Assessment of sFRP4 as a bio-marker for predicting aggressiveness and recurrence of growth hormone-secreting pituitary adenomas

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Abstract. The association of sFRP4 expression with aggressiveness and recurrence of growth hormone (GH)-secreting pituitary adenomas was investigated. Ten normal pituitary and 52 GH-secreting pituitary adenoma specimens were classified into three groups: normal pituitary (control) group, non-aggressive group, and aggressive group, according to preoperative evaluation by magnetic resonance imaging (MRI)/computed tomography (CT). Expression of sFRP4 was determined by quantitative real-time polymerase chain reaction (qRT-PCR), western blot analysis, and tissue microarrays, to assess the association between sFRP4 and aggressiveness. Follow-up information of all 52 patients was collected to evaluate the impact of sFRP4 expression on the recurrence/progression of GH-secreting pituitary adenomas. qRT-PCR results showed a lower level of sFRP4 mRNA in the aggressive group, as compared to that in the non-aggressive group (P=0.001). A similar trend was observed on western blot analysis for sFRP4 protein expression (P=0.004). On analysis by tissue microarrays, weak sFRP4 expression was detected in the aggressive group (10/15, 66.7%). Univariate analysis showed a significant relationship between low sFRP4 expression and aggressiveness (P=0.024). On multivariate analysis weak sFRP4 expression was found to be an independent factor of recurrence/progression (odds ratio: 0.063, P=0.026). Methylation of the sFRP4 promoter was increased in low sFRP4 staining group compared to that in the high sFRP4 staining group (P<0.001). In this study, weak sFRP4 expression appeared to predict aggressive behavior, and was associated with recurrence/progression of GH-secreting pituitary adenomas. Methylation of the sFRP4 promoter may account for the low sFRP4 expression.

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Abbreviations: sFRPs, secreted frizzled-related proteins; GH, growth hormone; CT, computed tomography; MRI, magnetic resonance imaging; NFPs, non-functioning pituitary adenomas; PCR, polymerase chain reaction; TMA, tissue microarray; IHC, immunohistochemistry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Key words: sFRP4 expression, GH-secreting pituitary adenoma, aggressiveness, recurrence

Introduction

Pituitary adenomas are relatively common neoplasms that account for 10-15% of all intracranial tumors. More than two-thirds of pituitary adenomas are characterized by excessive hormonal secretion, which manifests in the form of distinctive clinical syndromes (1,2). Based on the clinical presentation, pituitary adenomas are classified as growth hormone- (GH), adrenocorticotropic hormone- (ACTH), prolactin- (PRL), and thyroid-stimulating hormone (TSH)-secreting pituitary adenomas, or clinically non-functional adenomas. The most common hormone-secreting adenomas are prolactinomas, followed by growth hormone (GH) secreting and mixed GH and prolactin-producing adenomas (3).

Surgical resection is the primary therapeutic modality, although there are medical treatments available for GH-secreting tumors (4). Indeed, in patients with intrasellar microadenomas, microsurgical removal of the tumor alone is sufficient for biochemical control as the procedure is associated with normalization of IGF-I in 75-95% of patients. However, in macroadenomas or aggressive adenomas, surgery alone is not expected to achieve disease control or hormonal remission in most cases (5,6). Surgical treatment of aggressive GH-secreting pituitary adenomas carries a high risk of recurrence/progression. These patients almost always need further therapy to achieve biochemical control of the disease. Currently, there is neither an effective methodology to distinguish non-aggressive GH-secreting pituitary adenomas from aggressive adenomas, nor is there an efficient way to predict the recurrence/progression after surgery. Hence, research on bio-markers associated with aggressive pituitary adenomas and prognosis is a key imperative.

The Wnt signaling pathway is involved in several developmental processes, including, tissue differentiation, proliferation

and apoptosis (7,8). Aberrant regulation of the Wnt signaling pathway is thought to play a role in tumorigenesis (9), especially in pituitary adenomas (10,11). The secreted frizzled related protein (sFRP) family, coded by a class of pro-apoptotic genes, is thought to play an important role in tumorigenesis by inhibiting Wnt signaling pathway (12). Upregulation of sFRPs expression, and in particular that of sFRP4, has been shown to correlate with apoptosis in various tissues (13-17). Further, the restoration or upregulation of sFRPs expression in cancer cells has been shown to attenuate grades and invasive growth characteristics of tumors such as, prostate, ovarian, and cervical cancer, which suggests that activities of sFRPs are fundamental for tissue homeostasis (18-23).

Elston *et al* (24) reported significant downregulation of sFRP4 in pituitary adenomas. Our previous study on gene microarrays also demonstrated significant downregulation of sFRP4 in aggressive NFPAs, as compared to that in non-aggressive NFPAs and normal pituitary tissues (25). Similar observations were forthcoming from yet another study conducted by our group, where we demonstrated a negative correlation of sFRP4 with aggressiveness of NFPAs (26).

Promoter hypermethylation often accounts for the loss of expression of tumor suppressor genes (27). Dense CpG islands flank the first exons of *sFRP1, sFRP2, sFRP4* and *sFRP5*, but not *sFRP3*. These sequences have been reported to be hypermethylated in several types of carcinoma, and particularly in colorectal, gastric, mammary, prostate, renal cell, and chronic lymphocytic leukemia (22,28-35).

In this study, we investigated the difference in sFRP4 expression between the aggressive GH pituitary adenoma and the non-aggressive GH pituitary adenoma through quantitative real-time polymerase chain reaction (qRT-PCR), western blot analysis, and tissue microarrays. The objective was to investigate the potential role of sFRP4 as a prognostic biomarker for GH-secreting pituitary adenomas, in terms of predicting aggressiveness and the risk of recurrence. Furthermore, methylation analysis of the sFRP4 promoter region was performed to figure out the underlying mechanism for abnormal expression of sFRP4.

Materials and methods

Ethical approval. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committee of Beijing Tiantan Hospital, Beijing, China, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Sample collection. A total of 52 patients with GH-secreting pituitary adenoma (age range, 13-75 years) were enrolled at the Beijing Tiantan Hospital (2008-2012). None of the patients received somatostatin analog therapy in the preoperative period due to economic reasons.

The inclusion criteria were: i) availability of sufficient pituitary tissue specimen to allow for analysis by tissue microarrays; ii) no history of pre-operative radiation; iii) availability of clinical data, including endocrinological evaluation results and imaging results; iv) minimum follow-up duration of 3 years.

The diagnosis of biological behavior of the tumor was made on the basis of preoperative magnetic resonance imaging (MRI) and computed tomography (CT). Pituitary adenomas that were classified as grade III or IV according to Hardy or Knops classification were defined as aggressive.

Ten specimens of normal pituitary glands were obtained from a donation program. The donors included 6 men and 4 women aged 21-45 years (mean age, 35 years). All of the donors had died of non-neurological diseases. Written informed consent was obtained from all donors prior to their enrollment; the study protocol was approved by the Ethics Committee at the Beijing Tiantan Hospital, Beijing, China.

Specimens were categorized into three groups: the normal pituitary control group, non-aggressive group and aggressive group. Pituitary tumors were removed by trans-sphenoidal surgery and immediately 'flash-frozen' in liquid nitrogen until further analyses. Suitable parts of each sample were embedded in paraffin.

All patients were diagnosed based on clinical symptoms, preoperative sellar MRI and postoperative histopathological examination. Postoperative sella MRI scans were performed within 72 h after surgery, to evaluate the residual mass. Two neuroradiologists, and one neurosurgeon blinded to the patients' characteristics conducted the evaluation. The same were repeated at 6-month intervals in the first 2 postoperative years. To investigate for tumor recurrence, serial sella MRI scans were performed at 1-year intervals. However, in the presence of clinical symptoms, sella MRI scan was performed immediately. Recurrence/progression was defined on the basis of 1 or more of the following parameters: i) presence of a new tumor in patients with a total resection, based on the first post-operative MRI scan; ii) evidence of new growth of an incompletely resected tumor on serial postoperative MRI scans versus the immediate postoperative MRI scan; iii) clinical deterioration or recurrence of symptoms after surgery, and associated with elevated serum hormone levels.

Preoperative serum GH levels were recorded to estimate the association of serum GH level with aggressiveness and recurrence/progression of tumors. The results were categorized as high level (≥ median level) and low level (< median level).

Quantitative real-time polymerase chain reaction. Total RNA was isolated from frozen pituitary adenomas and normal pituitaries (100-150 mg) using TRIzol reagent (Invitrogen Life Technologies, 15596-026). The housekeeping gene coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Relative quantification of gene expression was determined using the $2^{-\Delta\Delta CT}$ method as described by Livak and Scmittgen (36). The primers used in the Qrt-PCR assay are listed as follows: sFRP4 (forward, TGGCAACGTATCTCAGCAAA; reverse, GGATGGGTGA TGAGGACTTG), GAPDH (forward, ACCACAGTCCATGC CATCACT; reverse, GTCCACCACCCTGTTGCTGTA). The specificity of Qrt-PCR products was verified by performing dissociation reaction plots.

Protein extraction and western blot analysis. Protein extraction and western blot analysis (WB) were performed as described elsewhere (37), using antibodies for anti-sFRP4

(1:5,000, Abcam, Cambridge, UK). Rabbit anti-GAPDH (1:1,000, Sigma, St. Louis, MO, USA) was used as an internal control. Horseradish peroxisdase-conjugated secondary antibodies (1:5,000, Sigma) were used followed by enhanced chemiluminescence development (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The final data were subjected to grayscale scanning and semi-quantitative analysis using Quantity One Software (Bio-Rad, Hercules, CA, USA).

TMA construction and immunohistochemistry. Formalin-fixed paraffin-embedded tissue blocks were sliced and eosin-stained (H&E) slides were prepared. Three core biopsies, 2.0-mm diameter, were selected from the paraffin-embedded tissue. The cores were transferred to tissue microarrays using the Leica Bond-III fully automated arrayer from Leica Biosystems (Aperio, CA, USA). The locations of the core samples were in random order, and the pathologist were blinded to the identity of the TMA slides. The tissue micro-arrays were cut into 4-μm sections using a serial microtome and placed in a water bath at 50˚C, followed by its application onto positively-charged glass slides. Slides were dewaxed and then rehydrated through graded alcohols into water. After mounting, the slides were dried at room temperature for 24-48 h and stored at 4˚C until further testing. To minimize loss of antigenicity, the microarray slide was processed within 1 week of cutting.

All TMA slides were evaluated in advance using an H&E stain to assess tumor content and quality. The TMAs were placed in the Leica BOND-III instrument, which is a fully automated, random and continuous access slide-staining system that processes IHC tests simultaneously. IHC protocol F was selected in the machine, and 3 min with ER1 (epitope retrieval) was set for heat-induced epitope retrieval (HIER) parameter. Bond™ Ploymer Refine Detection (Leica Biosystems, DS9800) was used for detection of primary antibodies. The slides were scanned into digital pictures and expression was examined using Aperio AT2 (Leica Biosystems). Primary antibodies, anti-sFRP4 (1:5,000, Abcam) were used. The optimal titer of the primary Abs for the remainder was determined based on pre-experiment results.

Evaluation of immunohistochemical staining. Staining for sFRP4 was present in the cytoplasm and nucleus. The results were calculated using Aperio AT2 (Leica Biosystems) with digital slide viewing software. The staining intensity of sFRP4 was scored as follows: 0, no; 1, weak; 2, moderate; and 3, high intensity. The distribution of positively stained cells was scored on a scale of 0-5 as follows: 0, no staining; 1, <20%; 2, 20-40%; 3, 40-60%; 4, 60-80%; and 5, 80-100%. The total score was calculated as staining intensity x distribution (score ≤6: weak expression; score >6, strong expression).

Methylation and sequencing analysis. DNA was extracted from 37 fresh-frozen non-aggressive pituitary adenomas (15-50 mg), using TRIzol reagent according to the manufacturer's protocol (Invitrogen). DNA concentration and purity were measured by UV absorbance at 260/280 nm (Nanodrop ND-1000, USA). Before carrying out the methylation analysis, we quantified the yield of extracted DNA from the samples. The extracted DNA was of sufficient quality to allow successful amplification. Bisulfite treatment of DNA was performed using The EZ-96 DNA Methylation kit (Zymo Research, Orange, CA, USA). Two overlapping PCRs were performed to amplify a 1,924-bp area (-820 to +1,104, relative to the translation start site) of the sFRP4 promoter containing 134 CpG dinucleotides. The RNase-A enzyme (Sequenom, USA) was added to cleave the *in vitro* transcripts (T-cleavage assay). For every cleaved CpG site, the mass spectra were collected using MassARRAY Compact MALDI-TOF (Sequenom) and the spectra methylation ratios were generated by EpiTYPER software v1.0 (Sequenom).

Cell culture, DAC treatment and western blot analysis of cell lines. GH3 pituitary adenoma cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum, at 37˚C in a humidified atmosphere containing 5% CO_2. The demethylating agent, DAC (5-aza-2-deoxycytidine; Sigma), was freshly prepared in ddH_2O. GH3 cells ($3x10^5$ cells/well) in exponential growth phase were seeded in 6-well plates. After 24 h of culture, cells were treated with DAC at concentration of 0, 10, 20, 30 and 40 nM for 72 h. The culture medium was replaced every 24 h with fresh media containing DAC. Total proteins were extracted for WB analysis, which was performed using the standard procedure, and the proteins were identified using anti-sFRP4 (1:5,000, Abcam), rabbit anti-GAPDH (1:1,000, Sigma) antibodies.

Cell viability assay (MTS). Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using MTS reagent (CellTiter 96 AQueous One Solution Cell Proliferation assay, Promega). Briefly, $2x10^4$ exponentially growing GH3 cells were seeded in 96-well culture plates with DAC at concentration of 0, 10, 20, 40 and 80 nM. After 24, 48, 72 of incubation at 37˚C, 20 μl of MTS was added to each well, and the samples incubated for a further 3 h at 37˚C. Plates were analyzed on a Tecan M200Pro multimode microplate reader at 492 nm. The cell inhibition rate was calculated as follows: Inhibition rate (%) = (OD of control group - OD of drug group)/ OD of control group x 100%.

Statistical analysis. Differences between subgroups were analyzed using the Student's t-test for normally distributed continuous values, and Mann-Whitney U test for non-normally distributed continuous values. The χ^2 test was used to analyze categorical variables. Variables that showed association with aggressiveness and recurrence of GH-secreting pituitary adenomas were subjected to univariate and multivariate analyses. Two-sided P-values of <0.05 were considered statistically significant. SPSS software version 17.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses.

Results

Clinical and pathological features. Details of the GH pituitary adenoma specimens included in the study are shown in Table I. Fifty-two GH-secreting pituitary adenomas met the inclusion criteria. Patient age ranged from 13 to 75 years (mean, 40.3 years; median, 39.5 years) at the time of the first

Table I. Clinico-pathological characteristics of 52 patients with GH-secreting pituitary adenomas.

Variables	N	Percentage (%)
Gender		
Male	28	53.8
Female	24	46.2
Age (years)		
Mean	40.3	
Median	39.5	
Aggressiveness		
Aggressive	15	28.8
Non-aggressive	37	71.2
Preoperative serum GH level (ng/ml)		
Mean	22.82	
Median	21.35	
Surgical extent		
Gross total resection	35	67.3
Residual	17	32.7
Recurrence (within 42 months)		
Yes	11	21.2
No	41	78.8

GH, growth hormone.

surgical treatment. Out of the 52 patients, 24 patients (46.2%) were female, and 28 (53.8%) were male. There were 4 (26.7%) females and 11 (73.3%) males in the aggressive group (N=23), and 20 (54.1%) females and 17 (45.9%) males in the non-aggressive group (N=25). Gross total resection was found in 35 (67.3%) out of the 52 patients. The preoperative serum GH results ranged from 0.13 to 48 ng/ml (mean, 22.82 ng/ml, median, 21.35 ng/ml).

Quantitative-PCR and western blot analysis. qRT-PCR revealed a significant downregulation of sFRP4 mRNA level in both aggressive- as well as non-aggressive GH-secreting pituitary adenomas, as compared to that in the normal controls (0.031±0.006 vs. 1.35±0.19, P<0.001, N=15 vs. N=10; 0.981±0.111 vs. 1.35±0.19, P<0.001, N=37 vs. N=10) (Fig. 1A). The sFRP4 mRNA level was significantly lower in aggressive group as compared to that in the non-aggressive group (0.031±0.006 vs. 0.981±0.111, P=0.001, N=15 vs. N=37) (Fig. 1A).

The sFRP4 protein expression in the three groups was assessed by WB (Fig. 1B). There was a significantly lower sFRP4 protein level in the non-aggressive group as compared to that in the normal pituitary tissues (6.82±0.45 vs. 14.65±1.38, P<0.001, N=37 vs. N=10); the expression was even lower in the aggressive group as compared to that in the non-aggressive group (4.13±0.49 vs. 6.82±0.45, P=0.004, N=15 vs. N=37) (Fig. 1C).

Tissue microarrays analysis. sFRP4 expression was detected in all specimens by TMA (Fig. 2). On univariate analysis, sFRP4 expression showed no association with age, gender, and preoperative serum GH levels. However, a significant relationship was found between aggressive behavior and sFRP4 expression (P=0.024) (Table II).

On comparing the total score for sFRP4 staining with the aggressive behavior, the sFRP4 expression was downregulated in most of the aggressive GH-secreting pituitary adenomas (10/15, 66.7%) (Table III). Only 12 (32.4%) pituitary adenomas expressed weak sFRP4 expression among the 37 non-aggressive GH-secreting pituitary adenomas. On univariate analysis a significant association was found between sFRP4 expression and aggressiveness of tumors (P=0.024) (Table III). However, age, gender and preoperative serum GH level were not found to be associated with aggressiveness (Table III).

Follow-up data were available for all 52 patients. Patients were followed up for 42 months. During follow-up, 11 patients (21.2%) experienced recurrence (Table I). Out of the 11 recurrent adenomas, 10 (90.9%) had weak sFRP4 expression (Table IV). Weak sFRP4 expression was found only in 16 of 41 patients (39%) with non-recurrent GH-secreting pituitary adenomas. On univariate analysis, weak sFRP4 expression (P=0.002), increased aggressiveness (P=0.034), and surgical

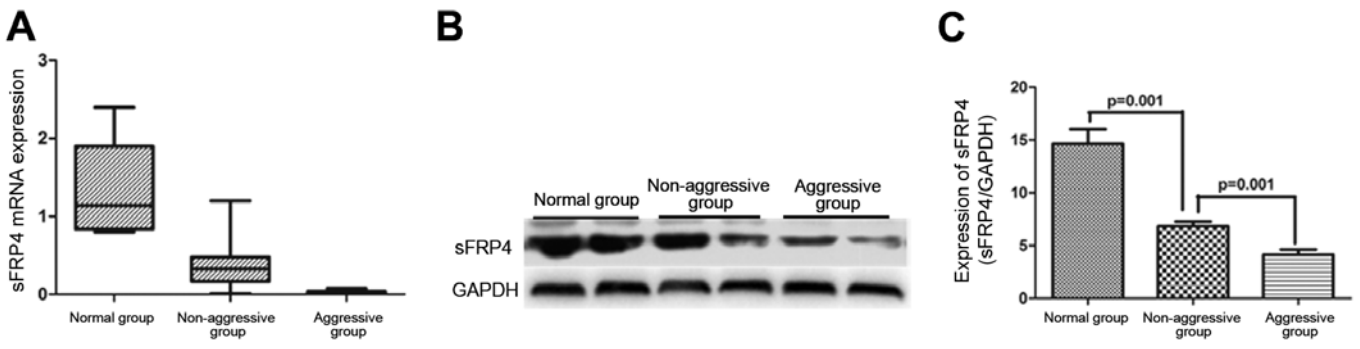

Figure 1. (A) mRNA levels of sFRP4 in normal pituitary tissues, non-aggressive and aggressive GH-secreting pituitary adenomas were assessed by qRT-PCR. (B) Protein expression of sFRP4 and GAPDH in normal pituitary tissues, non-aggressive, and aggressive pituitary adenomas were detected by western blotting. GAPDH was used as an internal control. (C) Quantitative analyses of western blot results are shown. Data are expressed as mean ± SEM. qRT-PCR, Quantitative real-time polymerase chain reaction; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; SEM, standard error of the mean.

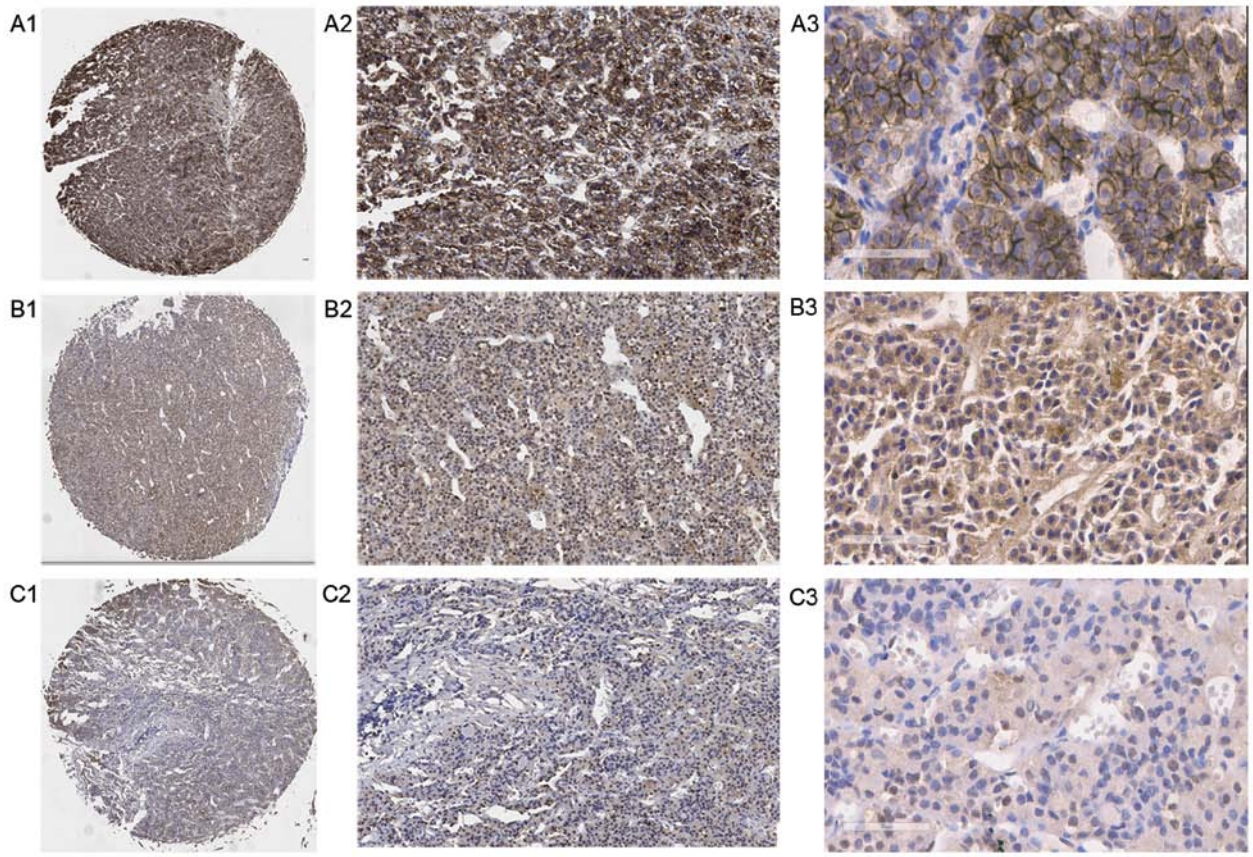

Figure 2. Expression of sFRP4 was assessed by TMA. (A) Weak sFRP4-positive cells from normal pituitary tissues (A1, x40, A2, x100 and A3, x400 magnification). (B) Weak sFRP4-positive cells from non-aggressive adenoma (B1, x40, B2, x100 and B3, x400 magnification). (C) Strong sFRP4-positive cells from aggressive pituitary adenomas (C1, x40, C2, x100 and C3, x400 magnification). TMA, tissue microarray.

Table II. Association between sFRP4 (TMA) expression and clinico-pathological characteristics.

Variables	sFRP4 expression		Univariate analysis	
	Weak (N=22)	Strong (N=30)	χ^2	P-value
Gender				
Male	14 (63.6%)	14 (46.7%)	1.471	0.225
Female	8 (36.4%)	16 (53.3%)		
Age				
≤39	11 (53.8%)	15 (46.2%)	0	1
>39	11 (46.2%)	15 (53.8%)		
Aggressiveness				
Yes	10 (45.5%)	5 (16.7%)	5.125	0.024
No	12 (54.5%)	25 (83.3%)		
Preoperative serum GH level				
High	10 (45.5%)	13 (43.3%)	0.023	0.879
Low	12 (54.5%)	17 (56.7%)		

TMA, tissue microarray; GH, growth hormone.

Table III. Univariate and multivariate analyses for the clinico-pathological correlates of aggressive GH secreting pituitary adenomas.

Variables	Aggressiveness N (%)		Univariate analysis	
	No (N=37)	Yes (N=15)	χ^2	P-value
Gender				
Male	17 (45.9%)	11 (73.3%)	3.221	0.073
Female	20 (54.1%)	4 (26.7%)		
Age				
≤39	17 (45.9%)	9 (60%)	0.843	0.358
>39	20 (54.1%)	6 (40%)		
sFRP4				
Yes	25 (67.6%)	5 (33.3%)	5.125	0.024
No	12 (32.4%)	10 (66.7%)		
Preoperative serum GH level				
Low	23 (62.2%)	6 (40%)	2.125	0.145
High	14 (37.8%)	9 (60%)		

TMA, tissue microarray; GH, growth hormone.

Table IV. Univariate and multivariate analyses for the clinico-pathological correlates of recurrence/progression-free survival.

	Recurrence (within 42 months)		Univariate analysis		Multivariate analysis	
Variables	Yes (N=11)	No (N=41)	χ^2	P-value	Odds ratio (95% CI)	P-value
Gender						
Male	6 (54.5%)	22 (53.7%)	0.003	0.958		
Female	5 (45.5%)	19 (46.3%)				
Age						
≤39	5 (45.5%)	21 (51.2%)	0.115	0.734		
>39	6 (54.5%)	20 (48.8%)				
sFRP4 (TMA)						
Strong	1 (9.1%)	25 (61%)	9.339	0.002	0.063 (0.06-0.722)	0.026
Weak	10 (90.9%)	16 (39%)				
Aggressiveness						
Yes	6 (54.5%)	9 (22%)	4.489	0.034	2.367 (0.14-39.82)	0.55
No	5 (45.5%)	32 (78%)				
Preoperative serum GH level						
Low	5 (45.5%)	24 (58.5%)	0.602	0.438		
High	6 (54.5%)	17 (41.5%)				
Surgical extent						
Gross total resection	4 (36.4%)	31 (75.6%)	6.071	0.014	0.139 (0.009-2.15)	0.155
Partial resection	7 (63.6%)	10 (24.4%)				

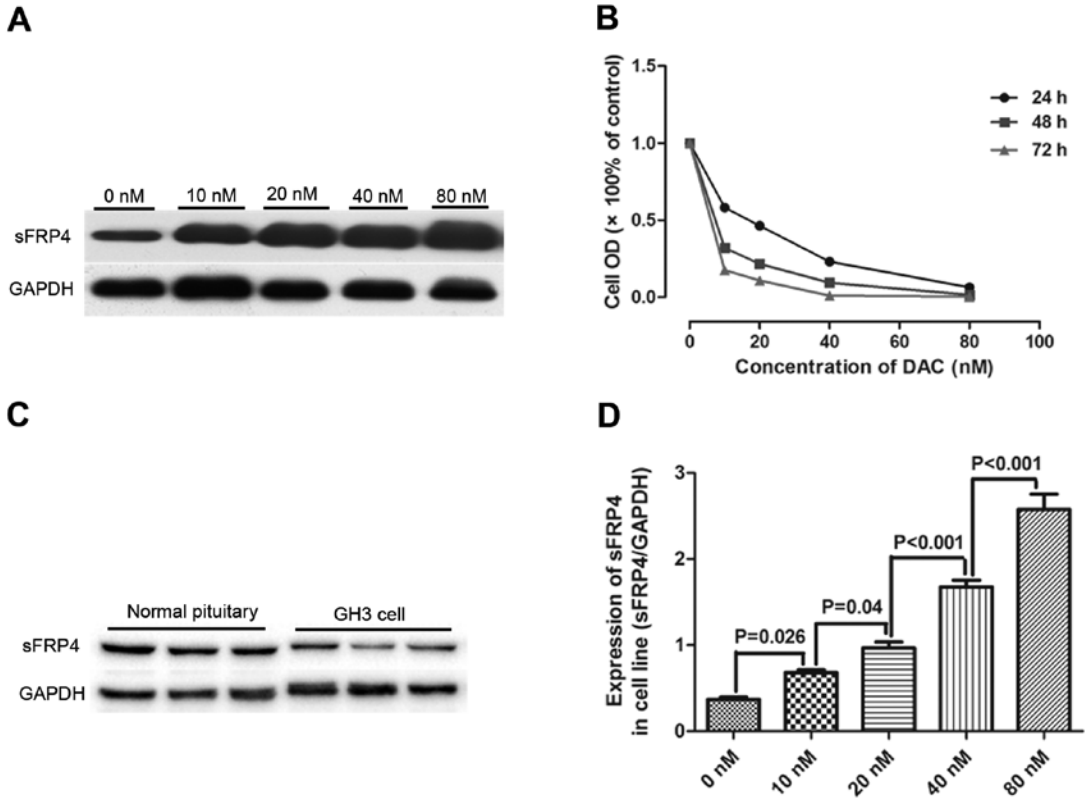

Figure 3. (A) sFRP-4 protein expression in pituitary adenoma cell lines (GH3) and normal human pituitary protein by western blot analysis. (B) Relative optical density (OD) of GH3 pituitary adenoma cells at 24, 48 and 72 h after DAC treatment at a range of concentrations. OD values are relative to the control (the concentration of DAC: 0 nM), and are determined using the MTS assay. (C) Western blots showing protein expression of sFRP4 and GAPDH in GH3 pituitary adenoma cells at 72 h after treatment with DAC at a concentration of 40 nM. GAPDH was used as an internal control. (D) Quantitative analyses of western blot analysis results. Data are presented as mean ± SEM. DAC, 5-aza-2-deoxycytidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SEM, standard error of the mean.

extent (P=0.014) were significantly associated with recurrence/progression (Table IV). Gender, age, and preoperative serum GH levels were not associated with recurrence (Table IV).

On multivariate analysis, only decreased sFRP4 expression was found to be independently associated with increased aggressiveness [odds ratio (OR): 0.063, P=0.026] (Table IV).

Methylation analysis of the sFRP4 promoter region. Methylation analysis was performed to investigate the contribution of hypermethylation to the loss of sFRP4 expression. The 52 samples were classified into two groups: weak sFRP4 staining and strong sFRP4 staining groups, according to the total TMA score. One CpG site (+351 bp) was found to be hypermethylated in pituitary adenomas. The methylation ratio of the CpG site in the weak sFRP4 staining group was higher than that in strong sFRP4 staining group (11.83±1.12 vs. 5.53±0.58%, P<0.001, N=22 vs. N=30).

Western blot and MTS analysis of cell lines. The sFRP4 protein expressions in pituitary adenoma cell lines (GH3), as well as in normal human pituitary tissue were assessed by western blotting. As shown in Fig. 3A, sFRP4 expression was significantly downregulated in pituitary adenoma cell lines (GH3) as compared to that in the normal human pituitary (0.32±0.03 vs. 0.7±0.05, P=0.023, N=9 vs. N=9). MTS analysis showed a dose- and time-dependent inhibition of GH3 cell proliferation induced by DAC. A maximal inhibition of 87.6±2.2% at a concentration of 80 nM after 72 h was observed. The cells were significantly inhibited at all concentrations of DAC used in this study (0, 10, 20, 40 and 80 nM) after 3-day exposure. Growth curves for GH3 cells for various DAC concentrations are shown in (Fig. 3B), indicating that DAC suppressed cell growth in a dose-dependent and time-dependent manner. We evaluated the level of sFRP4 protein in GH3 cells after DAC treatment by WB analysis (Fig. 3C). GH3 cells after DAC treatment at the concentration of 10 nM exhibited upregulation of sFRP4 protein compared with GH3 cells without DAC treatment (0.32±0.03 vs. 0.7±0.05, P=0.023, N=8 vs. N=8). There was a progressive increase in sFRP4 expression with the increase in DAC concentration (Fig. 3D). Both MTS and WB analysis revealed that DAC inhibits the growth of GH3 cells possibly by upregulating the expression of sFRP4 in GH3 cells.

Discussion

In this study, we compared the expression of sFRP4, both at the mRNA and protein level, in normal pituitary tissue, and in non-aggressive and aggressive GH-secreting pituitary adenomas. We documented a significant downregulation of sFRP4 mRNA levels in aggressive GH-secreting pituitary adenomas as compared to that in both normal controls and in the non-aggressive GH-secreting pituitary adenomas. This was further confirmed by WB and tissue microarray analyses. Expression of sFRP4 was negatively linked to aggressiveness and recurrence/progression of tumors, indicating that sFRP4 may be used as biomarker for predicting aggressiveness and recurrence/progression of GH-secreting pituitary adenomas. However, no significant association of sFRP4 expression with gender, age and preoperative serum GH level, was observed. Methylation and sequencing analysis of the sFRP4 promoter region showed it to be densely methylated in the weak sFRP4 staining group, whereas there was significantly decreased methylation in strong sFRP4 staining group. In addition, treatment with demethylation agent (5-Aza-dc) and histone deacetylase inhibitor (TSA) restored sFRP4 mRNA expression in pituitary adenoma cell lines (GH3). Our results indicate that downregulation of sFRP4 expression in the weak sFRP4 staining group correlate with CpG methylation of the sFRP4 promoter.

To the best of our knowledge, this study is the first to demonstrate correlation of sFRP4 expression with aggressiveness of GH-secreting pituitary adenomas. Similar results have been reported elsewhere in other cancers (21,38). Even though we showed a negative relationship between weak sFRP4 expression and aggressiveness, we did not find any relationship between gender, age, preoperative serum GH level and aggressiveness of the tumors. So weak sFRP4 maybe serve as an independent factor of aggressiveness in GH-secreting pituitary adenomas.

Accumulated evidence indicates an association of low sFRP4 expression with recurrence/progression in several cancers (39,40). In this study, 52 patients were followed up for a period of 42 months with clinical and imaging data, and is one of the few studies to report recurrence rates for GH-secreting pituitary adenomas after surgery. In our study, the univariate analysis showed a significant association between low sFRP4 expression, surgical extent, tumor aggressiveness and recurrence/progression of tumors. However, on multivariate analysis, only low sFRP4 expression was an independent predictor of recurrence/progression of tumor, and tumor aggressiveness and surgical extent were not significantly associated with the recurrence/progression. This observation is in contrast to findings of some other reports that tumor resection and tumor aggressiveness were predictive factors for recurrence of pituitary adenomas (41,42). The relatively short duration of follow-up may, at least partially, explain this difference.

Further, we used logistic regression analysis instead of survival analysis in consideration of potential censoring caused by low recurrence rate, so survival time was not included in our study. All of these factors could have led to the contradictory results. These differences highlight the continued need for further research with a larger sample size and longer duration of clinical follow-up.

Hypermethylation of the sFRP4 gene has been reported in various cancers, and is associated with tumor progression and malignancy (22,43). In our study, silencing of SFRP4 expression correlated with the promoter methylation. At cellular level, we observed a restoration of SFRP4 expression in pituitary adenoma cells (GH3) after treatment with the demethylating agent, 5-aza-2 V-deoxycytidine. These findings are consistent with studies conducted on various other malignancies, including gastric, cervical, hepatocellular, pancreatic, oral squamous cell, breast, colon, and bladder cancers (44-49).

In conclusion, in this study weak sFRP4 expression appeared to be a predictive factor for aggressive behavior of GH-secreting pituitary adenomas. Furthermore, weak sFRP4 expression was also associated with post-operative tumor recurrence/progression. We believe that methylation of the

sFRP4 promoter could account for the decreased sFRP4 expression.

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