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ORIGINAL ARTICLE

Alternative splicing of placental lactogen (CSH2) in somatotroph pituitary adenomas

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Abstract

OBJECTIVES: Somatotroph adenomas secrete supraphysiological amounts of GH, causing acromegaly. We have previously shown epithelial splicing regulator 1 (ESRP1) to play a role in epithelial mesenchymal transition (EMT) progression in these adenomas and account for poor treatment response. We evaluated if the mRNA levels of the GH/CSH gene cluster in somatotroph adenomas are associated with an epithelial phenotype and response to SA treatment.
METHODS: We investigated the associations between *ESRP1* and the growth hormone/chorionic somatomammotropin (*GH/CSH*) gene cluster by RNA sequencing (RNAseq). *CSH2* isoform 3 mRNA was further evaluated in 65 somatotroph adenomas and associations with disease severity and treatment response.
RESULTS: mRNA for all genes in the *GH/CSH* cluster were expressed, however, only *chorionic somatomammotropin 2/placental lactogen 2* (*CSH2*) displayed an alternative splicing pattern. *CSH2* isoform 3 was associated with a dense granulation pattern and an epithelial phenotype with high levels of *ESRP1* and *E-cadherin* expression. Further, *CSH2* isoform 3 was associated with reduced serum GH and IGF-I levels after somatostatin analog treatment.
CONCLUSIONS: Attenuated *CSH2* isoform 3 was associated with mesenchymal phenotype and a blunted clinical response to somatostatin analog treatment in patients with acromegaly.

Abbreviations:

CDH1	- E-cadherin
CSH	- Chorionic somatomammotropin
EMT	- Epithelial mesenchymal transition
ESRP1	- Epithelial splicing regulator 1
FDR	- False discovery rate
GH	- Growth hormone
IGF	- Insulin growth factor
PL	- Placental lactogen
SA	- Somatostatin analog

INTRODUCTION

In humans the genes for growth hormone and placental lactogen are clustered on chromosome 17 (q22-24) containing the five related genes; the growth hormone 1 gene (*GH1*), the chorionic somatomammotropin (human placental lactogen, hPL) genes (*CSH1* and *CSH2*), placental GH gene (*GH2*), and a CSH-like gene (*CSHL1*) (Figure 1). GH1 is reported to be produced by somatotrophs in the pituitary while *GH2*, *CSH1*, *CSH2* and *CSHL1* are produced by the syncytiotrophoblasts in the placenta (Day *et al.* 2004). hPL is structurally and functionally similar to GH1 with 80–90% sequence homology, but they activate the prolactin receptor and have a lower affinity for the GH receptor, in contrast to GH1 (Walsh & Kossiakoff 2006). Hormones are mainly secreted into the bloodstream and have biological actions in a wide variety of target tissues (Handwerger & Freemark 2000), but local, paracrine or autocrine actions of hormones independently of their systemic effects have also shown to be important (Corbacho *et al.* 2002).

Alternative splicing produces multiple isoforms of the genes in the GH/CSH cluster (Mannik *et al.* 2012; Zhan *et al.* 2005). *CSH2* consists of three different isoforms, where isoform 1 has 5 exons, isoform 2 contains the intron between exon 4 and 5, while isoform 3 lacks exon 3 and 4. hPL is produced by the placenta during pregnancy and increases during the last trimester. Its major function is to modulate maternal carbohydrate and lipid metabolism (Handwerger & Freemark 2000). Expression of hPL has been reported in breast, ovarian and testicular malignant tumors (Fukunaga & Ushigome 1993; Monteiro *et al.* 1983; Sesterhenn & Davis, Jr. 2004; Tuttle *et al.* 2014) and circulating levels have been associated with poor outcome (Horne *et al.* 1976; Sheth *et al.* 1977).

Epithelial splicing regulator (ESRP) 1 and -2 have been proposed as master regulators of epithelial mesenchymal transition (EMT) and are down regulated in cells undergoing EMT by changing the network of alternative splicing and thereby contributing to EMT (Tavanez & Valcarcel 2010; Warzecha *et al.* 2009; Warzecha *et al.* 2010). We recently found the *CSH2* gene to be expressed and alternatively spliced between pituitary adenomas with different levels of ESRP1 expression, from patients with acromegaly (somatotroph adenomas). To further explore the CSH2 and GH/CSH gene cluster we 1) investigated the levels of the isoforms in the GH/CSH gene cluster by mRNA sequencing (RNAseq), 2) investigated if alternatively spliced isoforms were correlated with EMT progression and clinical indices of disease activity and treatment response.

MATERIAL AND METHODS

Patients and samples

Sixty-five patients with acromegaly were included, of whom 38 were treatment naïve and 27 treated with a somatostatin analogue (SA) before transsphenoidal adenomectomy in the period 1996–2011 (Lekva *et al.* 2013). Median treatment duration with SA was 6 months (range 2–32 months). Table 1 provides an overview of the study population. The study was approved by the Regional Committee for Medical and Research Ethics, South-East, Norway and conducted according to the Declaration of Helsinki II. Written informed consent was obtained from all patients.

Biochemical measurements

Blood samples were drawn after an overnight fast and serum isolated. Serum insulin growth factor 1 (IGF-I) was measured by RIA (Nichols Institute, Nijmegen, The Netherlands) or Immulite 2000 (Siemens, Munich, Germany) calibrated to the WHO standard IS 87/518, and mean daytime (3–5 times) GH (detection limit 0.3 mU/l), by AutoDelfia (Wallac Oy, Turku, Finland) and after 2005 Immulite 2000 (Siemens) calibrated to the WHO standard IS 98/574. When the methods were changed, cross-calibration was performed, as described previously (Fougner *et al.* 2008). An acute somatostatin test was performed in 59 patients prior to any treatment, measuring the reduction in serum GH concentration 2–4 hours after the test dose. Mean GH values before injection were compared with mean GH levels measured after injection and the percentage reduction of GH calculated for each patient (Fougner *et al.* 2010).

Tumor volume and invasiveness

Based on data from a previously publication (Lekva *et al.* 2013), an experienced neuroradiologist estimated tumor volume with the formula width x height x length x 0.5. For each tumor, the SIPAP (suprasellar, infrasellar, parasellar, anterior, and posterior) grading score was determined, as previously reported (Fougner *et al.* 2010). In relation to patients treated primarily with SA, MR scans were available for 26 patients before and after SA treatment calculating tumor reduction.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue blocks were available for 57 of the 65 adenomas. For granulation status, immunohistochemical analyses with a CAM5·2 antibody (catalogue No. 345779; Becton Dickinson, San Jose, CA, USA) were performed on whole sections from formalin-fixed, paraffin-embedded tissue blocks from all 57 somatotroph adenomas using the Dako EnVision Flex+ System (K8012; Dako, Glostrup, Denmark) and Dako Autostainer, results previously reported (Fougner *et al.* 2012). Tumors were classified semiquantitatively based on the criteria by Obari *et al.* (Obari *et al.* 2008) as: (i) Densely granulated (DG) when perinuclear pattern cells were over 70% and dot-like pattern cells were <10% irrespective of the percentage of transitional pattern cells, (ii) Sparsely granulated (SG) when dot-pattern cells were over 70% irrespective of the percentage of perinuclear and transitional pattern cells and (iii)

transitional type when not fitting into the above two categories. Adenoma classification was performed by two independent pathologists blinded for the clinical data (Fougner *et al.* 2012).

RNA isolation and real time RT-qPCR

Extraction of total RNA was performed using Trizol (Invitrogen, Carlsbad), as previously reported (Lekva *et al.* 2013). RNA was purified using QIAGEN RNeasy micro kit (Qiagen, Valencia, CA). The integrity was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and concentrations determined by OD readings on a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies,Wilmington, DE). Reverse transcription was performed using a High-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantification of mRNA was performed using the standard curve method of the ABI Prism 7500 (Applied Biosystems). For real-time RT-PCR, sequence-specific exon-exon spanning oligonucleotide primers for *CSH2* isoform 3 (forward primer: 5`-CGTTATCCAGGCTTTTTGACCA-`3 and reverse primer: 5`-TCTTCCAGCCTAAACTCCTGGTAG-`3) were designed using Primer Express software version 2.0 (Applied Biosystems). Transcript expression was normalized to GAPDH mRNA levels in human pituitary samples and expressed as relative mRNA levels (data was log or square root transformed to normal distribution in human pituitary adenomas). We were unable to make specific primers for *CSH2* isoform 1 because of the strong sequence similarity between this isoform and *CSH1* and different isoforms of *GH1/2*.

RNAseq

The study is based on data from a previous publication (Lekva *et al.* 2013). Six adenomas with no pre-treatment were selected based on low (n=3) and high (n=3) *ESRP1* levels in both microarray and RT-qPCR analyses. Sequencing libraries were prepared from 500 ng of total RNA using TruSeq RNA sample prep reagents (Illumina) according to manufacturer's instructions, with fragmentation for 4 min at 94 °C. The libraries were sequenced by 100 bp paired-end sequencing on an Ilumina HiSeq 2000. We obtained an average of 23.6 million (range 22.3–25.1) paired reads per sample. Sequence reads were mapped to the reference genome (hg19) with Bowtie (Langmead *et al.* 2009) and TopHat (Trapnell *et al.* 2009), and transcripts quantified using Cufflinks (Trapnell *et al.* 2012). CuffDiff was used to calculate the differential expression between high and low ESRP1 groups at gene and transcript levels, and identify alternative splicing. Due to excess expression levels of *GH1* in the previous RNAseq analysis (Lekva *et al.* 2013), we have reanalyzed our data, setting the value of max reads to a higher number (max-bundle-frags 10 000 000) due to a HIDATA error for the GH1 gene in initial analysis. The R package CummerBund (http://compbio.mit.edu/cummeRbund/) and the Integrative Genome Viewer (IGV) were used for visualization (Robinson *et al.* 2011).

Statistics

Differences in patient demographics and circulating CSH2 levels were analyzed by Mann-Whitney U-test. The transcripts analyzed in all patients by RT-qPCR were not normally distributed and were transformed (log or square root) prior to statistical analysis. For analyzing differences in transcript levels *in vivo* and *in vitro*, unpaired T-tests were used for two groups while one-way ANOVA with bonferroni adjusted post-hoc tests were used when comparing 3 groups. A two-sided p-value <0.05 was considered significant. Differentially expressed genes, isoforms and splicing between groups from RNAseq were identified using CuffDiff according to Trapnell *et al.* (2012). Cufflinks and Cuffdiff implement a linear statistical model to estimate an assignment of abundance to each transcript that explains the observed reads with maximum likelihood. The false discovery rate (FDR) was set to 5% and used to adjust p-values for each test after Benjamini-Hochberg correction for multiple-testing. The proportion of the isoforms was calculated based on the average of the six samples from RNAseq.

RESULTS

Clinical characteristics of the study population are given in Table 1.

GH/CSH gene cluster isoforms in somatotroph pituitary adenomas

Figure 1A shows the location of the genes in the GH/CSH cluster, with the number of isoforms ranging from 1 to 5. Figure 1B shows the abundant expression levels of *GH1* compared to the other mRNA species within the cluster. The proportion of isoforms were as follows; *GH1*, isoform 1 (86.6%) > isoform 2 (9.8%) > isoform 3 (3.3%) > isoform 4 (0.2%). *GH2*, isoform 4 (99.9%) > isoform 2 (0.1%) while no expression of isoform 1 and 3. *CSH2*, isoform 3 (50.0%) > isoform 1 (47.5%) > isoform 2 (2.5%). *CSHL1* was expressed at low levels; isoform 3 (75.4%) > isoform 1 (24.6%), with no expression of isoforms 2 and 4. *CSH1* has only one isoform. Figure 1C shows the distribution of isoforms within the GH/CSH cluster according to expression levels of *ESRP1*. *CSH2* isoform 1 and 3 where alternatively spliced (p=0.002, 5% FDR) in tumors characterized by low and high expression levels of the splicing regulator *ESRP1*, respectively, as previously reported (Lekva *et al.* 2013), while there was no significant difference in distribution of isoforms for *GH1, GH2, CSH1* or *CSHL1*. Differential transcription of the *CSH2* gene between adenomas with low and high *ESRP1* expression, modified from IGV, is shown in Figure 1D. Figure 1E shows the RNAseq analysis of the 3 isoforms of *CSH2* in low and high

Tab. 1. Demographics of the study population.

Groups	Total (n=65)	Direct surgery (n=38)	Preoperative SA (n=27)
Age (yr)	47 (41, 57)	49 (42, 56)	47 (37, 60)
Women/men (n)	28/37	16/22	12/15
Tumor size (cm^3)	1.21 (0.54, 3.12)	0.68 (0.46, 2.08)†	2.19 (0.81, 5.40)
Granulation status (densely/transition/sparsely)	25/28/10	16/13/7	9/15/3
Biochemistry			
Serum GH (mU/L)	32 (20, 70)	28 (15, 46)†	53 (24, 108)
Serum IGF-I (nmol/L)	106 (83, 131)	96 (83, 125)	109 (90, 139)
SA response			
GH reduction (%) n= 59#	88 (69, 92)	84.2 (62, 90)	89 (69, 95)
IGF-I reduction (%) n=27*			54 (12, 62)
GH reduction (%) n=27*			73 (41, 95)
Tumor size reduction (%) n= 26*			26 (5, 43)

Unless stated, data are given as median (25th, 75th percentile). †$p<0.05$ vs. Preoperative SA.
During acute octreotide test in patients not treated preoperatively with SA;
*After median 6 months (range 2-32 months) preoperative SA treatment.

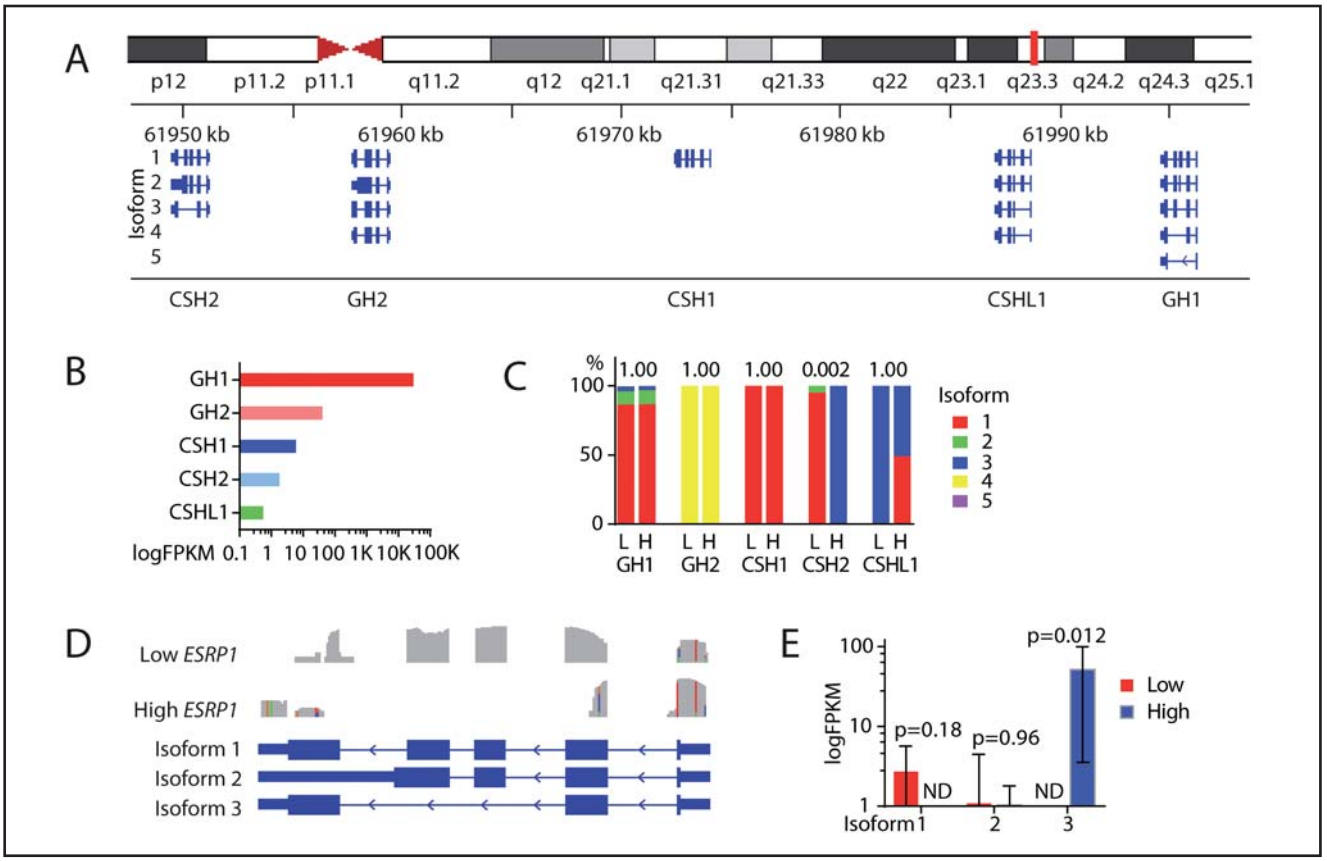

Fig. 1. RNAseq analysis. Six adenomas with no SA treatment characterized by low (n=3) and high (n=3) *ESRP1* expression, based on microarray and qPCR analyses, were selected for RNAseq analysis, as previously reported. A, Presentation of the genes in the GH/CSH gene cluster on chromosome 17. Modified from IGV, human hg19. B, The expression levels of the genes from the six adenomas in the GH/CSH gene cluster presented as log fragments per kilobase of exon per million fragments mapped (FPKM) values from the RNAseq analysis. C, Proportion of the isoforms of the genes in the GH/CSH gene cluster based on low (L) and high (H) *ESRP1* expression. Numbers represents *p*-values (5% FDR) of alternative splicing of the genes between adenomas with low and high *ESRP1* expression. D, Differential transcription of the *CSH2* gene between adenomas with low and high ESRP1 expression, modified from IGV. Upper panel shows read depth for each exon. E The three isoforms of *CSH2* presented between low and high ESRP1 expression groups by FPKM values from RNAseq analysis (modified from the R package CummeRbund).

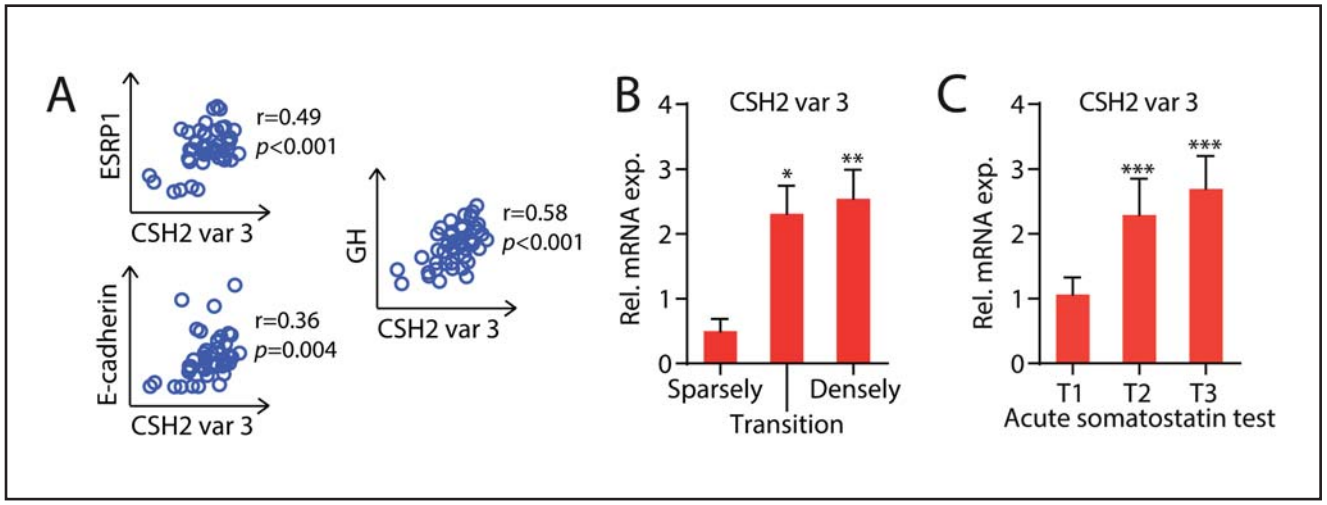

Fig. 2. *CSH2* isoform 3 validated by qPCR and associated with disease severity and SA treatment response. A, Correlation matrix between mRNA expression of *CSH2* isoform 3 and mRNAs of ESRP1, E-cadherin and GH in the entire cohort (n= 65) measured by qPCR and expressed as relative mRNA levels normalized to GAPDH. B, The mRNA expression of *CSH2* isoform 3 divided by the granulation pattern (sparsely, densely, and intermediate) of the adenomas (n=61). C, The mRNA expression of *CSH2* isoform 3 divided by tertiles (T1-low, T2-medium, T3-good response) of acute somatostatin analog test (n=57). **p*<0.05, ***p*<0.01, ****p*<0.001.

ESRP1 expression groups by fragments per kilobase of exon per million fragments mapped (FPKM, modified from CummeRbund). Significantly higher FPKM levels were observed for isoform 3 comparing high and low ESRP1 expression.

CSH2 isoform 3, disease severity and treatment response

We next measured CSH2 isoform 3 mRNA levels in adenomas from our total population by RT-qPCR and identified clinical and molecular determinants including verifying the association with *ESRP1* mRNA levels as identified by the RNAseq analysis. As shown in Figure 2A, CSH2 isoform 3 mRNA levels were correlated with several features characterizing EMT progression. Patients with high mRNA levels of *CSH2* isoform 3 were characterized by higher mRNA expression of *ESRP1* (n=63), *E-cadherin* (n=63) and *GH* (n=46), and more frequently a dense granulation pattern (Figure 2B, n=61, $p<0.001$), all indices of an epithelial phenotype, not committed to EMT progression. No association was found with age or gender and these factors did not impact the associations above. Further, there was no association between *CSH2* isoform 3 mRNA levels and tumor size (n=62, r=–0.19, $p=0.15$) and SIPAP score (n=54, r=–0.14, $p=0.31$).

The main goal of treating acromegaly is to normalize GH and IGF-I levels and to control tumor volume. We found that enhanced *CSH2* isoform 3 mRNA levels were correlated with reduced circulating GH (n=27, r=0.39, $p=0.042$) and IGF-I (n=27, r=0.40, $p=0.042$) in patients pretreated with SA. As shown in figure 2C, an increased response to the acute somatostatin analog test was observed in patients with high *CSH2* isoform 3 mRNA expression (n=57). No association with tumor size reduction (n=26, r=0.32, $p=0.11$) was observed.

DISCUSSION

In this study we investigated splicing patterns of genes in the GH/CSH gene cluster in somatotroph adenomas from patients with acromegaly. Our main findings were *i)* All the genes in the GH/CSH gene cluster were expressed in the somatotroph adenomas *ii)* An alternative splicing pattern was found for *CSH2* dependent on *ESRP1* mRNA abundance and elevated mRNA levels of isoform 3 was associated with indices of an epithelial phenotype and good treatment response.

Several previous studies have demonstrated distinct splicing patterns of genes in the GH/CSH gene cluster in different tissues, and further shown that mRNA levels of different isoforms are associated with different clinical responses. In particular, in placental tissue, where EMT progression may be a frequently regulated event, differential expression patterns of GH/CSH genes have been reported and associated with metabolic complications such as pre-eclampsia, gestational diabetes mellitus and birth size (Mannik *et al.* 2012). In the present study we detected transcript levels of all genes in the GH/CSH2 cluster with an abundance of *GH1* compared to *GH2* and *CSH1/2*. The *GH1* isoform mRNA proportions were similar to the protein proportions previously reported in somatotroph adenomas (Zhan *et al.* 2005). To our knowledge there has so far been no report of *GH2*, *CSH1*, *CSH2* or *CSHL1* expression in somatotroph adenomas, possibly due to strong sequence similarity and homology between these genes, making it difficult to distinguish between the various genes, isoforms and in particular proteins.

ESRPs are components of an epithelial gene signature and are down-regulated in cells undergoing EMT (Tavanez & Valcarcel 2010), which corresponds to

splicing changes in several ESRP-regulated exons. Of the genes in the GH/CSH gene cluster, only *CSH2* displayed an alternative splicing pattern dependent upon ESRP1 levels in our study. The association between *CSH2* isoform 3 and *ESRP1* was verified in the total population showing a strong correlation, and enhanced levels of this isoform was further associated with E-cadherin, a dense granulation pattern and a higher *GH* mRNA expression, all indices of an epithelial phenotype not committed to EMT progression. Furthermore, increased *CSH2* isoform 3 mRNA expression was associated with a good response to the acute SA test and reduction in circulating GH and IGF-1 following SA treatment. Together these data may suggest a role for ESRP1 driven CSH2 abnormal splicing in EMT progression that may impact treatment responses in patients with acromegaly, although caution is needed when interpreting these results since correlations do not necessarily imply a causal relationship.

We were not able to measure mRNA levels of the CSH2 isoform 1, possibly due to strong sequence homology with the other genes. Thus, we can only speculate about the effects and functions of the alternative splicing of *CSH2* expression in somatotroph adenomas. CSH1 has been shown to inhibit apoptosis, both in insulinoma cells and human islets, by the phosphorylation of AKT protein (Lombardo *et al.* 2011). The *CSH1* and *CSH2* isoform 1 have almost identical sequences and could have similar functions. Adenomas with low ESRP1 levels, and increased expression of *CSH2* isoform 1, could support survival of the cells via AKT phosphorylation. It has also been reported that hPL (CSH1 and CSH2) both have angiogenic and anti-angiogenic functions (Corbacho *et al.* 2002). The full length isoform stimulate angiogenesis and the 16kDa isoform inhibits angiogenesis and a similar mechanism may apply within the somatotroph adenomas where high expression of the isoform 3, the 16kDa isoform, could inhibit angiogenesis in the adenomas with high levels of ESRP1.

A number of growth factors and receptors have previously been identified as targets of ESRP1 and would therefore be more feasible candidates to alter pituitary growth and function. However, our focus on the *GH/CSH* in the present study is based on our original screening experiment in adenomas characterized by high and low ESRP1 mRNA expression, where only a limited number of spliced candidates, including CSH2, were identified. Limitations to our study include lack of functional and protein data. A functional role of ESRP1 directly in rodent GH3 cells is difficult to achieve since studying alternative splicing and isoforms in GH3 cells is difficult because they lack many of the different isoforms of the spliced genes investigated, possibly due to the evolution of genes from rats to humans, with an increasing frequency of splicing events (Modrek & Lee 2003). Moreover, the rat *Csh2* mRNA consists only of one isoform. Several studies have detected endogenous CSH mRNA expression, but have failed to identify hPL protein production, possibly indicating that the *CSH* gene is not translated into protein in these cells, or due to problems with non-specific antibodies for hPL (Tuttle *et al.* 2014).

In conclusion, we found all the genes in the GH/CSH gene cluster to be expressed in somatotroph adenomas. We further found that low mRNA expression levels of *CSH2 isoform3* were associated with EMT progression and poor response to treatment, encouraging further studies on the role of *CSH2* in the regulation of EMT progression.

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Conflict of interest

The authors declare that they have no conflict of interest.

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