and specificity (Fig. 2d lower).

fgSSA include two prescriptions, Octeotride LAR (Octeotride, $n = 5$ patients) and Lanreotide Autogel (Lanreotide, $n = 13$ patients). We separated patients receiving each treatment and found that, again, ARF expression was able to separate Group1 from Group 0 for both treatments (Supplementary Fig. 2 h). We performed statistics in the Lanreotide group of patients, and again differences in ARF levels were clearly significant between Group 0 (Resistant) and Group 1 (Responsive), in spite of their reduced number.

GFRA4 was the only negatively correlated gene during follow-up (Fig. 2b). When all samples were taken into account there were significant higher expression levels in Group 0 than Group 1, although the gene was not a perfect classifier (Fig. 2f left). Selecting samples that were not cured with surgery and treatment with fgSSA (Resistant Group 0, Responsive Group 1), GFRA4 expression was again significantly higher in Group 0, with a cutoff ≥0·1 (Fig. 2E right). There was a single sample in Group 0 that did not follow this rule (yellow dot = ACRO36), which was from a reoperation following radiotherapy in the same patient as ACRO27. This could be because radiotherapy or fgSSA

Fig. 1. mRNA expression characterizing ACRO and the RET pathway. a) Quantitative RNA expression comparing mean ± SEM ACRO ($n = 32$, blue bars) and NFPA ($n = 56–63$, pink bars). In every sample, gene expression was normalized to a commercial pool of pituitary poly-A mRNA (technical control; white bars). Genes most highly expressed in ACRO were characteristic of functional pituitary somatotrophs, related to GH secretion (GH^{Taq} (22 KDa), GH^{Sybr} (22, 20 and 17 KDa)), or PIT1 and hypothalamic regulation (GHRHR, SSTR2, SSTR5). However, the RET receptor (RETN), its ligand GDNF and genes in the RET pathway regulated at the RNA level (ARF and PIT1) were significantly more abundant in ACRO than NFPA. p53 was slightly upregulated in ACRO. SF-1 was characteristic of NFPA, and T-PIT of ACTH-secreting adenomas (orange bar). (Mann-Whitney test). b) Cartoon representing isoforms of the RET receptor, differing at the C-terminal tail (long RETL and short RETS), its four ligands and its GFR-alpha co-receptors (GFRA1–4). Although there is preference of a ligand for a co-receptor, cross-interaction exists. In somatotrophs in the absence of ligand, RET is processed by Caspase-3 generating an intracellular fragment (IC-RET) that triggers a cell-death pathway through overexpression of PIT1 gene inducing p14ARF expression, p53 accumulation and apoptosis. When the ligand is present, RET dimerizes and activates its cytoplasmic tyrosine kinase activity leading to AKT phosphorylation and survival. c) In ACRO, both RETL and RETS are expressed in approximately equal amounts. Although all four RET co-receptors were expressed, the most highly expressed was GFRA1 (high affinity for GDNF), followed by GFRA4. (ANOVA). d) GDNF was by far the most highly expressed ligand, with NTRN and PSPN also abundantly expressed. (ANOVA). e) Significant correlations among all the genes studied in ACRO ($r_s > 0·36$) revealed that GDNF expression was significantly and positively correlated with expression of RET isoforms and the other ligands. GDNF (and NRTN) were negatively correlated with PIT1 expression but positively correlated with PROP1. As expected, expression of both transcription factors PIT1 and PROP1 were negatively correlated. PROP1 was also correlated with GFRA1 and SF1. ARF expression was significantly correlated with the somatotroph phenotype (GH, GHRHR, AIP), T-PIT and the ligand ARTN. (Spearman test). Weakest correlations were lost ($p > .2$) when the naïve group ($N = 16$) was analysed separately from the group receiving pre-surgery therapy (N = 16). Yellow dots: correlations lost in pre-surgery therapy group; Blue dots: correlations lost in naïve group. f) Genes implicated in lost correlations were not differentially expressed between both groups except for PIT1 and GH that were significantly reduced in the pre-surgery therapy group (all Mann-Whitney test except ARF t-Test). ($p < 0·05$; **, $p < 0·01$; ***, $p < 0·001$; ****, $p < 0·0001$; 0·0000 means lower p).



affected expression of GFRA4, making its value as a prognostic marker for response to fgSSA limited to adenomas not cured by a first operation.

To test whether the results obtained for gene expression were verifiable with protein expression we studied relevant protein expression in a sub-set of samples (Fig. 2f). We compared one resistant sample (ACRO27) with five responsive, either cured by surgery (ACRO7, 8) or responsive to fgSSA therapy (ACRO9, 16 and 15). Only ACRO8 and ACRO27 were naïve samples that had not been pre-surgically treated with fgSSA, at least for a short time while waiting for the surgery (Supplementary Table 1). As shown in Fig. 2g, ACRO27 contained high levels of RET protein but negligible or low levels of PIT1, ARF and p53. Only ACRO7, 8 and 27 had detectable GFRA4 expression, but the proteins detected were of three different molecular weights. It has previously been shown that GFRA4 has a canonical protein GPI isoform that makes a GPI link on the extracellular aspect of the plasma membrane and functions as a RET co-receptor, as well as a longer TM isoform with a transmembrane domain, and a shorter secreted isoform [40]. Thus we could ascertain that ACRO7 expressed the secreted GFRA4 isoform but ACRO8 the TM isoform. Only ACRO27 presented the canonical GPI isoform able to function as a RET co-receptor. Quantitative ARF protein expression, relative either to ACTB or GAPDH as loading controls, was well correlated to ARF mRNA levels (Fig. 2g bottom).

3.3. Tyrosine Kinase Inhibition as potential therapy for SSA Resistant Acromegaly

Taken together, the data to this point indicated that the RET/PIT1/ARF/p53 apoptosis pathway is maintained in ACRO that are responsive to first-line therapy, but blocked and re-oriented towards survival in tumors that are resistant to first-line therapy. To counteract apoptosis, RET must bind GDNF, the main ligand in ACRO, dimerize and activate survival pathways through its tyrosine kinase (TK) activity [15–17,31–37]. Next, we therefore explored the possibility that any of the TK inhibitors (TKI) currently used to treat neuroendocrine tumors might be able to block the RET/GDNF survival pathway.

To do so we needed to develop a primary cell culture system that would allow repeated experiments and dose-response analyses, while maintaining characteristics relevant to the disease. Previously, we and others have performed acute experiments with cell dispersions of human ACRO or rat pituitaries in culture conditions that do not allow cell growth or proliferation [15–17,25,26]. The few pituitary cell lines with a somatotroph phenotype are rat, and not human, and do not maintain the full secretory phenotype, expressing Gh and Pit1 but lacking essential receptors like Ghrhr or very reduced Ret expression (Supplementary Fig. 3). In 2012, we established new culture conditions for human thyroid, h7H, in which all components were adapted to physiological concentrations found in human serum [23]. Through culturing normal and neoplastic follicular cells, h7H has led to the discovery of specific mechanisms underlying human thyroid cancer [24]. We adapted h7H medium for growing human pituitary adenomas by varying the hormone mixture (Supplementary Table 6). We successfully (about 75% success) grew 8 NFPA and 3 ACRO (indicated as P-NFPA or P-ACRO, Fig. 3a), which maintained their phenotype for at least twenty-one passages (range 21–33, longer passages achieved for P-NFPA). Cultures showed the same genetic identity profiles to the original pituitary tissue, which was distinct from any known human cell line (Supplementary Fig. 4A). Cells grew slowly (with a passage 1:2 every two weeks) and were positive for cytokeratins, GH, RET and GDNF assayed immunocytochemically (Supplementary Fig. 4B). Human GH was detected by western blot in the culture medium of the three P-ACRO only after incubation, showing that it was being secreted during culture (Fig. 3b, left). Quantification of the hGH secretion with a kit was specific for ACRO cultures while undetectable in NFPA or medium with no incubation with the cells (Fig. 3b, centre). Normalizing the amount secreted to the cell number on the dish values were normalized for the three ACRO cultures to 1000–6000 pg/mL/10^4 cells/day (Fig. 3b, right).

The phenotype was maintained through passages as shown for mRNA expression of P-ACRO28 p3 to p15 (Fig. 2a), for hGH secretion P-ACRO28 p6-p15, P-ACRO30 p6-p17 and P-ACRO32 p5-p6-p7-p12 (Fig. 2b). After passage 21, it arrived one passage where the cells stop growing, increased size and reduced hGH secretion as seen for ACRO32 p24 (Fig. 2b, centre).

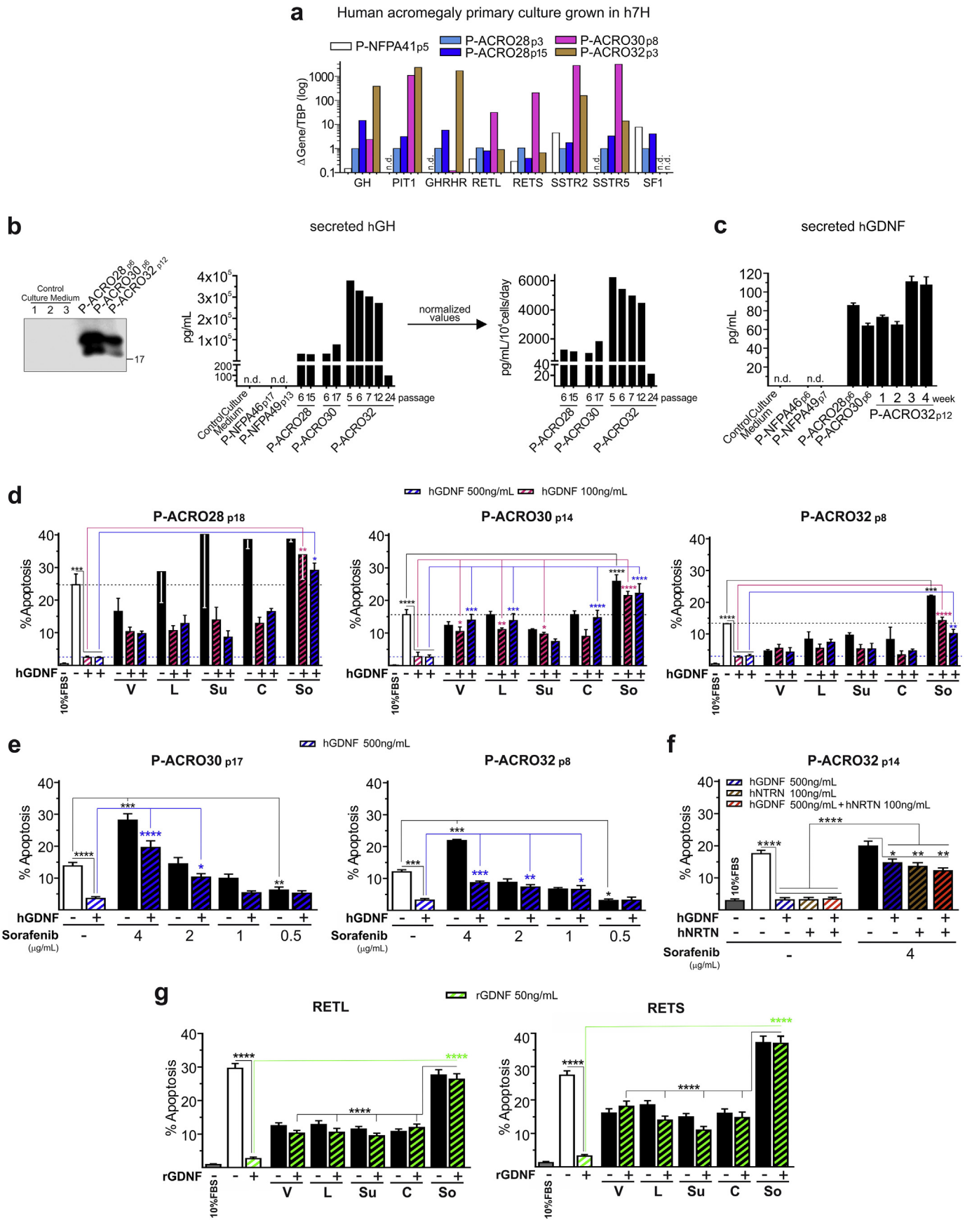
Since GDNF was the main RET ligand expressed in ACRO (Fig. 1a), we also measured its secretion into the culture medium. Fig. 3c shows the concentration of human GDNF (hGDNF) secreted by the three P-ACRO, while no hGDNF was detected in either P-NFPA or medium cultured in empty wells. In P-ACRO32 medium GDNF concentration increased as the culture grew.

We selected five TKI, Vandetanib (V), Lenvatinib (L), Sunitinib (Su), Cabozantinib (C) and Sorafenib (So), all FDA approved for treatment of neuroendocrine cancers and known to inhibit RET kinase (Supplementary Table 4). A key step in our assay was to decide which TKI concentrations tested in vitro were relevant to their in vivo actions. We therefore performed an in silico study to ascertain relevant mean concentrations at the steady-state (Cm_{ss}), as detected in sera from patients treated with the TKIs in Phase II/III clinical trials (Supplementary Table 4). Sorafenib was used at half this concentration since 10–20% of the patients required their dose to be halved due to serious side effects.

As shown in Fig. 3D, the three cultured P-ACRO maintained the RET apoptotic pathway and presented marked apoptosis when deprived of GDNF (by washing and replacing with low-serum medium) that was inhibited by re-addition of GDNF (100 or 500 ng/mL). In the absence of GDNF different TKIs affected RET-dependent apoptosis to different extents. Only Sorafenib caused significant and appropriate apoptosis in the presence of GDNF, neutralizing the survival effect of both 100 and 500 ng/mL GDNF in the three cultures (Fig. 3D). We performed a dose-response curve of Sorafenib in two of the cultures. In both P-ACRO30 and P-ACRO32 2 and 4 microg/mL led to significant inhibition of the GDNF survival effect (Fig. 3E), suggesting that lower doses than those required in cancer could be effective in acromegaly treatment. Some ACRO showed a combined expression of GDNF and NRTN, the second most important ligand for RET (Figs. 1B and 2F) which can cross-activate RET through GFRA1 [33–36]. In accordance with this, in our culture system NRTN alone or combined with GDNF was also a survival factor following ligand deprivation (Fig. 3F). We therefore tested whether Sorafenib was able to counteract the survival effect of GDNF or NRTN or the combination of both, and indeed found that it could neutralize the survival action of each factor alone or in combination (Fig. 3F).

ACRO tumors expressed both isoforms of the RET receptor, RETL and RETS (Fig. 1C and 2F), which have distinct thyrosines and differentially activating signal transduction pathways, either quantitatively (intensity, duration) or qualitatively (activated signaling cascades) [10]. P-ACRO maintained expression of both RET isoforms in culture

Fig. 2. ARF mRNA expression as a prognostic marker for ACRO resistant to first-line combined treatment (sugery + fgSSA). GFRA4 as a prognostic marker only for those not cured by surgery. a-b: Significant correlations (Spearman test, rs > 0·5) between mRNA expression and clinical variables (diagnostic, pathologic and prognostic/follow-up) (See also Supplementary Table 1). Dots: blue, correlations not existing in naïve samples; yellow: correlations lost in the pre-surgery therapy group. a) Left: serum GH at diagnosis was correlated with GH, SSTR2 (only if pre-treated), AIP and p53. Centre: Pre-surgery therapy was negatively correlated with GH and PIT1. ARF was negatively correlated with the negative characteristics of the tumor (invasiveness (only if naïve), Knosp grade, residual tumor). Right: TPIT was negatively correlated with negative tumor characteristics (diameter, volume (only if pre-treated), invasiveness and Knosp) but positively correlated with surgical cure. b) Left: ARF was the strongest positive marker for response to adjuvant treatment with fgSSA (rs > 0·8). GFRA4 was the second-strongest marker but negatively correlated with response (rs > 0·7). GH, TPIT and SSTR2 (only if naïve) showed some positive correlation with response to analogs (rs 0·5–0·6). Right: Combining first-line treatments (surgery+fgSSA) into a single category, ARF (rs > 0·7) and GFRA4 (rs = 0·5) were the most significant positively and negatively correlated markers, respectively, followed by TPIT (rs = 0·6) as a positive marker. c-f: Patients were categorized into two groups, Group 0 (Resistant: Not cured by surgery and resistant to analogs) and Group 1 (Responsive to first-line treatment, either cured by surgery or controlled with analogs). A subanalysis of patients not cured by surgery was also performed. c) ARF expression was a good discriminator of Group 0 and Group 1, both in the series as a whole ($p < 0·0001$) and in patients not cured by surgery ($p = 0·0002$). d) Samples were categorized with different cutoffs for non-mutated (GNASwt cutoff 0·1) or mutated (GNASmut, cutoff 0·06) GNAS; Chi-squared tests classified all samples. e) A subanalysis of naïve patients and those with pre-surgery therapy including categorizing by GNAS demonstrated the ability of ARF to separate Group 0 from Group 1 in either group of patients with similar cutoffs. f) Left: Enhanced GFRA4 expression was not a good classifier for ACRO as some in Group 1 (Responsive, high ARF) had high GFRA4 expression. Right: In the group of non-surgically cured, GFRA4 was a good classifier in the opposite way to ARF, with high levels in Group 0 (Resistant to first-line therapy). Coloured dots show reoperations after radiotherapy (Group 0, yellow; Group 1, orange). g) Western blots of RET pathway (RET, PIT1, ARF, p53) and ligand (GDNF, NRTN) proteins with controls (GH, GAPDH, ACTB) from ACRO tissue extracts (Group 1 (Responsive) and Group 0 (Resistant)). ARF and GFRa4 protein levels corroborated mRNA levels described above. ACRO27 (Group 0) expressed high RET but low PIT-1 protein levels, correlating with absence of p14ARF and p53. GFRA4 showed three different molecular weights corresponding to distinct isoforms. The RET co-receptor (GPI, canonical isoform) was highly expressed in ACRO27 (Group 0) while ACRO7 and ACRO8 (Group 1) expressed different non-RET co-receptor isoforms. Quantification of ARF protein band intensity respect to controls, ACTB (yellow bars) or GAPDH (orange bars), in relation to the ARF mRNA expression (blue bars). (c-e-f Mann-Whitney test; d Chi-square test. *, p < 0·05; **, p < 0·01; ***, p < 0·001; ****, p < 0·0001; 0·0000 means lower p).


a
Human acromegaly primary culture grown in h7H
P-NFPA41p5
P-ACRO28p3
P-ACRO28p15
P-ACRO30p8
P-ACRO32p3
ΔGene/TBP (log)
GH
PIT1
GHRHR
RETL
RETS
SSTR2
SSTR5
SF1
b
secreted hGH
Control Culture Medium
P-ACRO28p6
P-ACRO30p8
P-ACRO32p12
pg/mL
normalized values
pg/mL/10⁴cells/day
passage
P-NFPA46p6
P-NFPA49p7
P-ACRO28
P-ACRO30
P-ACRO32
c
secreted hGDNF
week
d
hGDNF 500ng/mL
hGDNF 100ng/mL
P-ACRO28 p18
P-ACRO30 p14
P-ACRO32 p8
% Apoptosis
hGDNF
10%FBS
V
L
Su
C
So
e
hGDNF 500ng/mL
P-ACRO30 p17
P-ACRO32 p8
Sorafenib (μg/mL)
f
P-ACRO32 p14
hGDNF 500ng/mL
hNTRN 100ng/mL
hGDNF 500ng/mL + hNRTN 100ng/mL
hNRTN
g
rGDNF 50ng/mL
RETL
RETS
rGDNF

(Fig. 3A). We investigated the anti-survival action of Sorafenib on each RET isoform by using either RETS or RETL transfection into the rat pituitary cell line GH4C1 that expresses GH, PIT1 and GDNF, but RET at very low levels (Supplementary Fig. 3) [15,16]. In this set-up, Sorafenib was able to block the survival action of rat GDNF (rGDNF) in the presence of either human isoform RETL or RETS, much more effectively than any of the other TKIs tested (Fig. 3G). The other TKIs (V, L, Su, C) were able to counteract GDNF survival, but also affected the RET apoptotic pathway in the absence of ligand (Fig. 3G).

We next investigated which signal transduction pathways activated by RET/GDNF were being affected by Sorafenib. AKT has been implicated in GDNF/RET survival in normal somatotrophs and acromegaly [15,17]. We therefore performed an antibody array with extracts of two primary acromegaly cultures, P-ACRO30 and P-ACRO32, treated for one hour with/without GDNF plus/minus Sorafenib (Fig. 4A). Arrays were scanned and quantified (Fig. 4B). Kinases significantly activated by GDNF were AKT and mTOR. Significantly inhibited by GDNF were the cleavage of Caspase-3 (cl-CASP3) and PARP (cl-PARP), two enzymes activated by cleavage, as well as Ser15 phosphorylation of p53 (p-p53) a site well-known for p53 stability and activity, all of which are implicated in apoptosis in somatotrophs [15,17]. Sorafenib blocked GDNF-induced AKT/mTOR activation and increased cl-Caspase-3 and cl-PARP and p-p53. Moreover, Sorafenib caused activation of p-AMPKa per se, which was augmented in the presence of GDNF (Fig. 4B). To validate the array results we performed western blots with extracts from P-ACRO32, also treated with GDNF/Sorafenib for one hour. Lysates were obtained with buffers optimised for enrichment in the plasma membrane or cytoplasm. In parallel, whole cell extracts were treated for 24 h, the time at which apoptosis or survival was clearly detectable (see Fig. 3). As expected, GDNF induced RET phosphorylation at the membrane (Fig. 4C). Sorafenib blocked this phosphorylation and even reduced total RET expression. Previous studies have demonstrated that in the absence of GDNF the RET apoptotic pathway is initially activated after a complex between RET and Caspase-3 is formed at the membrane, resulting in both RET processing and Caspase-3 activation by cleavage (IC-RET and cl-CASP3 fragments, respectively) [15]; this also occurred in our experiment (Fig. 4C and D). When the cells are deprived of serum/GDNF cl-CASP3 increases but this was blocked in the presence of GDNF as described [15,17]. GDNF in the presence of Sorafenib recovered and even enhanced cl-Casp3 levels (Fig. 4C).

At the cytoplasmic level (Fig. 4D), GDNF induced phosphorylation of AKT at (mainly) Thr450 and Ser473, which was blocked by Sorafenib. As a corollary, GDNF-induced mTOR phosphorylation was also blocked by Sorafenib, as was the mTOR-dependent phosphorylation of S6 kinase (Fig. 4D). Deprivation induced RET processing with appearance of the cytoplasmic Caspase-3 processed IC-RET fragment; RET processing is inhibited by GDNF but recovered by Sorafenib (Fig. 4D). On the other hand, Sorafenib alone induced AMPKa activating phosphorylation; this activation was enhanced in the presence of GDNF. Similarly, Sorafenib induced the activating phosphorylation of p53 that was enhanced – and not inhibited- in the presence of GDNF (Fig. 4D).

After 24 h of treatment (Fig. 4E), PIT1 accumulated in serum-deprived cells, leading to an increase in p14ARF. GDNF reduced both PIT1 and ARF levels, and this was prevented in the presence of Sorafenib. In parallel, serum deprivation induced p53 accumulation which was blocked by GDNF. Sorafenib led to recovery of p53 protein levels and to apoptosis (Fig. 3).

4. Discussion

The data from this molecular study of sporadic acromegaly tumors demonstrate that expression of elements of the RET pathway are hallmarks of acromegaly. GDNF, in particular, is strongly enriched in ACRO compared with normal pituitary and NFPA. The two RET isoforms RETL and RETS are equally expressed within ACRO. GFRA1 is the main co-receptor expressed in ACRO, followed by GFRA4. NRTN is also expressed in some ACRO. Correlations among expression of various ligands and RET indicate redundancy of the RET survival pathway that completely inhibits the RET apoptotic pathway. Pre-treatment of the samples with fgSSA reduces expression of PIT1 and GH but do not alter particularly other genes of the RET pathway.

The most striking result of our work is that ARF mRNA expression is a sensitive and specific marker of good prognosis. High ARF levels guarantee response while low ARF predict resistance to first-line therapy (surgery plus adjuvant therapy with fgSSA). ARF predictive ability is not altered in samples from patients submitted to pre-surgery therapy with fgSSA. The confidence level reaches 100% if GNAS mutation is used to slightly adjust the cutoff (≥0·1 for non-mutated GNAS and ≥ 0·06 for mutated GNAS). This cutoff was initially obtained using TBP as the control gene to adjust for overall expression, and could also be obtained using another control gene such as ACTB. We also obtained results at the protein level in tissues from tumors that fully corroborated the mRNA level data.

mRNA expression is directly related to protein expression for ARF since it is an mRNA isoform of the CDKN2A gene, that also encodes p16INK4 [41]. The two isoforms have different promoters and exon 1; exons 2 and 3 are shared but with different reading frames of translation, giving rise to two different proteins [41,42]. Initial studies proposed ARF as a marker of good prognosis in cancer [43], although this idea was subsequently discarded as aggressive cancers either mutate p53, becoming independent of ARF, or methylate the ARF promoter [43]. However, whole genome sequencing has repeatedly shown, that somatotropinomas are benign adenomas with non-mutated p53 (as well as RET and ARF) [44–47]. Moreover, mouse and cell culture models have demonstrated an essential role for ARF and p53 in the regulation of pituitary cellularity [48,49]. Furthermore, inhibitors of the interaction between MDM2 and p53, which degrades p53, induces senescence in pituitary adenomas [50].

Current acromegaly treatment takes a trial-and-error approach: [51,52] first surgery is followed by evaluation at 4–16 weeks, and a trial with fgSSA is evaluated after 6–12 months. After that, 20–25% of patients (as in our series) undergo expensive second-line SSA therapy to which they are also frequently resistant while the peripheral disease continues unabated due to high GH/IGF1 levels and continued tumor growth. Risk factors related to a poor prognosis are age and male sex, size and volume of the adenoma, Knosp grade and extent of invasion

Fig. 3. Primary cultures of human acromegaly in humanized conditions (h7H) unveiled Sorafenib as a potential new treatment. a) Acromegaly (P-ACRO28, P-ACRO30 and P-ACRO32) and NFPA (P-NFPA41) cultured in h7H conditions grew for many passages while maintaining mRNA expression of key genes from Fig. 1: in ACRO, GH, PIT1, GHRHR, RETL and RETS isoforms, SSTR2 and 5; in NFPA, SF1. Data are normalized to the first acromegaly P-ACRO28. Passage was performed by splitting the culture in two (shown by small 'p'). b) Left: Human GH (hGH) was secreted by cultured ACRO into the medium as demonstrated by western blot. Centre: Quantitative hGH (pg/mL) measurements performed in whole medium taken just before passaging. Right: Secretion was normalized by cell count and length of incubation with the cells (3–4 days). c) Human GDNF (hGDNF) was secreted into the medium by ACRO, but not by NFPA, as demonstrated by ELISA. Secretion was increased as cells grew, as shown for ACRO32. d) When cells were deprived of GDNF, RET processing induced apoptosis (white bar) that was blocked by addition of GDNF (hatched white bars). Five TKIs used for other neuroendocrine tumors (Vandetanib (V), Lenvatinib (L), Sunitinib (Su), Cabozantinib (C) and Sorafenib (So)) were tested against the survival action of GDNF at clinically relevant concentrations. Three independent cultures are shown: P-ACRO28, P-ACRO30 and P-ACRO32. Some of the inhibitors enhanced RET-dependent apoptosis in the absence of GDNF (black bars) but did not have a strong effect on GDNF-induced survival (black hatched bars). Sorafenib was the only TKI that potently blocked the GDNF survival effect in the three ACRO without exacerbating RET apoptosis in the absence of GDNF (which we assumed to be a toxic effect). e) Dose-response curve of Sorafenib in P-ACRO30 and P-ACRO32, demonstrating a GDNF-counteracting effect at lower doses than those used in cancer treatment. f) Sorafenib could also block the survival effect of NRTN and combined GDNF+NRTN. g) Sorafenib could block the survival effect of GDNF on both human RETL and RETS isoforms, transfected in the non-RET-expressing rat pituitary somatotroph cell line GH4C1. (n.d. = not detected). (d-g: Mean ± SEM. ANOVA test, all bars compared to white bar –deprived in the absence of GDNF-) (*, $p < 0·05$; **, $p < 0·01$; ***, $p < 0·001$; ****, $p < 0·0001$).

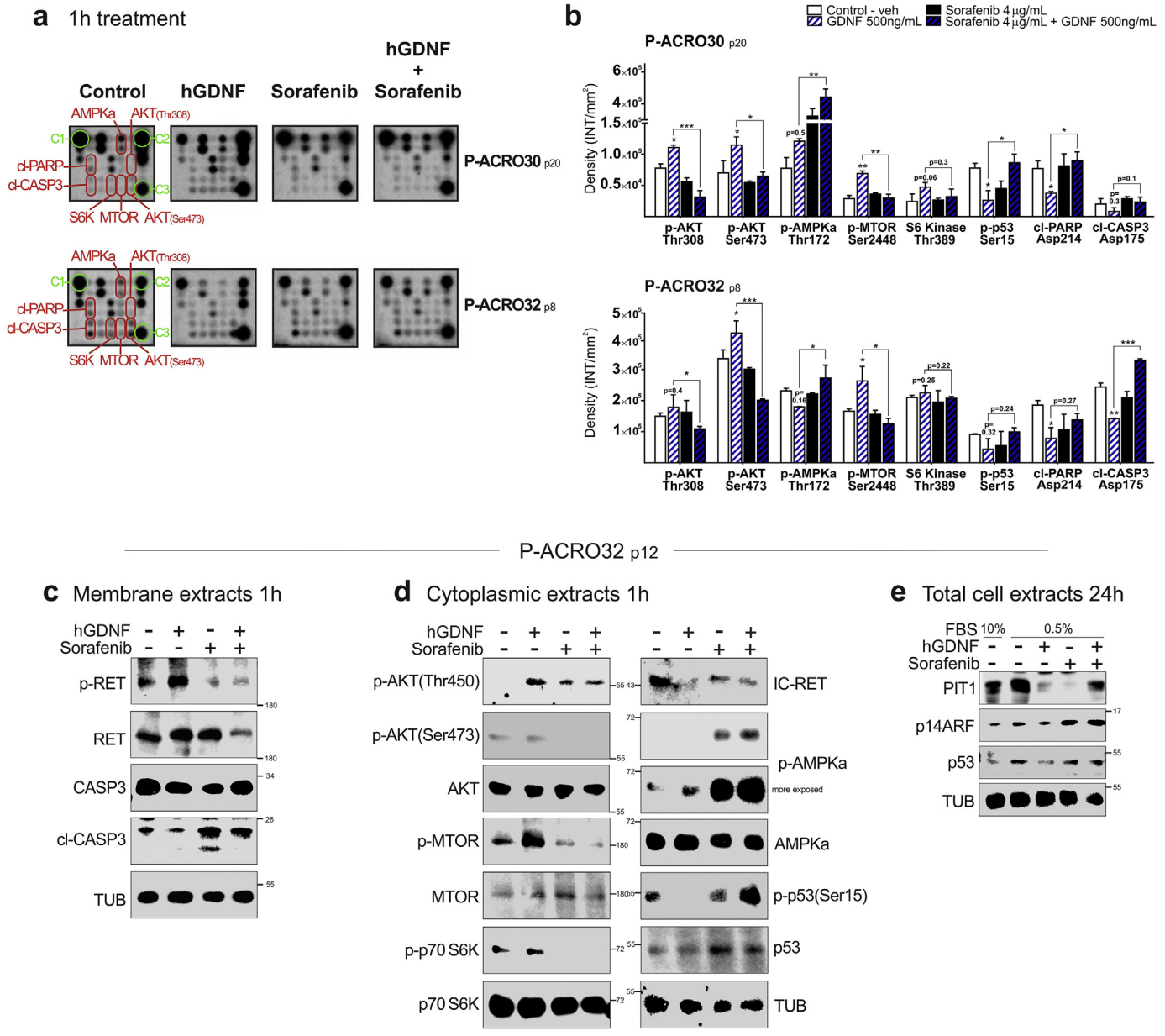


Fig. 4. Blocking of RET signal transduction by Sorafenib exchanged the RET-GDNF survival pathway for the RET apoptotic pathway. a) Antibody array performed with extracts from P-ACRO30 and P-ACRO32 following one- hour incubations in each of four conditions: deprivation (no GDNF), GDNF (500 ng/mL), Sorafenib (4 microg/mL) or Sorafenib+GDNF. b) Signal quantification (Mean ± SEM. ANOVA test): GDNF induced AKT/MTOR phosphorylation, and downregulated active p-p53 (Ser15 p-p53), as well as cleaved PARP (cl-PARP) and Caspase-3 (cl-CASP3), hallmarks of apoptosis. Sorafenib blocked the AKT pathway, inducing phosphorylation of AMPK, p53 and apoptotic markers. c-e) Western blots with different cell extracts confirmed the results of the array. c) At 1 h in membrane extracts, GDNF induced full-length RET phosphorylation and blocked Caspase-3 cleavage; both were prevented by Sorafenib. d) At 1 h in cytoplasmic extracts, GDNF phosphorylated AKT, mTOR and, less intensely, S6K while preventing p53 phosphorylation. In the presence of Sorafenib, GDNF was unable to activate the AKT/MTOR pathway, but induced phosphorylation of AMPK and p53. As expected, the caspase-processed cytoplasmic fragment of RET (IC-RET) was strongly expressed in deprived cells in the absence of GDNF, reduced in the presence of GDNF and recovered with Sorafenib. e) In cells deprived of GDNF for 24 h, PIT1 was induced, activating ARF expression and p53 accumulation, thus leading to apoptosis. GDNF reduced PIT1, ARF and p53 expression, all of which recovered in the presence of Sorafenib. (*, $p < 0{\cdot}05$; **, $p < 0{\cdot}01$; ***, $p < 0{\cdot}001$).

[4]. However, these risk factors are more related to whether the surgery is curative without causing hypopituitarism than to the biology of the tumor per se: a larger tumor impinges on and invades surrounding structures, making surgery less likely to be successful. Importantly, however, not all the macroadenomas or invasive tumors are resistant to therapy. Thus, none of these factors, nor the combination of them, can predict with precision the response to adjuvant fgSSA therapy or to combined first-line treatment.

Several molecular factors have been proposed for early identification, at the time of initial surgery, of SSA-resistant tumors. SSTR2 receptor expression has been the most widely studied, using immunohistochemistry and mRNA expression, as it mediates SSA action on somatotroph cells [53–58]. SSTR2 protein expression is correlated with a response to SSA. However, while the predictive negative value is remarkable, there is no clear cutoff for SSTR2 expression levels to separate resistant from responsive tumors, both because levels can be affected by pre-surgical treatment and resistance might be mediated by a post-receptor mechanism. This is also seen in our current series in relation to SSTR2 expression which does not discriminate between Responsive and Resistant'. Although tumor granulation (positiveness for CAM5.2) is also related to increased expression of SSTR2 pre-surgical treatment and other factors affect its immunostaining too much for it to be a clear marker.

Other proposed molecular markers include SSTR5, the other SSA receptor expressed in somatotrophs, dopamine receptors or a

combination of the two [59,60], and non-receptor proteins such as ZAC1, E-cadherin, B-arrestin, AIP and Ki67 [61–65]. However, none of these have reached the consistency or sensitivity of a reliable prognostic marker [5]. Regarding the new marker, we propose here, while ARF appears to have potential as a marker from the current series, ultimately this will also depend on other prospective studies in more series.

The early identification of SSA-resistant patients will also inform the search for other anti-ACRO pharmacological therapies. In the current study, we have explored the possibility that TKIs, already used to treat endocrine cancers, might have an effect against the RET/GDNF/survival pathway and could be useful in acromegaly. To that end, we have developed a new culture system, adapted from a previous study of human thyroid tumors [23] that has enabled us to amplify the number of adenoma cells and perform functional and signaling experiments in ACRO cultures while maintaining the cellular phenotype. Importantly, TKIs were tested at relevant concentrations with respect to serum levels in patients. Of the TKIs tested, only Sorafenib was able to inhibit the survival action of GDNF through RET tyrosine kinase activation without affecting the normal RET/PIT1/ARF/p53 apoptotic pathway in the absence of GDNF. Lenvatibib, Cabozantinib, Sunitinib and Vandetanib seemed to affect both apoptosis and survival pathways, and thus to have no net effect. It could be that these four TKIs are affecting kinases required by the RET/apoptotic pathway, such as PKCdelta or JNK, which phosphorylates and recruits CREB and c/EBPa, permanently activating the PIT1 promoter and leading to apoptosis [15]. Sorafenib, on the other hand, had a dose-response effect and seemed sufficiently potent against GDNF to function at lower doses than those required in cancer therapy. In our in vitro studies, we have used half the concentration of that found in serum of the patients in Phase III studies since a relevant percentage of patients needed a dose reduction due to adverse effects (Supplementary table 4). Moreover, the dose-response curve indicated that even lower doses were effective. In any case, if the treatment were effective for acromegaly, inducing apoptosis, it may be expected a limited treatment for a period of time until resumption or reduction of the adenoma.

As mentioned previously, NRTN is also able to activate RET through the GFRA1 co-receptor [33–36], and some ACRO also express NRTN. However, Sorafenib not only blocks the survival action of GDNF but also that of NRTN and the combination GDNF+NRTN present in some tumors. Finally, both RET isoforms, RET-L and RET-S, were expressed in both ACRO tumors and cultures. Sorafenib was also able to block survival without affecting apoptosis in rat pituitary cells transfected specifically with RETL or RETS.

At the molecular level, Sorafenib was able to directly (after a one-hour treatment) inhibit GDNF-induced RET phosphorylation, promoting RET degradation. As a consequence, GDNF-induced AKT and mTOR phosphorylation were also blocked by Sorafenib. Sorafenib activated AMPKa kinase, which has previously been demonstrated to be by the direct action of Sorafenib on mitochondria by which it reduces the ATP/AMP quotient [66]. However, in the ACRO cultures Sorafenib AMPKa activation was potentiated in the presence of GDNF, suggesting that the mTOR blockade might be involved. AMPKa activation has previously been proposed as essential for Sorafenib activity [67]. AMPKa activation is also implicated in cellular autophagy, although it is not clear whether Sorafenib's anti-tumor activity is necessarily mediated by autophagy [68]. The possibility remains that other AKT/mTOR related survival pathways inhibited by Sorafenib could play important roles. The overall consequence of AMPKa activation or AKT/mTOR inhibition or both was Caspase-3 activation with RET processing obtaining the cytopkasmic IC-RET fragment, an accumulation of PIT1, ARF and p53, and apoptosis, despite the presence of GDNF.

Other data that could be of interest in the future included expression of PROP1 in ACRO and the role of the GFRA4 co-receptor. PROP1 is a transcription factor characteristic of embryonic pituitary progenitors and maintained in postnatal pituitary stem cells [69,70]. During differentiation, PROP1 activates PIT1 while itself being repressed [38,70]. In ACRO, GDNF was negatively correlated with PIT1, but positively with PROP1 expression, suggesting a role for GDNF in inducing a more progenitor-like/less differentiated phenotype in adenoma somatotroph cells. Previous studies in the mouse have demonstrated that three different Gfra4 isoforms expressing GPI, soluble or transmembrane proteins are present in the thyroid and pituitary [40]. Only the Gfra4 GPI and soluble isoforms, and not the transmembrane isoform, are able to act as RET co-receptors [71]. Our results in ACRO, measuring total GFRA4 mRNA and detecting specific protein isoforms, suggest that the GFRA4 GPI isoform specifically is associated with resistance to adjuvant SSA therapy in acromegaly. Future studies should be performed to assess this specific isoform. Also for future work is the relations between the Somatostatin pathway, repressing PIT1 and GH expression, and the RET pathway including its ligands. Although we did not find significant differences in the pre-surgery therapy group of samples, some correlations with NRTN were lost. But this was a small sample size of pre-surgically treated samples and future studies will require collecting a larger number of samples.

In summary, we propose that tumor ARF mRNA expression measured at the time of the surgery is a prognosis factor in acromegaly, and that a new pharmacotherapy with Sorafenib could be effective. Other candidate pharmacological agents are the very specific anti-RET TKIs, LOXO-292 and BLU-667, currently undergoing phase I/II and phase I clinical trials, respectively, for the treatment of neuroendocrine cancers and potentially applicable to acromegaly in the future. Although the current series will require validation with other series, our study included 32 ACRO cases of three different hospitals, was prospective and analysed fresh tissue obtained at initial surgery. Ultimately, the ability to identify patients who are resistant to first-line therapy and the trial of new pharmaceuticals should improve outcomes in acromegaly.

Acknowledgments

We thank Monica Marazuela, Manel Puig, Justo Castaño and Carlos Dieguez for helpful discussions on GH regulation and clinical and biological aspects of acromegaly. We thank Ana Estany for her statistical revision. We thank Miguel Lopez and Ruben Nogueiras for helpful discussion on AMPK and mTOR pathways and their generosity with antibodies.

Author contributions

MC, ARGR, SPR, MSF and CVA collected tissue samples, performed molecular and cellular studies, carried out statistical analyses and constructed figures. IARG, RS, RVT, EFR, AGA, JMCA, RMAS, CAE and IB recruited patients and collected clinical data at the hospitals specified. JRG analyzed secreted hormones. PVL performed data interpretation and wrote the manuscript. IB and CVA designed the study, obtained financial support, and wrote the manuscript.

Competing interests

Dr. Alvarez reports grants from Agencia Estatal de Investigación (AEI) with European FEDER funds, other from Novartis, during the conduct of the study; grants from Medical Research Council (UK), personal fees and other from PFIZER, other from NOVARTIS, outside the submitted work; In addition, Dr. Alvarez has a patent P17567EP00 pending. Dr. BERNABEU reports grants from Instituto Investigación Carlos III (ISCIII) with FEDER funds, grants from PFIZER_Investigator Initiated Research (IIR) Program, grants from PFIZER Foundation, during the conduct of the study; grants and personal fees from PFIZER Inc., outside the submitted work; In addition, Dr. BERNABEU has a patent P17567EP00 pending.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.04.007.

References

[1] Ezzat S, Asa SL, Couldwell WT, Barr CE, Dodge WE, Vance ML, et al. The prevalence of pituitary adenomas: a systematic review. Cancer 2004;101:613–9.
[2] Hernández-Ramírez LC, Gabrovska P, Dénes J, Stals K, Trivellin G, Tilley D, et al. Landscape of familial isolated and young-onset pituitary adenomas: prospective diagnosis in AIP mutation carriers. J Clin Endocrinol Metab 2015;100:E1242–54.
[3] Pivonello R, Auriemma RS, Grasso LF, Pivonello C, Simeoli C, Patalano R, et al. Complications of acromegaly: cardiovascular, respiratory and metabolic comorbidities. Pituitary 2017;20:46–62.
[4] Terzolo M, Reimondo G, Berchialla P, Ferrante E, Malchiodi E, De Marinis L, et al. Italian Study Group of Acromegaly. Acromegaly is associated with increased cancer risk: a survey in Italy. Endocr Relat Cancer 2017;24:495–504.
[5] Gadelha MR, Kasuki L, Lim DS, Fleseriu M. Systemic complications of acromegaly and the impact of the current treatment landscape: an update. Endocr Rev 2018. https://doi.org/10.1210/er.2018-00115.
[6] Melmed S, Bronstein MD, Chanson P, Klibanski A, Casanueva FF, Wass JAH, et al. A consensus statement on acromegaly therapeutic outcomes. Nat Rev Endocrinol 2018;14:552–61.
[7] Giustina A, Chanson P, Kleinberg D, Bronstein MD, Clemmons DR, Klibanski A, et al. Expert consensus document: a consensus on the medical treatment of acromegaly. Nat Rev Endocrinol 2014;10:243–8.
[8] Colao A, Auriemma RS, Lombardi G, Pivonello R. Resistance to somatostatin analogs in acromegaly. Endocr Rev 2011;32:247–71.
[9] Garcia-Lavandeira M, Diaz-Rodriguez E, Garcia-Rendueles ME, Rodrigues JS, Perez-Romero S, Bravo SB, et al. Functional role of the RET dependence receptor, GFRa co-receptors and ligands in the pituitary. Front Horm Res 2010;38:127–38.
[10] Mulligan LM. RET revisited: expanding the oncogenic portfolio. Nat Rev Cancer 2014;14:173–86.
[11] Ibanez CF, Andressoo JO. Biology of GDNF and its receptors - relevance for disorders of the central nervous system. Neurobiol Dis 2017;97:80–9.
[12] Urbano AG, Suarez-Penaranda JM, Dieguez C, Alvarez CV. GDNF and RET-gene expression in anterior pituitary-cell types. Endocrinology 2000;141:1893–6.
[13] Japon MA, Urbano AG, Saez C, Segura DI, Cerro AL, Diéguez C, et al. Glial-derived neurotropic factor and RET gene expression in normal human anterior pituitary cell types and in pituitary tumors. J Clin Endocrinol Metab 2002;87:1879–84.
[14] Negulescu AM, Mehlen P. Dependence receptors - the dark side awakens. FEBS J 2018;285:3909–24.
[15] Canibano C, Rodriguez NL, Saez C, Tovar S, Garcia-Lavandeira M, Borrello MG, et al. The dependence receptor Ret induces apoptosis in somatotrophs through a Pit-1/p53 pathway, preventing tumor growth. EMBO J 2007;26:2015–28.
[16] Diaz-Rodriguez E, Garcia-Lavandeira M, Perez-Romero S, Senra A, Cañibano C, Palmero I, et al. Direct promoter induction of p19Arf by Pit-1 explains the dependence receptor RET/Pit-1/p53-induced apoptosis in the pituitary somatotroph cells. Oncogene 2012;31:2824–35.
[17] Diaz-Rodriguez E, Garcia-Rendueles AR, Ibanez-Costa A, Gutierrez-Pascual E, Garcia-Lavandeira M, Leal A, et al. Somatotropinomas, but not nonfunctioning pituitary adenomas, maintain a functional apoptotic RET/Pit1/ARF/p53 pathway that is blocked by excess GDNF. Endocrinology 2014;155:4329–40.
[18] Luque RM, Ibanez-Costa A, Sanchez-Tejada L, Rivero-Cortés E, Robledo M, Madrazo-Atutxa A, et al. The molecular registry of pituitary adenomas (REMAH): a bet of Spanish endocrinology for the future of individualized medicine and translational research. Endocrinol Nutr 2016;63:274–84.
[19] McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 2005;97:1180–4.
[20] Katznelson L, Laws Jr ER, Melmed S, Molitch ME, Murad MH, Utz A, et al. Acromegaly: an endocrine society clinical practice guideline. J Clin Endocrinol Metab 2014;99:3933–51.
[21] Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun 2004;313:856–62.
[22] Lossos IS, Czerwinski DK, Wechser MA, Levy R. Optimization of quantitative real-time RT-PCR parameters for the study of lymphoid malignancies. Leukemia 2003;17:789–95.
[23] Bravo SB, Garcia-Rendueles ME, Garcia-Rendueles AR, Rodrigues JS, Perez-Romero S, Garcia-Lavandeira M, et al. Humanized medium (h7H) allows long-term primary follicular thyroid cultures from human normal thyroid, benign neoplasm, and cancer. J Clin Endocrinol Metab 2013;98:2431–41.
[24] Garcia-Rendueles A, Rodrigues JS, Garcia-Rendueles M, Suarez-Fariña M, Perez-Romero S, Barreiro F, et al. Rewiring of the apoptotic TGF-beta-SMAD/NFkappaB pathway through an oncogenic function of p27 in human papillary thyroid cancer. Oncogene 2017;36:652–66.
[25] Coya R, Alvarez CV, Perez F, Gianzo C, Dieguez C. Effects of TGF-beta1 on prolactin synthesis and secretion: an in-vitro study. J Neuroendocrinol 1999;11:351–60.
[26] Garcia A, Alvarez CV, Smith RG, Dieguez C. Regulation of Pit-1 expression by ghrelin and GHRP-6 through the GH secretagogue receptor. Mol Endocrinol 2001;15:1484–95.
[27] Gatto F, Feelders RA, Franck SE, van Koetsveld PM, Dogan F, Kros JM, et al. In vitro head-to-head comparison between Octreotide and Pasireotide in GH-secreting pituitary adenomas. J Clin Endocrinol Metab 2017(6):2009–18.
[28] Gupta P, Rai A, Mukherjee KK, Sachdeva N, Radotra BD, Punia RPS, et al. Imatinib inhibits GH secretion from somatotropinomas. Front Endocrinol 2018;9:453.
[29] Al-Massadi O, Porteiro B, Kuhlow D, Köhler M, Gonzalez-Rellan MJ, Garcia-Lavandeira M, et al. Pharmacological and genetic manipulation of p53 in brown fat at adult but not embryonic stages regulates thermogenesis and body weight in male mice. Endocrinology 2016;157:2735–49.
[30] Petrossians P, Daly AF, Natchev E, Maione L, Blijdorp K, Sahnoun-Fathallah M, et al. Acromegaly at diagnosis in 3173 patients from the Liege Acromegaly Survey (LAS) Database. Endocr Relat Cancer 2017;24:505–18.
[31] Jing S, Wen D, Yu Y, Holst PL, Luo Y, Fang M, et al. GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. Cell 1996;85:1113–24.
[32] Treanor JJ, Goodman L, de Sauvage F, Stone DM, Poulsen KT, Beck CD, et al. Characterization of a multicomponent receptor for GDNF. Nature 1996;382:80–3.
[33] Baloh RH, Tansey MG, Golden JP, Creedon DJ, Heuckeroth RO, Keck CL, et al. TrnR2, a novel receptor that mediates neurturin and GDNF signaling through ret. Neuron 1997;18:793–802.
[34] Jing S, Yu Y, Fang M, Hu Z, Holst PL, Boone T, et al. GFRalpha-2 and GFRalpha-3 are two new receptors for ligands of the GDNF family. J Biol Chem 1997;272:33111–7.
[35] Baloh RH, Tansey MG, Lampe PA, Fahrner TJ, Enomoto H, Simburger KS, et al. Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRα3–RET receptor complex. Neuron 1998;21:1291–302.
[36] Milbrandt J, de Sauvage FJ, Fahrner TJ, Baloh RH, Leitner ML, Tansey MG, et al. Persephin, a novel neurotrophic factor related to GDNF and neurturin. Neuron 1998;20:245–53.
[37] Sidorova YA, Matlik K, Paveliev M, Lindahl M, Piranen E, Milbrandt J, et al. Persephin signaling through GFRalpha1: the potential for the treatment of Parkinson's disease. Mol Cell Neurosci 2010;44:223–32.
[38] Olson LE, Tollkuhn J, Scafoglio C, Krones A, Zhang J, Ohgi KA, et al. Homeodomain-mediated beta-catenin-dependent switching events dictate cell-lineage determination. Cell 2006;125:593–605.
[39] Cushman LJ, Watkins-Chow DE, Brinkmeier ML, Raetzman LT, Radak AL, Lloyd RV, et al. Persistent Prop1 expression delays gonadotrope differentiation and enhances pituitary tumor susceptibility. Hum Mol Genet 2001;10:1141–53.
[40] Lindahl M, Timmusk T, Rossi J, Saarma M, Airaksinen MS. Expression and alternative splicing of mouse Gfra4 suggest roles in endocrine cell development. Mol Cell Neurosci 2000;15:522–33.
[41] Quelle DE, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell 1995;83:993–1000.
[42] Duro D, Bernard O, Della Valle V, Berger R, Larsen CJ. A new type of p16INK4/MTS1 gene transcript expressed in B-cell malignancies. Oncogene 1995;11:21–9.
[43] Kotsinas A, Papanagnou P, Evangelou K, Trigas GC, Kostourou V, Townsend P, et al. ARF: a versatile DNA damage response ally at the crossroads of development and tumorigenesis. Front Genet 2014;5:236.
[44] Valimaki N, Demir H, Pitkanen E, Kaasinen E, Karppinen A, Kivipelto L, et al. Whole-genome sequencing of growth hormone (GH)-secreting pituitary adenomas. J Clin Endocrinol Metab 2015;100:3918–27.
[45] Ronchi CL, Peverelli E, Herterich S, Weigand I, Mantovani G, Schwarzmayr T, et al. Landscape of somatic mutations in sporadic GH-secreting pituitary adenomas. Eur J Endocrinol 2016;174:363–72.
[46] Bi WL, Horowitz P, Greenwald NF, Abedalthagafi M, Agarwalla PK, Gibson WJ, et al. Landscape of genomic alterations in pituitary adenomas. Clin Cancer Res 2017;23:1841–51.
[47] Bi WL, Greenwald NF, Ramkissoon SH, Abedalthagafi M, Coy SM, Ligon KL, et al. Clinical identification of oncogenic drivers and copy-number alterations in pituitary tumors. Endocrinology 2017;158:2284–91.
[48] Tsai KY, MacPherson D, Rubinson DA, Nikitin AY, Bronson R, Mercer KL, et al. ARF mutation accelerates pituitary tumor development in Rb+/− mice. Proc Natl Acad Sci U S A 2002;99:16865–70.
[49] Chesnokova V, Zonis S, Rubinek T, Yu R, Ben-Shlomo A, Kovacs K, et al. Senescence mediates pituitary hypoplasia and restrains pituitary tumor growth. Cancer Res 2007;67:10564–72.
[50] Chesnokova V, Zhou C, Ben-Shlomo A, Zonis S, Tani Y, Ren SG, et al. Growth hormone is a cellular senescence target in pituitary and nonpituitary cells. Proc Natl Acad Sci U S A 2013;110:E3331–9.
[51] Puig Domingo M. Treatment of acromegaly in the era of personalized and predictive medicine. Clin Endocrinol (Oxf) 2015;83:3–14.
[52] Kasuki L, Wildemberg LE, Gadelha MR. Management of Endocrine Disease: personalized medicine in the treatment of acromegaly. Eur J Endocrinol 2018;178:R89-100.
[53] Taboada GF, Luque RM, Bastos W, Guimarães RF, Marcondes JB, Chimelli LM, et al. Quantitative analysis of somatostatin receptor subtype (SSTR1-5) gene expression levels in somatotropinomas and non-functioning pituitary adenomas. Eur J Endocrinol 2007;156:65–74.
[54] Ferone D, de Herder WW, Pivonello R, Kros JM, van Koetsveld PM, de Jong T, et al. Correlation of in vitro and in vivo somatotropic adenoma responsiveness to somatostatin analogs and dopamine agonists with immunohistochemical evaluation of somatostatin and dopamine receptors and electron microscopy. J Clin Endocrinol Metab 2008;93:1412–7.
[55] Taboada GF, Luque RM, Neto LV, Machado Ede O, Sbaffi BC, Domingues RC, et al. 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. Eur J Endocrinol 2008;158:295–303.
[56] Neto LV, Machado Ede O, Luque RM, Taboada GF, Marcondes JB, Chimelli LM, et al. Expression analysis of dopamine receptor subtypes in normal human pituitaries, nonfunctioning pituitary adenomas and somatotropinomas, and the association between dopamine and somatostatin receptors with clinical response to octreotide-LAR in acromegaly. J Clin Endocrinol Metab 2009;94:1931–7.

[57] Casar-Borota O, Heck A, Schulz S, Nesland JM, Ramm-Pettersen J, Lekva T, et al. Expression of SSTR2a, but not of SSTRs 1, 3, or 5 in somatotroph adenomas assessed by monoclonal antibodies was reduced by octreotide and correlated with the acute and long-term effects of octreotide. J Clin Endocrinol Metab 2013;98:E1730–9.
[58] Gatto F, Feelders RA, van der Pas R, Kros JM, Waaijers M, Sprij-Mooij D, et al. Immunoreactivity score using an anti-sst2A receptor monoclonal antibody strongly predicts the biochemical response to adjuvant treatment with somatostatin analogs in acromegaly. J Clin Endocrinol Metab 2013;98:E66–71.
[59] Duran-Prado M, Saveanu A, Luque RM, Gahete MD, Gracia-Navarro F, Jaquet P, et al. A potential inhibitory role for the new truncated variant of somatostatin receptor 5, sst5TMD4, in pituitary adenomas poorly responsive to somatostatin analogs. J Clin Endocrinol Metab 2010;95:2497–502.
[60] Venegas-Moreno E, Vazquez-Borrego MC, Dios E, Gros-Herguido N, Flores-Martinez A, Rivero-Cortés E, et al. Association between dopamine and somatostatin receptor expression and pharmacological response to somatostatin analogues in acromegaly. J Cell Mol Med 2018;22:1640–9.
[61] Theodoropoulou M, Tichomirowa MA, Sievers C, Yassouridis A, Arzberger T, Hougrand O, et al. Tumor ZAC1 expression is associated with the response to somatostatin analog therapy in patients with acromegaly. Int J Cancer 2009;125:2122–6.
[62] Gatto F, Biermasz NR, Feelders RA, Kros JM, Dogan F, van der Lely AJ, et al. L ow beta-arrestin expression correlates with the responsiveness to long-term somatostatin analog treatment in acromegaly. Eur J Endocrinol 2016;174:651–62.
[63] Fougner SL, Lekva T, Borota OC, Hald JK, Bollerslev J, Berg JP. The expression of E-cadherin in somatotroph pituitary adenomas is related to tumor size, invasiveness, and somatostatin analog response. J Clin Endocrinol Metab 2010;95:2334–42.
[64] Denes J, Kasuki L, Trivellin G, Colli LM, Takiya CM, Stiles CE, et al. Regulation of aryl hydrocarbon receptor interacting protein (AIP) protein expression by MiR-34a in sporadic somatotropinomas. PLoS One 2015;10:e0117107.
[65] Kasuki L, Wildemberg LE, Neto LV, Marcondes J, Takiya CM, Gadelha MR. Ki-67 is a predictor of acromegaly control with octreotide LAR independent of SSTR2 status and relates to cytokeratin pattern. Eur J Endocrinol 2013;169:217–23.
[66] Ross FA, Hawley SA, Auciello FR, Gowans GJ, Atrih A, Lamont DJ, et al. Mechanisms of paradoxical activation of AMPK by the kinase inhibitors SU6656 and Sorafenib. Cell Chem Biol 2017;24:813–824.e4.
[67] Fumarola C, Caffarra C, La Monica S, Galetti M, Alfieri RR, Cavazzoni A, et al. Effects of sorafenib on energy metabolism in breast cancer cells: role of AMPK-mTORC1 signaling. Breast Cancer Res Treat 2013;141:67–78.
[68] Prieto-Dominguez N, Ordonez R, Fernandez A, García-Palomo A, Muntané J, González-Gallego J, et al. Modulation of autophagy by Sorafenib: effects on treatment response. Front Pharmacol 2016;7:151.
[69] Garcia-Lavandeira M, Quereda V, Flores I, Saez C, Diaz-Rodriguez E, Japon MA, et al. A GRFa2/Prop1/stem (GPS) cell niche in the pituitary. PLoS One 2009;4:e4815.
[70] Sornson MW, Wu W, Dasen JS, Flynn SE, Norman DJ, O'Connell SM, et al. Pituitary lineage determination by the prophet of pit-1 homeodomain factor defective in Ames dwarfism. Nature 1996;384:327–33.
[71] Lindahl M, Poteryaev D, Yu L, Arumae U, Timmusk T, Bongarzone I, et al. Human glial cell line-derived neurotrophic factor receptor alpha 4 is the receptor for persephin and is predominantly expressed in normal and malignant thyroid medullary cells. J Biol Chem 2001;276:9344–51.