CLINICAL STUDY

SAGE analysis highlights the putative role of underexpression of ribosomal proteins in GH-secreting pituitary adenomas

Deison Soares de Lima¹, Clarissa Silva Martins¹, Beatriz Maria de Carvalho Paixao¹, Fernando Colbari Amaral¹, Leandro Machado Colli¹, Fabiano P Saggioro², Luciano Neder², Helio Rubens Machado³, Anemari Ramos Dinarte dos Santos⁴, Daniel G Pinheiro⁴, Ayrton Custodio Moreira¹, Wilson Araújo Silva Jr⁴ and Margaret Castro¹

Departments of ¹Internal Medicine, ²Pathology, ³Anatomy and Surgery and ⁴Genetics, School of Medicine of Ribeirao Preto, University of Sao Paulo, Avenida Bandeirantes 3900, 14049-900 Ribeirao Preto, Sao Paulo, Brazil

(Correspondence should be addressed to M Castro; Email: castrom@fmrp.usp.br)

Abstract

Background: Although the molecular pathogenesis of pituitary adenomas has been assessed by several different techniques, it still remains partially unclear. Ribosomal proteins (RPs) have been recently related to human tumorigenesis, but they have not yet been evaluated in pituitary tumorigenesis.

Objective: The aim of this study was to introduce serial analysis of gene expression (SAGE), a high-throughput method, in pituitary research in order to compare differential gene expression.

Methods: Two SAGE cDNA libraries were constructed, one using a pool of mRNA obtained from five GH-secreting pituitary tumors and another from three normal pituitaries. Genes differentially expressed between the libraries were further validated by real-time PCR in 22 GH-secreting pituitary tumors and in 15 normal pituitaries.

Results: Computer-generated genomic analysis tools identified 13 722 and 14 993 exclusive genes in normal and adenoma libraries respectively. Both shared 6497 genes, 2188 were underexpressed and 4309 overexpressed in tumoral library. In adenoma library, 33 genes encoding RPs were underexpressed. Among these, *RPSA*, *RPS3*, *RPS14*, and *RPS29* were validated by real-time PCR.

Conclusion: We report the first SAGE library from normal pituitary tissue and GH-secreting pituitary tumor, which provide quantitative assessment of cellular transcriptome. We also validated some downregulated genes encoding RPs. Altogether, the present data suggest that the underexpression of the studied RP genes possibly collaborates directly or indirectly with other genes to modify cell cycle arrest, DNA repair, and apoptosis, leading to an environment that might have a putative role in the tumorigenesis, introducing new perspectives for further studies on molecular genesis of somatotrophinomas.

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Introduction

The molecular pathogenesis of GH-secreting pituitary adenoma initiation and progression remains partially unexplained. The involvement of different cyclin D1 alleles, p16 amplification, and methylation are still obscure. Classic proto-oncogenes, such as *NRAS*, *MYCL1*, *MYCN*, *HRAS* (*HRAS1*), *BCL1* (*CCND1*), *FGF4*, *Sea*, *KRAS2* (*KRAS*), *C-erbB-2*, and *FOS*, and tumor suppressors such as retinoblastoma gene (*RBI*) and *P53* do not appear to have a significant role in pituitary adenoma pathogenesis (1, 2). Activating mutations, promoter insertions, deletions, or point mutations of *PTTG* (*PTTG1*) as well as clear correlation between *PTTG* expression and mitotic index in human pituitary tumors have not been described. It remains

unclear whether overexpression of normal *PTTG* has an active role in human pituitary tumorigenesis (3).

Four genetic syndromes have provided insights into the molecular basis of pituitary tumorigenesis. Multiple endocrine neoplasia type 1 (MEN1), caused by germline mutations in *MEN1* (menin) or rarely in *CDKN1B* (cyclin-dependent kinase (CDK) inhibitor) genes, is an autosomal dominant condition associated with pituitary adenomas in 40–60% of patients (4). In Carney complex (CNC), unlike MEN1, only GH-secreting adenomas have been described. In this condition, the normal allele of the regulatory subunit type 1 of the cAMP-dependent protein kinase A gene (*PRKAR1A*) is mutated or lost (5). Pituitary adenomas of all types can occur in a familial setting in the absence of MEN1 and CNC – this phenotype is termed familial isolated

pituitary adenomas (6). Germline inactivating mutations in the *AIP* gene, which maps at 11q13 and encodes the aryl hydrocarbon receptor interacting protein, have been found in some of these families (7). McCune–Albright syndrome, which has been associated with GH-secreting pituitary adenomas, results from somatic mutations in the adenylate cyclase-stimulating G alpha protein gene (*GNAS1* (*GNAS*)) (8). In addition, in ~40% of isolated somatotroph adenomas, activating mutations of the *GNAS1* were identified and remain the most important pathogenic mechanism yet defined for human pituitary adenomas (9).

New concepts in biology point out that microRNAs (10) and ribosomal proteins (RPs) (11, 12) can act as gene expression regulators, controlling multiple biological processes, including cancer. RPs are core proteins that come together with rRNA in the nucleolus to form the small and the large subunits (40S and 60S) of the ribosome (11). Several eukaryotic RPs have also been linked to extra-ribosomal functions, including DNA replication, transcription, splicing, cell growth, proliferation, apoptosis, and DNA repair. Recently, RP gene mutations or altered expression were found in many human tumors such as breast, prostate, uterine cervix, esophagus, and liver carcinoma as well as glioblastoma (11).

Serial analysis of gene expression (SAGE) is a powerful high-throughput genomic approach, which is based on generating, cloning, and sequencing concatenated short sequence tags, each representing a single transcript derived from target tissue mRNA (13). It has a significant advantage over microarray-based analysis as it allows detection of the expression of thousands of genes in a quantitative manner without previous knowledge of their coding sequences. It has been applied to identify a number of genes that are over- or underexpressed in the transcriptome of different types of cancer (14).

This study reports the first SAGE library from normal pituitary tissue and GH-secreting pituitary tumors. We also described and validated differentially expressed RP genes in both tissues, which could be involved in the pathogenesis of somatotrophinomas.

Materials and methods

Subjects

This study was approved by the Ethics Committee of Hospital of School of Medicine of Ribeirao Preto, University of Sao Paulo, and according to the Declaration of Helsinki requirements, and written informed consent was obtained from all participants.

Twenty-two patients (13 females, nine males; mean age, 42 ± 10.5 years) were diagnosed as having acromegaly based on the failure of the GH suppression of $<1 \mu\text{g/l}$ after an oral glucose tolerance test (oGTT) and a high insulin-like growth factor 1 (IGF1) level,

presented as the upper limit of normal range for sex- and age-matched controls (15) (Table 1). Plasma GH was determined by a solid-phase immunofluorometric assay in duplicate, calibrated against the WHO 2nd International Standard 98/574 Reference Preparation for 22 kDa rhGH (somatropin; $1 \mu\text{g/l}$ corresponding to 3 mU/l) acquired from the National Institute for Biological Standards and Control (NIBSC; Hertfordshire, UK). The limit of detection for GH was $0.095 \mu\text{g/l}$ and the intra- and interassay coefficients of variation (CV) were 5.5 and 11.7% respectively. IGF1 was determined in duplicate by immunoradiometric assay after acid extraction (Diagnostic System Laboratories, Inc., Webster, TX, USA). The limit of detection for IGF1 was $10 \mu\text{g/l}$, and the intra- and interassay CV were 5 and 11.3% respectively (15). All patients underwent visual field evaluation and magnetic resonance imaging.

GH-secreting pituitary tumor samples were obtained during transsphenoidal surgery; part of the sample was processed for routine histopathological and immunohistochemical studies, and part was snap-frozen and stored at -70°C for molecular biology studies. All samples were microdissected by an experienced pathologist in order to separate any nontumoral tissue and disrupted using a Polytron homogenizer. Fifteen normal pituitaries were obtained during autopsies from subjects who had natural death without previous evidence of any endocrine disease or neurological abnormality.

RNA and DNA extraction

Total RNA and DNA were isolated from frozen tissues using TRIzol reagent (Invitrogen Life Technologies, Inc.). The quality of the RNA and DNA was evaluated by spectrophotometry and agarose gel electrophoresis.

GNAS and AIP mutation screening

Codons 201 and 227 of the *GNAS* gene and the entire *AIP* gene were directly sequenced. Primers and PCR conditions to detect activating mutations of *GNAS* gene (*gsp* oncogene) and *AIP* mutations in tumoral DNA were previously described (7, 16).

SAGE library construction

Two SAGE cDNA libraries were constructed, one using a pool of mRNA obtained from five GH-secreting pituitary tumors and another from a pool of mRNA obtained from three normal pituitaries. A total of $23 \mu\text{g}$ of purified RNA from a pool of tumoral or normal samples were used for the construction of each library using I-SAGE kit (Invitrogen Life Technologies, Inc.). Binding mRNA to oligo (dT) magnetic beads, cDNA synthesis and all subsequent steps of the SAGE procedure were essentially performed as described previously (13). After synthesis, cDNA was digested with *Nla*III as anchoring enzyme.

Table 1 Clinical, biochemical, imaging findings, and follow-up of patients with acromegaly.

Patient	Gender	Age (years)	Tumor diameter (MRI; cm)	IHC	IGF1 (% ULNR)	GNAS mutation	GH (oGTT; ng/ml)	Remission after surgery	SRL therapy	Disease control
1	F	28	2.4	GH, PRL, FSH, LH	480	p.Q227L	10.4	No	Yes	Yes
2	M	29	3.0	GH, PRL	210	No	52.0	No	Yes	No
3	F	43	1.0	GH, PRL, LH	205	No	17.0	Yes	No	Yes
4	M	42	1.5	GH, PRL, LH, FSH, TSH	921	No	22.3	Yes	No	Yes
5	F	55	0.8	GH, PRL	680	p.R201C	1.7	Yes	Yes	Yes
6	M	34	1.3	GH, PRL	119	p.R201C	5.4	No	Yes	Yes
7	F	50	1.2	GH	NA	No	22.0	Yes	No	Yes
8	F	53	1.1	GH	232	p.R201H	3.4	No	Yes	Yes
9	F	30	1.9	GH	410	p.R201C	7.1	No	Yes	Yes
10	F	37	3.0	GH, PRL	297	No	67.0	No	Yes	Yes
11	M	36	1.6	GH	404	No	32.4	No	Yes	No
12	F	50	1.1	GH, PRL	246	p.R201C	NA	No	Yes	NA ^b
13	M	57	2.0	GH, PRL	758	No	28.9	No	Yes	No
14	M	39	2.7	GH, PRL	360	No ^a	17.0	No	Yes	No ^b
15	F	52	3.4	GH, PRL, LH	376	No	19.5	No	Yes	Yes
16	M	33	2.0	GH, TSH, PRL, LH	485	p.R201C	8.5	No	Yes	No
17	F	44	2.0	GH, PRL	835	No	1.6	No	Yes	No
18	F	57	1.8	GH	492	NA	1.3	No	Yes	Yes
19	F	54	2.8	GH	542	No	19.2	No	Yes	No
20	M	42	2.0	GH	NA	p.Q227L	104.0	Yes	No	Yes ^b
21	F	31	2.1	GH, PRL, TSH	337	No	117.0	No	Yes	No
22	M	26	5.8	GH, TSH, FSH, PRL	NA	p.R201H	338.0	No	No ^b	NA ^b

F, female; M, male; IGF1, insulin-like growth factor 1; ULNR, upper limit of normal range; MRI, magnetic resonance imaging; IHC, immunohistochemistry; PRL, prolactin; SRL, somatostatin receptor ligand; NA, not available.

^aPatient presenting *AIP* p.R304X mutation.

^bPatients without long-term follow-up.

The 3'-terminal cDNA fragments were captured using magnetic stand. SAGE cDNA tags (~10 bp attached to the linker) were generated by binding of linkers to the NlaIII-compatible sticky ends and then were digested by BsmFI (site present in linkers). After blunting of the linker-cDNA products, dimerization, and PCR amplification (30 cycles) were performed using primers directed against linker sequences, which were excised by BsmFI digestion. The released ditag was purified from the excised linkers by PAGE and bound to long multimers of ditags (concatemers) separated by NlaIII sites. Size separation by PAGE was performed and a 300–500 bp fraction was excised from gel, purified, and cloned into the SphI site of pZero-1 (Invitrogen Life Technologies, Inc.). The resulting colonies were gridded in 96-well microtiter dishes containing Low Salt LB+Zeocin (100 µg/ml), grown overnight at 37 °C, and stored at 4 °C until further use. Sequencing was performed using the DYEnamic ET Dye Terminator Kit (GE Healthcare, Piscataway, NJ, USA) and a MegaBACE DNA Analysis System (GE Healthcare).

The sequences were further analyzed using SAGE2000 Software (Invitrogen, Carlsbad, CA, USA) and, briefly, the analysis steps were as follows: locate the NlaIII sites (i.e. CATG 'punctuation signals') within the ditag concatemer; extract ditags of 20–26 bases, length, which are located between the sites; remove repeat occurrences of ditags, including repeat occurrences in the reverse-complemented orientation; define tags as the end-most ten bases of each ditag, reverse-complementing the

right-handed tag; remove tags corresponding to linker (i.e. TCCCCGTACA and TCCCTATTAA), as well as those with unspecified bases (i.e. bases other than A, C, G, or T); and for each tag, count its number of occurrences. The product of this processing is a list of tags with their corresponding count values and thus is a digital representation of cellular gene expression (17).

Tag frequency tables were constructed from data by the SAGE analysis software, with the minimum tag count set at 1 and the maximum ditag length set at 28 bp, and the parameters were set as default. The annotation was based on the SAGEmap Database (<http://www.ncbi.nlm.nih.gov/SAGE>). Statistical analysis was carried out with the H2G Software (<http://gdm.fmrp.usp.br>) for the comparison of two SAGE libraries, using as reference the normal pituitary library to evaluate over- and underexpressed genes.

RT-PCR and real-time PCR quantification

Genes differentially expressed between the libraries were further validated by real-time PCR in 22 GH-secreting pituitary tumors and in 15 normal pituitaries, including those previously used in library construction. Approximately 1 µg total RNA was used in a RT reaction of 10 µl using 2.5 M oligo (dT), 5.5 mM MgCl₂, 2.0 mM dNTP, 20 U/l RNase inhibitor, 50 U/l MultiScribe TaqMan, and 10× RT buffer in a first-strand cDNA synthesis kit (TaqMan RT reagents; Applied Biosystems, Branchburg, NJ, USA). The RT-PCR cycle sequence was

25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min. Control reactions were performed to exclude genomic DNA contamination.

RPSA, *RPS3*, *RPS7*, *RPS14*, and *RPS29* genes were identified among the differently expressed genes between somatotroph adenoma and normal pituitary libraries. cDNA of each RP gene and of *GUSB*, *TBP*, and *PGK1*, used as housekeeping genes, was separately amplified in duplicates, in a total volume of 12 µl, in real-time PCR assays, using Applied Biosystems 7500 Real-Time PCR System (Foster City, CA, USA). In addition, by real-time PCR, we also evaluate *SSTR2* gene expression in all samples. We used Applied Biosystems-customized assay by-design for *RPS3* (TaqMan Gene Expression Assay 79710183), *RPS7* (TaqMan Gene Expression Assay 79686327), *RPS14* (TaqMan Gene Expression Assay 79685288), *RPS29* (TaqMan Gene Expression Assay 79685936), assay on demand for *RPSA* (TaqMan Gene Expression Assay Hs01080364_m1), and inventoried assay for *SSTR2* (TaqMan Gene Expression Assay Hs00990356_m1). For endogenous controls, we used *PGK1* (TaqMan Gene Expression Assay Hs99999906_m1), *TBP* (TaqMan Gene Expression Assay Hs99999910_m1), and *GUSB* (TaqMan Gene Expression Assay Hs99999908_m1).

Reactions were incubated in a 96-well optical plate at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The cycle threshold (Ct) was defined as the fractional cycle number at which the fluorescence surpasses the fixed threshold. The Ct data were performed using default threshold settings.

Data were presented as the Ct of each sample of each target gene normalized by the endogenous controls (*GUSB*, *TBP*, and *PGK1*), Ct-median expression, and calibrated by ΔCt -median value obtained from all normal pituitary tissues. Each gene expression in normal pituitary and tumoral tissue samples are presented as mean, s.d., median, and range. Relative quantification of gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (18). Fold change of gene expression observed in somatotroph adenomas compared with normal pituitaries was determined by the median of $2^{-\Delta\Delta\text{Ct}}$ values of the former, related to median of $2^{-\Delta\Delta\text{Ct}}$ values of the latter.

Statistical analysis

Statistical analyses were carried out using Mann–Whitney *U* test for continuous variables. Fisher's exact test was used for categorical data such as the presence of *GNAS* mutation, tumor size, remission after transsphenoidal surgery, control after somatostatin receptor ligand (SRL) therapy, and the presence of exclusive GH staining on immunohistochemistry (IHC). Data were analyzed by GraphPad Prism 4 Software (San Diego, CA, USA) and differences were considered significant at $P < 0.05$. For analysis of correlation between gene expression of RP genes and *SSTR2*, the Pearson's correlation coefficient was used.

Results

Subjects

Table 1 shows the clinical, biochemical, and imaging findings, and follow-up of patients with GH-secreting pituitary tumors. Magnetic resonance imaging diagnosed macroadenoma in 21 out of 22 patients. Exclusive GH staining on IHC was observed in seven patients (31.8%), GH and prolactin (PRL) staining in eight patients (36.4%), and GH, PRL, and glycoprotein hormones (LH, FSH, and TSH) in seven patients (31.8%).

Disease control was defined by GH level suppressed by < 1 ng/ml after oGTT and normal IGF1 levels for sex- and age-matched controls. Seventeen patients (77.2%) had no clinical or biochemical remission after transsphenoidal surgery and were submitted to complementary therapy with SRL. Among these patients, seven (41.1%) achieved control. Patient 19 achieved disease control after SRL associated with dopamine agonist, while patients 2, 11, 13, and 16 underwent second surgery, radiotherapy, and treatment with dopamine agonist.

Among 22 patients, DNA of one patient was not available for *GNAS* and *AIP* mutation screening. Somatic activating *GNAS* mutations (p.R201C, p.R201H, and p.Q227L) were found in nine out of 21 patients (42.8%). Only one patient harbored somatic *AIP* p.R304X mutation (4.7%) and it was not associated with *GNAS* mutation.

SAGE analysis and selection of genes to validation by real-time PCR

Data of SAGE libraries constructed from samples of normal pituitary and GH-secreting pituitary tumor were included in the Genome Data Mining Website for normal pituitary cDNA library, <http://gdm.fmrp.usp.br/h2g/library/695> and for GH pituitary tumor cDNA library, <http://gdm.fmrp.usp.br/h2g/library/1145> of the Molecular Genetics and Bioinformatics Laboratory of the Department of Genetics of School of Medicine of Ribeirao Preto, University of Sao Paulo. The libraries analysis were performed using a web-based tool S3T (Score System for Sequence Tags) available for free at <http://gdm.fmrp.usp.br/s3t/> (19). The analysis of normal and adenoma libraries identified 21 030 and 22 329 unique SAGE tags respectively. Computer-generated genomic analysis tools (<http://gdm.fmrp.usp.br>) identified 13 722 exclusive genes in normal pituitary and 14 993 in somatotroph adenoma cDNA library. Both libraries shared 6497 genes, 2188 under-expressed and 4309 overexpressed in tumoral library. Of interest, *PTTG* gene was not expressed in tumor library generated by the present SAGE. Differential expression of genes related to cell adhesion, cell differentiation, cell cycle, and apoptosis were also demonstrated between

normal pituitary tissue and GH-secreting pituitary tumors (Table 2).

In the normal library, SAGE also showed overexpression of *POMC* (62-fold), *LH β* subunit (54-fold), *TSH* (24-fold), *PRL* (fivefold), and exclusive *FSH β* subunit expression compared with tumoral library. On the other hand, GH-secreting adenoma library showed higher expression of *GH2* gene (3.2-fold).

In addition, SAGE data revealed 33 under- and 14 overexpressed RP tags in tumors. Among the underexpressed RP genes, five of them – *RPSA*, *RPS3*, *RPS7*, *RPS14*, and *RPS29* – were selected for validation by real-time PCR based on Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com). We observed underexpression of *RPSA* (–1.4-fold; $P=0.004$), *RPS3* (–1.8-fold; $P=0.03$), *RPS14* (–1.8-fold; $P=0.001$), and *RPS29* (–2.9-fold; $P=0.002$). *RPS7* expression showed only a tendency to underexpression (–1.2-fold; $P=0.08$) in GH-secreting pituitary tumor compared with normal pituitaries samples (Table 3).

We also observed no difference between *SSTR2* expression by real-time PCR in GH-secreting pituitary tumors and normal pituitaries ($P=0.4$). However, *SSTR2* overexpression in tumoral samples was associated with disease control after treatment with SRL ($P=0.01$).

There was no relationship between *RPSA*, *RPS3*, *RPS14*, and *RPS29* expression and the presence of *GNAS* mutation, IGF1 or GH levels, tumor size, exclusive GH staining on IHC, remission after surgery, or disease control with SRL therapy. There was no correlation between RP gene expression and *SSTR2* expression.

Discussion

This study reports the first SAGE library from normal pituitary tissue and GH-secreting pituitary tumor. SAGE data generated will be available at Gene Expression Omnibus website (<http://www.ncbi.nlm.nih.gov/geo/>) and will be accessed through GEO accession GSM 912557 (somatotrophinomas) and GSM912556 (normal pituitary). Our entire data will be available at this database, providing information on differently expressed genes in somatotroph adenomas for further research.

Until now, most available information on the pituitary tumor transcriptome was based on microarray studies (20, 21). SAGE has some advantages over microarrays because it allows detection of all expressed sequences in a tissue, including those not previously described (13). Similar to microarray, there is limitation in using SAGE method in order to compare the monoclonal somatotroph adenoma and the normal pituitary, which contains various cell types. The fact that normal pituitary tissue is composed of more than 50% somatotrophic cells might, however, decrease this limitation. It is also important to point out that

all high-throughput methods have exclusive advantages and limitations. Therefore, integrating different data sets will provide complementary knowledge on pituitary tumorigenesis.

The molecular profile of pituitary tumor exhibited several similarities and some differences compared with previously published data using microarray. Genes related to pituitary physiological hormone secretion, such as *POMC*, *LH β* subunit, *TSH*, and *PRLm* were differently expressed in normal tissue library. On the other hand, GH-secreting pituitary adenoma library showed higher expression of *GH2* gene (3.2-fold). Morris *et al.* (21), using microarray, also found that *GH2* as well as GH-releasing hormone receptor (*GHRHR*) genes were overexpressed in GH pituitary tumor tissue. Interestingly, *PTTG* gene was not expressed in tumor library generated by the present SAGE and, in the microarray study (21), its expression was similar in normal pituitary and GH-secreting tumors. These data diverge from those previously reported by comparative RT-PCR, which demonstrated ubiquitous and prevalent expression of *PTTG* in pituitary tumors, mainly in invasive hormone-secreting tumors (3).

In this study, differential expression of genes related to cell adhesion, cell differentiation, cell cycle, and apoptosis were also demonstrated between normal pituitary tissue and GH-secreting pituitary tumors by SAGE. These findings have not yet been validated. However, they introduce new perspectives for further studies on molecular genesis of somatotrophinomas. Additional experiments and functional studies are required to confirm these data and the involvement of these signaling pathways in GH-secreting pituitary tumors.

SAGE also identified 33 under- and 14 overexpressed RP genes in the adenoma library, which might be involved in the poorly understood pathogenesis of GH-secreting pituitary tumors, as many extra-ribosomal functions in other cellular processes have been suggested. Indeed, RP gene mutations or altered expression have been found in many human tumors (22, 23). In this study, based on Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com), we chose for validation five of them – *RPSA*, *RPS3*, *RPS7*, *RPS14*, and *RPS29* – all involved in cell cycle regulation, apoptosis, and DNA repair. We validated, by real-time PCR, four of the five RP genes studied. Although the role of RPs in pituitary tumorigenesis has never been proposed, research at seed site analysis (<http://www.ncbi.nlm.nih.gov/geo/>) enables us to find a differential expression profile of all these RPs in four pituitary adenoma subtypes (GH, *PRL*, *ACTH*-secreting pituitary adenomas, and nonfunctioning pituitary adenoma), reinforcing the putative role of RPs in pituitary tumor pathogenesis (21).

RP genes have been identified in human diseases, especially in genetic disorders (24). Recently,

Table 2 Under- and overexpressed genes in GH-secreting pituitary adenoma library compared with normal pituitary library, obtained by SAGE and involved in functional pathways. Under- and overexpressed genes described in this table refer to fold change inferior to -2.5 and superior to 2.5 respectively.

SAGE tag	Unigene	Gene symbol	Gene description	Fold change
Apoptotic pathways				
GCAGGGTGGG	Hs.631535	<i>AKT2</i>	V-akt murine thymoma viral oncogene homolog 2	-5.02
TCACTGTGGG	Hs.372331	<i>SPTAN1</i>	Spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)	-4.02
AAGCATTAAG	Hs.520851	<i>PRKAR1B</i>	Protein kinase, cAMP-dependent, regulatory, type I, beta	-3.52
AAAATAAAGA	Hs.73722	<i>APEX1</i>	APEX nuclease (multifunctional DNA repair enzyme) 1	-3.52
CCTGCCCCCC	Hs.861	<i>MAPK3</i>	Mitogen-activated protein kinase 3	-3.01
AAAAAGCAGA	Hs.443914	<i>SOD1</i>	Superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1)	-2.34
AAGCCTTGCT	Hs.444356	<i>GRB2</i>	Growth factor receptor-bound protein 2	2.99
CTGCTGCACT	Hs.271510	<i>GSR</i>	Glutathione reductase	3.98
CCAGTGCACT	Hs.220950	<i>FOXO3</i>	Forkhead box O3	3.98
TGAATCTGGG	Hs.436687	<i>SET</i>	SET translocation (myeloid leukemia associated)	4.98
Cell differentiation pathways				
CCTAAGGCTA	Hs.108371	<i>E2F4</i>	E2F transcription factor 4, p107/p130 binding	-3.01
TATGACTTAA	Hs.413812	<i>RAC1</i>	Ras-related C3 botulinum toxin substrate 1	-2.58
CTCTGAGGTA	Hs.435714	<i>PAK1</i>	P21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	-2.51
TGGCTGGCCA	Hs.654394	<i>CSF1R</i>	Colony stimulating factor 1 receptor, (v-fms) oncogene homolog	2.99
Cell adhesion pathways				
AAAGGGTCAC	Hs.21422	<i>NRCAM</i>	Neuronal cell adhesion molecule	-8.04
TGTGGCGTAT	Hs.556600	<i>MYLK</i>	Myosin light chain kinase	-4.02
TTGCTGACTT	Hs.474053	<i>COL6A1</i>	Collagen, type VI, alpha 1	-3.52
TGGCCTAATA	Hs.1501	<i>SDC2</i>	Syndecan 2	-3.45
TGTTTATATT	Hs.49774	<i>PTPRM</i>	Protein tyrosine phosphatase, receptor type, M	-3.01
TGAAAACACTAC	Hs.347270	<i>HLA-DPA1</i>	Major histocompatibility complex, class II, DP alpha 1	-3.01
GCATTGCTCA	Hs.365689	<i>CADM3</i>	Cell adhesion molecule 3	-3.01
TCTTCGTCCT	Hs.493919	<i>MPZL1</i>	Myelin protein zero-like 1	-3.01
TAATATTTTT	Hs.270291	<i>ACTN4</i>	Actinin, alpha 4	-3.01
GTGGAGAAAA	Hs.694732	<i>ITGA4</i>	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	-3.01
CAGATTGCTG	Hs.705383	<i>PPP1CB</i>	Protein phosphatase 1, catalytic subunit, beta isoform	-2.51
GACCGCAGGA	Hs.17441	<i>COL4A1</i>	Collagen, type IV, alpha 1	2.99
CAAGAGTTTT	Hs.503878	<i>NCAM1</i>	Neural cell adhesion molecule 1	2.99
TTTGTTTTTC	Hs.489142	<i>COL1A2</i>	Collagen, type I, alpha 2	2.99
TCAAAAAAAA	Hs.445827	<i>COL5A2</i>	Collagen, type V, alpha 2	3.65
TGGAAATGAC	Hs.172928	<i>COL1A1</i>	Collagen, type I, alpha 1	4.98
CTCCAATAAA	Hs.471014	<i>TLN1</i>	Talin 1	4.98
GGGCGGAGCT	Hs.632717	<i>MYL6</i>	Myosin, light chain 6, alkali, smooth muscle and non-muscle	6.97
ACCCTCTTCC	Hs.181244	<i>HLA-A</i>	Major histocompatibility complex, class I, A	12.94
Cell cycle pathways				
CTTAATCTTG	Hs.519162	<i>BTG2</i>	BTG family, member 2	12.06
TGAGAGGGTG	Hs.74405	<i>YWHAQ</i>	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHA) theta polypeptide	-6.03
GAATTAACAT	Hs.513851	<i>YWHAH</i>	(YWHA) epsilon polypeptide	-4.47
AACTCCAGT	Hs.110571	<i>GADD45B</i>	Growth arrest and DNA damage inducible, beta	-4.02
AAAGTCTAGA	Hs.523852	<i>CCND1</i>	Cyclin D1	-3.01
TAATTAAGAAG	Hs.40582	<i>CDC14B</i>	CDC14 cell division cycle 14 homolog B (<i>Saccharomyces cerevisiae</i>)	-3.01
CCTAAGGCTA	Hs.108371	<i>E2F4</i>	E2F transcription factor 4, p107/p130 binding	-3.01
TCAATCAAGA	Hs.226755	<i>YWHAH</i>	(YWHA) eta polypeptide	-2.68
TGCATATCAT	Hs.370770	<i>XPO1</i>	Exportin 1 (CRM1 homolog, yeast)	2.99
GCTGAAGGGG	Hs.483635	<i>FGF1</i>	Fibroblast growth factor 1 (acidic)	2.99
CTGCACTTAC	Hs.438720	<i>MCM7</i>	Minichromosome maintenance complex component 7	3.48
AATGTGAGTC	Hs.520974	<i>YWHAH</i>	(YWHA) gamma polypeptide	3.98
CGTCCCGGAG	Hs.654838	<i>MAD1L1</i>	MAD1 mitotic arrest deficient-like 1 (yeast)	3.98
TCCTGCTGGC	Hs.149957	<i>RPS6KA1</i>	Ribosomal protein S6 kinase, 90 kDa, polypeptide 1	3.98
GACAAGGTTC	Hs.279562	<i>MYT1</i>	Myelin transcription factor 1	5.97
CCCTCCTCTC	Hs.534307	<i>CCND3</i>	Cyclin D3	6.97

Table 3 Ribosomal protein genes in GH-secreting adenoma and normal pituitary cDNA libraries evaluated by SAGE and validated by qPCR. Frequencies A and B correspond to the number of each tag, obtained by SAGE, in normal and GH-secreting pituitary cDNA libraries, respectively, and normalized to 300 000 total tags.

SAGE tag	Unigene	Gene	Frequency A (normal library)	Frequency B (tumor library)	Fold change (SAGE)	Normal pituitary ($2^{-\Delta\Delta Ct}$)	Tumor ($2^{-\Delta\Delta Ct}$)	Fold change (qPCR)
GAAAAATGGT	Hs.449909	<i>RPSA</i>	963	434	−2.2	1.55 ± 0.82 ; 0.78–3.26	0.88 ± 0.44 ; 0.29–2.09	−1.4 ($P=0.004$)
GAGAGTGGGG	Hs.546286	<i>RPS3</i>	387	291	−1.3	1.28 ± 0.70 ; 0.47–3.17	0.72 ± 0.60 ; 0.17–2.81	−1.8 ($P=0.03$)
TCGTCTTTAT	Hs.546287	<i>RPS7</i>	99	19	−5.0	1.16 ± 0.69 ; 0.26–2.93	0.79 ± 0.38 ; 0.25–1.64	−1.2 ($P=0.08$)
TAAACTGTTT	Hs.381126	<i>RPS14</i>	114	34	−3.3	1.28 ± 0.69 ; 0.59–3.10	0.73 ± 0.52 ; 0.15–2.29	−1.8 ($P=0.001$)
ATAATTCTTT	Hs.156367	<i>RPS29</i>	1474	528	−2.8	1.52 ± 0.78 ; 0.41–3.30	0.81 ± 0.89 ; 0.09–3.78	−2.9 ($P=0.002$)

The results for gene expression ($2^{-\Delta\Delta Ct}$) in normal pituitaries and in tumors are expressed as median $\pm$ s.d. and range.

Ebert *et al.* (25) provided evidence that *RPS14* is a causal gene for the 5q-syndrome. The ribosomal gene *RPS14* is required for the maturation of 40S ribosomal subunits and maps to the deleted region in the 5q-syndrome, suggesting that this syndrome represents a disorder of aberrant ribosome biogenesis (26). However, it is conceivable that other genes (on 5q or elsewhere) collaborate with *RPS14* to cause the disease phenotype.

RPS29, which is also underexpressed in our study, is a mediator of cell death. The overexpression of *RPS29* cDNA by transfection into thymocytes and HeLa cells induce cell shrinkage, chromatin condensation, plasma membrane alteration, nuclear fragmentation, and a distinct internucleosomal DNA ladder, typical of apoptosis (27). Downregulation of the expression of apoptosis inhibitor proteins BCL2, BCL-XL, and survivin and upregulation of pro-apoptotic p53 and Bax was also observed, suggesting a role of *RPS29* in apoptosis modulation (28).

Besides *RPS14* and *RPS29*, we also observed underexpression of *RPSA* and *RPS3* and a tendency of underexpression of *RPS7* in somatotrophinomas compared with normal pituitaries. Mammalian cells respond to DNA damage signals by activating cell cycle checkpoints and DNA repair systems, or inducing apoptosis (29). *Drosophila* *RPS3* has been shown to have an enzymatic activity that cleaves DNA containing 8-oxoguanine residues and an associated apurinic and apyrimidinic (AP) lyase activity that cleaves phosphodiester bonds (30). The AP endonuclease activity is inseparable from activities acting upon u.v.-irradiated DNA, which cleaves a phosphodiester bond within a cyclobutane pyrimidine dimer. Moreover, these activities are apparently associated with *RPS3* (31). The ectopic expression of *RPS3* protein induces programmed cell death through the activation of caspase-8 and caspase-3, indicating that *RPS3* itself is a major determinant of cell fate (32).

Epigallocatechin-3-O-gallate (EGCG), found in green tea, has been shown to have cancer preventive activity

in a variety of human organ sites (33). The inhibitory effect of EGCG on tumor cell proliferation seems to be exerted by its binding to *RPSA* (or 67 kDa laminin receptor) (34, 35). In addition, RNAi-mediated silencing of *RPSA* results in abrogation of EGCG-induced apoptosis in myeloma cells by TNF α , activated Fas, and IFN γ , which are mediated by DAPK2, a key regulatory step between the formation of death-inducing signaling complex leading to the activation of caspases (36) and p53 pathway (37). The induction of p53-like proteins (p73 and p63) provides an important alternative mechanism of cell growth arrest in the absence of p53. These proteins are not only involved in the induction of apoptosis genes but also regulate genes responsible for cell cycle arrest and DNA repair. In addition, *RPSA* leads to the induction of CDK inhibitors, such as p16 and p18, which can induce cell cycle arrest by preventing retinoblastoma phosphorylation (38). Finally, eukaryotic translation elongation factor 1 alpha 1 (eEF1A), which is activated by *RPSA* (39) and was also underexpressed in our data, is an important component of the eukaryotic translation apparatus and is indispensable for mediating anticancer activity of EGCG. Therefore, the underexpression of *RPSA* and *eEF1A* in the GH-secreting pituitary adenomas suggest that these proteins might determine the efficiency of cell proliferation and a decrease in apoptosis control, probably contributing to pituitary cancer development.

As a major negative regulator of p53, murine MDM2 or its human analog HDM2 oncogene plays an important role in carcinogenesis and tumor progression. By catalyzing the ubiquitination of p53, MDM2 promotes p53 protein degradation and, in turn, MDM2 binds to the transcriptional activation domain of p53 and blocks its transactivation activities (40). Several subunit proteins of the ribosome, L5, L11, L23, S3, and S7, bind to MDM2, thus stabilizing p53 and rescuing its transactivational activities (41, 42, 43). More recently, *in vitro* and *in vivo* data suggest that

Figure 1 Summary of the possible interactions of ribosomal and other proteins involved in the control of cell cycle arrest based on Ingenuity Pathway Analysis. *RPS3* expression results in apoptosis initiated by activated Fas/FasL and mediated by DAPK2, leading to the activation of caspases and also by p53 pathway. It can also induce p53-like proteins (p73 and p63). The induction of cyclin-dependent kinase (CDK) inhibitors p16 and p18 also induce cell cycle arrest by preventing retinoblastoma phosphorylation. Eukaryotic translation elongation factor 1 alpha 1 (eEF1A) is also an important component of the eukaryotic translation apparatus and is indispensable for cell cycle arrest. The expression of *RPS3* protein induces programmed cell death through the activation of caspase-8 and caspase-3. In addition, as a major negative regulator of p53, MDM2 and its interaction with *RPS3* protect p53 from MDM2-mediated ubiquitination. S7, a protein of the small subunit of the ribosome, has the capacity to stabilize p53 by binding to MDM2. Ribosomal protein S29 induces downregulation of the expression of inhibitors of apoptosis proteins BCL2, BCL-XL, and survivin and upregulation of pro-apoptotic p53 and Bax. Therefore, the underexpression of *RPSA* and eEF1A, *RPS3* and S7, and S29 collaborate directly or indirectly with other genes to cause alterations on cell cycle arrest, DNA repair, and apoptosis, affecting the genomic integrity.

at least one biological end point of the interaction of *RPS3* with p53 and MDM2 is to protect p53 from MDM2-mediated ubiquitination (44, 45). In addition, *RPS7*, another protein of the small subunit of the ribosome, has the capacity to stabilize p53 by binding to MDM2 (42). Therefore, the underexpression of *RPS3* and *RPS7*, which act as a scaffold for the additional binding of MDM2 and p53, would affect the genomic integrity facilitating GH-secreting pituitary adenoma development. Indeed, the *RPS3* is located at 11q13.3–q13.5, a locus where *AIP*, a pituitary adenoma susceptibility gene, has been identified. Germline mutations in *AIP* cause a low-penetrance pituitary adenoma predisposition, with particularly high relative risk of GH-secreting pituitary adenomas. As *AIP* identification, a number of single-exon and partial/whole-gene deletions detected in several tumor suppressor genes have been reported to cause hereditary tumor susceptibility (7). *AIP* as well as *MEN1* gene are located from 61 897K to 64 592K, and *RPS3*

is closely located at 72 424K on 11q.13; therefore, the underexpression of *RPS3* gene could modulate *AIP* and/or *MEN1* genes or similar to them might act as a tumor suppressor gene.

In conclusion, we report the first SAGE libraries from normal pituitary tissue and GH-secreting pituitary tumor, which provide a quantitative assessment of the cellular transcriptome. Differential expression of genes related to cell adhesion, cell differentiation, cell cycle, and apoptosis were demonstrated by SAGE, introducing new perspectives for further studies. We validated some underexpressed genes that encode RPs. The data observed in this sample suggest that the underexpression of the studied RP genes possibly collaborates directly or indirectly with other genes, as summarized in Fig. 1, to modify cell cycle arrest, DNA repair, and apoptosis, leading to an environment that might have a putative role in the somatotrophinoma tumorigenesis.

Declaration of interest

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

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