Research Article Open Access

Xiaosong Shan, Qian Liu, Zhenye Li, Chuzhong Li, Hua Gao, Yazhuo Zhang*

Epithelial–Mesenchymal Transition Induced by SMAD4 Activation in Invasive Growth Hormone-Secreting Adenomas

SMAD4 and Epithelial–Mesenchymal Transition

https://doi.org/10.1515/chem-2018-0061
received February 7, 2018; accepted March 29, 2018.

Abstract: Background The detection and treatment of invasive growth hormone-secreting pituitary adenoma (GHPA) remains challenging. Several transcription factors promoting the epithelial–mesenchymal transition (EMT) can act as cofactors for the transforming growth factor-beta (TGF-β)/SMAD4. The goal of this study was to investigate the association of SMAD4 expression and clinicopathologic features using a tissue microarray analysis (TMA). The levels of SMAD4 and the related genes of EMT in GHPAs were analyzed by q-PCR and western blot. SMAD4 was strongly expressed in 15/19 cases (78.9%) of invasive GHPA and 10/42 cases (23.8%) of noninvasive GHPA (χ^2=10.887, p=0.000). In the high SMAD4 group, a headache was reported in 16/25 cases (64%) compared with 13/36 cases (36.1%) in the low SMAD4 group (χ^2=4.565, p=0.032). The progression-free survival (PFS) in the high group was lower than that in the low group (p=0.026). qRT-PCR and western blot analysis further revealed a significant downregulation of E-cadherin and upregulation of N-cadherin and vimentin in the invasive GHPA group. SMAD4 was associated with increased levels of invasion of GH3 cells, as determined by a transwell test. SMAD4 downregulated E-cadherin levels and increased the levels of N-cadherin and vimentin. Our data provide evidence that SMAD4 is a potential prognosis biomarker and a therapeutic target for patients with invasive GHPA.

Keywords: SMAD4; Growth Hormone-secreting Pituitary Adenoma; Invasive; Epithelial–Mesenchymal Transition; GH3 cells.

1 Introduction

Pituitary adenomas are relatively benign tumors that account for 10–15% of intracranial tumors [1, 2]. Growth hormone-secreting adenomas (GHPAs) are the second most common hormone-secreting pituitary adenomas and represent 10–15% of pituitary adenomas [3]. GHPAs are characterized by an excessive production of growth hormone and the consequent increase in insulin-like growth factor 1 (IGF-1). This condition typically results in acromegaly, a disorder characterized by enlargement of the hands, feet and facial lines [4]. Although GHPAs are usually regarded as benign lesions, many can invade the sphenoid, cavernous sinus, or dura mater becoming malignant. The goal of GHPA treatment is to normalize the growth hormone and IGF-1 levels in order to prevent tumor growth [5]. Surgery provides 60–80% biochemical remission in microadenomas or noninvasive macroadenomas [6–8]. However, cure rate in invasive GHPAs is low due to the failure of complete resection [9, 10]. The remission rate in invasive GHPAs remains unsatisfactory even when surgical intervention is followed by radiological or pharmacological therapy.

***Corresponding author: Yazhuo Zhang,** Beijing Neurosurgical Institute, Capital Medical University, Beijing, China; Beijing Institute for Brain Disorders, Brain Tumor Center, Beijing, China National Clinical Research Center for Neurological Diseases, Beijing, China, E-mail: zyz2004520@yeah.net
Xiaosong Shan, Qian Liu, Chuzhong Li, Hua Gao: Beijing Neurosurgical Institute, Capital Medical University, Beijing, China
Zhenye Li: Department of Neurosurgery, Beijing Tiantan Hospital, Capital Medical University, Beijing, China
Hua Gao: Beijing Institute for Brain Disorders, Brain Tumor Center, Beijing, China National Clinical Research Center for Neurological Diseases, Beijing, China

Over the past 20 years, major advances have been made in cancer research, especially in the understanding of the molecular mechanisms of tumor development and the role of IGF-1. The mechanisms mediating invasive behavior in pituitary adenomas are complex [11, 12]. Epithelial–mesenchymal transition (EMT), a process involved in tumor invasion, occurs when an epithelial cell differentiates into a more motile mesenchymal cell. EMT markers such as N-cadherin, vimentin, and Snail are significant indicators of the appearance of cystic lesions, tumor progression, bone destruction, and endocrine functions. N-cadherin is a well-known EMT biomarker for invasive tumors, including growth hormone adenomas. Healthy cells express the E-cadherin-catenin complex on their plasma membrane; this complex plays a key role in cellular adhesion by maintaining tissue structure. When E-cadherin switches to N-cadherin this function is lost, resulting in cell invasion and dissemination of carcinoma cells [13, 14].

The growth factor-β (TGF-β) performs a dual role in carcinogenesis, being a tumor suppressor in the early stages and a tumor promoter in the later stages of carcinogenesis [15, 16]. TGF-β/SMAD signaling pathway is believed to play a major role in EMT. In this pathway, the activated complex SMAD2/SMAD3 binds the mediator SMAD4 and is translocated to the nucleus, where it further binds regulatory elements and induce the transcription of key genes associated with EMT, such as Snail1, ZEB1/2, and Twist. Despite the importance of TGF-β/SMAD pathway in EMT, the roles of TGF-β and SMAD4 in tumor progression remain controversial. Consistent with the role of TGF-β as a tumor suppressor is the observation that mutation of SMAD4 [17, 18] stimulates thyroid carcinogenesis and progression. Loss or weak expression of SMAD4 strongly correlates with invasion, metastasis, high histopathological grade, and poor prognosis of patients with diverse cancers as well [19–23]. Conversely, other studies suggest a tumor-promoting activity of TGF-β [24]. Likewise, the precise mechanism underlying the role of SMAD4 in the oncogenic response remains unclear. SMAD4 has been shown to act as an oncogene in tumorigenesis in previous studies [25–27]. Disrupting the association between SMAD4 and SMAD receptors also leads to the inhibition of EMT in human mammary epithelial cells [28]. Furthermore, knockdown of SMAD4 through RNA interference results in the inhibition of EMT [29–31]. In our previous work, we have suggested that TGF-β/SMAD signaling may be associated with the development and invasion of nonfunctioning pituitary adenomas [32].

Elucidating the potential role of SMAD4 in GHPAs may contribute to the improved diagnosis and treatment of invasive GHPA patients. In the present study, we first assessed SMAD4 expression in a cohort of GHPA patients and investigated the correlation between the expression of SMAD4 and the clinicopathological features of GHPA. Then, the markers of EMT were analyzed to determine the underlying mechanism of SMAD4 overexpression in GH3 cells.

2 Materials and methods

2.1 Patients and sample collection

The study was carried out on 61 patients with GHPA admitted at the Department of Neurosurgery in Beijing Tiantan Hospital (Capital Medical University) from April 2009 to November 2014. GHPAs were classified histologically according to the 2007 World Health Organization histologic classification of pituitary adenomas [33]. The cases that we selected met the following strict criteria: i) none of the patients had undergone somatostatin-analog therapy or radiotherapy during the preoperative period; ii) patients with recurrence were excluded; iii) adequate anamnestic and clinical data were available; and iv) the follow-up duration was a minimum of 2 years. The present study did not include a normal pituitary control group because of insufficient cases. Specimens were classified into two groups: non-invasive and invasive. The biological behavior of tumors was assessed based on preoperative magnetic resonance imaging (MRI) and computed tomography. GHPAs that were classified as Knosp classification grade III-IV and Hardy classification grade III-IV were defined as invasive.

GHPAs were resected by trans-sphenoidal surgery. Part of each specimen was formalin-fixed and paraffin-embedded to allow for analysis by tissue microarray (TMA), and the remainder was immediately snap-frozen in liquid nitrogen prior to further study. Postoperative sella MRI scans were performed within 72 h of surgery to evaluate the residual mass. Serial sella MRI scans were performed at 6-month intervals in the first 2 postoperative years and at 12-month intervals thereafter. MRI scans were performed immediately whenever the clinical symptoms occurred or reoccurred. Recurrence was identified based on one or more of the following parameters: i) for patients with total resection: the presence of a new tumor in the first postoperative sella MRI; ii) for patients with subtotal resection: the emergence of a new growth area in the first

postoperative sella MRI compared with the sella MRI scan; and iii) recurrence or aggravation of postoperative clinical symptoms accompanied by rising levels of growth hormone.

The present study was performed in accordance with the ethical standards of the Ethics Committee of Beijing Tiantan Hospital (Beijing, China). Written informed consent was obtained from all patients for surgery and for participation in the study.

2.2 TMA construction

Formalin-fixed paraffin-embedded tissue blocks were sliced and stained with hematoxylin and eosin (H&E). Three 2.0-mm diameter core biopsies were selected from the paraffin-embedded tissue blocks. The cores were transferred to TMAs and randomly ordered using the Leica Bond-III fully automated arrayer from Leica Biosystems (Aperio, CA, USA). The pathologists were blinded to the identity of the TMA slides. The TMAs were cut into 4-μm sections using a serial microtome and placed in a water bath at 50°C. They were then applied onto positively-charged glass slides. The 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 in a freezer at −80°C until further testing. To minimize the loss of antigenicity, the microarray slides were processed within 1 week of cutting.

2.3 Immunohistochemistry (IHC)

Prior to IHC, all TMA slides were H&E stained to evaluate the tumor content and quality. IHC tests with rabbit monoclonal anti-SMAD4 antibody (1:100, Abcam) were performed on the sections from all TMAs using the Leica Bond-III (Aperio, CA, USA) automated, random, and continuous access slide-staining system. IHC protocol F was selected in the machine, and 3 min ER1 (epitope retrieval) was set as the HIER (heat-induced epitope retrieval) parameter. The primary antibodies were detected by the Bond™ polymer refine detection system (Leica Biosystems, DS9800). The expression of slides was examined by an Aperio AT2 digital scanner (Leica Biosystems). The optimal titer of the primary antibodies was determined based on the pre-experimental results.

2.4 Evaluation of immunohistochemical staining

The sections were examined and scored by two pathologists who were unaware of the clinical records of the patients. Staining for SMAD4 was present in the cytoplasm and nucleus, and staining in either location was considered to indicate positive expression. Five fields were randomly selected at a ×200 magnification. The results were calculated using the Aperio AT2 system (Leica Biosystems) with digital slide viewing software. The staining intensity of SMAD4 was scored as follows: 0, no; 1, weak; 2, moderate; and 3, high intensity. The proportion of positively stained cells was scored as follows: 0 (<5%), 1 (6–25%), 2 (26–50%), 3 (51–75%), and 4 (>75%). The total score was calculated by multiplying the positive proportion score by the intensity score.

2.5 Cell culture and plasmid transfection

Rat GHPA cells (GH3) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum in a humidified incubator at 37°C in 5% CO_2. The culture medium was replaced every other day. The experimental GH3 cells were divided into three groups based on transfection: (1) the SMAD4 transfection groups, in which GH3 cells were transfected with p-CMV6-AC-GFP-SMAD4 (1μg plasmid in $1*10^6$ GH3 cells); (2) the negative control transfection group, in which GH3 cells were transfected with p-CMV6-AC-GFP; and (3) the untransfected group. The transfection was established using Lipofectamine™ 3000 according to the manufacturer's protocol (Invitrogen, CA, USA). The SMAD4 transfection group of cells was harvested 24, 48, 72, and 96 h after the transfection for further analysis. The untransfected group and the negative control transfection group of cells were harvested 72 h after transfection. Plasmids p-CMV6-AC-GFP-SMAD4 (GFP-tagged) that expresses full-length human SMAD4 and p-CMV6-AC-GFP were purchased from OriGene Technologies (Beijing, China).

2.6 RNA extraction and quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) assays

Total RNA was extracted from the frozen GHPAs specimens (40–60 mg) and the experimental GH3 cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA)

according to the manufacturer's protocol. The quality of total RNA was examined by UV spectrophotometry. The cDNA was synthesized using the SuperScript First-Strand Synthesis system with SuperScript II reverse transcriptase following the manufacturer's instructions (Invitrogen Life Technologies). RT-PCR was performed using an Applied Biosystems 7500 Fast system and Platinum SYBR-Green/ROX qPCR supermix-UDG (Invitrogen Life Technologies). The qRT-PCR reactions were performed using a 20 μL mix comprising 2×master mix (10 μL), forward primers (1 μl, 10 μmol/L), reverse primers (1 μL, 10 μmol/L), sample cDNA (1 μL), and double distilled water (ddH_2O; 7 μL). The amplification conditions were 50°C for 120 sec and 95°C for 120 sec, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. The fluorescence intensity values of PCR products were read after completion of the extension step. The expression of mRNA was determined from the threshold cycle (TC). The relative expression levels of the tested genes were normalized relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and calculated from the TC value using the $2^{\Delta\Delta TC}$ method for quantification [34].

2.7 Protein preparation and western blot analysis

GHPA specimens or the experimental GH3 cells were homogenized in ice-cold radio immunoprecipitation assay (RIPA) lysis buffer containing protease and phosphatase inhibitor cocktails (Roche, Mannheim, Germany). The homogenate was centrifuged at 12,000 *g* and 4°C for 30 min, then protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). After denaturing at 95°C for 5 min in loading buffer, 40 μg of the protein samples per lane were separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gel, then transferred to nitrocellulose membranes. The membranes were incubated with blocking buffer made of 5% non-fat milk in Tris-buffered saline Tween-20 (TBST) at room temperature for 1 h. Different blots were incubated with the corresponding primary antibodies against SAMD4 (1:2000, CST, USA), E-cadherin (1:2000, CST), N-cadherin (1:2000, CST), Vimentin (1:2000, CST) and GAPDH (1:8000, Sigma) at 4°C overnight, followed by three 10 min washes with TBST. Subsequently, membranes were incubated with secondary antibodies tagged with horseradish peroxidase (Abcam, Cambridge, USA) at room temperature for 1 h. Blots were visualized by enhanced chemiluminescence, and densitometry was performed with an Amersham Imager 6000 (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), and GAPDH levels were assessed as a loading control.

2.8 Cell migration and invasion assays

The migration and invasion of GH3 cells were measured using modified Transwell chambers (Corning, USA) coated with fibronectin and Matrigel in 24-well culture plates (BD Biosciences). GH3 cells (2×10^5 cells) of the SMAD4 transfection group, the negative control transfection group, and the untransfected control group after transient transfection for 72 h in serum-free DMEM, were respectively added onto membranes coated with fibronectin and Matrigel. After 24 h, migratory cells adhering to the lower membrane were fixed in 4% paraformaldehyde and stained using Harris stain. The average number of migratory cells was quantified by counting five random high-powered fields (100×) under a fluorescent microscope (ZEISS, Germany). All experiments were performed in triplicate.

2.9 Statistical analysis

χ^2 and Fisher's tests were used to analyze the significance of categorical variables. Differences between subgroups were analyzed using the Student's t-test for normally distributed continuous values. Variables that showed a relationship between SMAD4 expression levels and clinicopathologic characteristics were subjected to univariate analyses. All P-values were two-sided and P-values <0.05 were considered statistically significant. SPSS software version 17.0 was used for statistical analyses.

3 Results

3.1 Sample characteristics of the cohort

In this study, we retrospectively analyzed the clinicopathological features of 61 GHPA patients by standard H&E staining and IHC using an anti-SMAD4 antibody, as shown in Table 1. The patients' ages ranged from 16 to 75 years (median age: 39.5 years) including 32 males and 29 females. The largest tumor diameter ranged from 1.1 to 7.1 cm (median volume: 4.2 cm). All specimens were classified into either the invasive GHPA

Table 1: Clinicopathological data in 61 GHPAs cases.

Variable		Invasive	Non-Invasive
Case		19	42
Age(years)	Range	19-59	16-75
	Mean±SD	38.94±8.85	43.9±3.34
Gender	Male	12	20
	Female	7	22
HGH (ng/mL)		23.32±14.3	22.72±12.9
Tumor size	cm^3	33.6±23.2	3.88±1.01
Headache		14	15
Recurrence		7	9
SMAD4	H-Score≥6	15	10
	H-Score<6	4	32

Table 2: Association of SMAD4 levels and clinicopathological data in 61 GHPAs patients.

Variable		SMAD4		P-Value
		H-Score≥6	H-Score<6	
Age	≥45	15	17	p=0.326
	<45	10	19	
Gender	Male	12	20	p=0.561
	Female	13	16	
HGH(ng/mL)		23.03±13.75	22.82±14.04	p>0.05
Tumor size		15.89±23.87	11.08±26.15	p>0.05
Headache	Yes	16	13	p=0.032
	No	9	23	
Invasion	Yes	15	4	p=0.000
	No	10	32	
Recurrence	Yes	9	7	p=0.148
	No	16	29	

group (19 cases) or the non-invasive GHPA group (42 cases) according to the Knosp and Hardy classification systems. Of the tumors, 40 were gross total resections and 21 were subtotal resections. The serum GH levels of the preoperative patients ranged from 0.13 to 40 ng/mL (22.9±13.22 ng/mL) and from 0.27 to 22.4 ng/mL (9.41±7.05 ng/mL) after surgery.

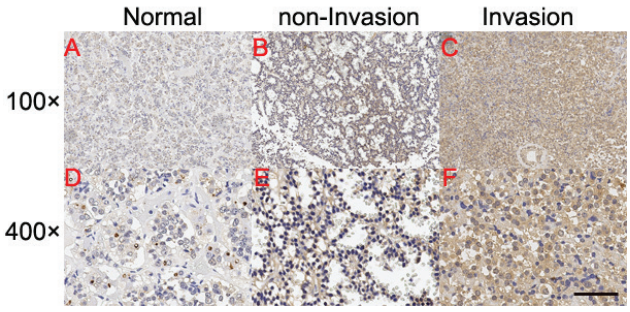

Figure 1: IHC analysis of SMAD4 expression in the GHPAs. A-C: 50×; D-F: 400× magnification. bar=60μm.

3.2 Tissue microarray analysis

SMAD4 protein expression was investigated in the 61 GHPA specimens by TMA, as shown in Figure 1. The results were categorized as either high level (H-Score ≥ 6) or low level (H-Score < 6) expression. Using univariate analysis, SMAD4 expression showed no association with age, gender, tumor size, or preoperative serum GH levels. High SMAD4 expression was detected in 15/19 (78.9%) invasive GHPA cases and 10/42 (23.8%) noninvasive GHPA cases according to the H-Score analysis (χ^2=16.44, p=0.000) (Table 2). In the high SMAD4 expression group, a headache occurred in 16/25 cases (64%), compared with only 13/36 cases (36.1%) in the low SMAD4 expression group (χ^2=4.565, p=0.033). The follow-up interval for the 61 cases ranged from 2 to 7 years (mean = 3.6 years). In 16 cases (26.3%), tumor recurrence was detected during the follow-up period.

3.3 qRT-PCR and western blot analysis of the expression of SMAD4 and the genes related to EMT in GHPA specimens

The mRNA levels of SMAD4 in the 19 invasive GHPA specimens were nearly three-fold higher than those of the 42 non-invasive specimens (p<0.001). To investigate the expression of EMT-related genes in the GHPAs, we analyzed the epithelial marker E-cadherin as well as the mesenchymal markers N-cadherin and vimentin. Our results showed a significant downregulation of E-cadherin mRNA (0.141-fold, p<0.001) and upregulation of both the N-cadherin (8.87-fold, p<0.001) and Vimentin (5.24-fold, p<0.001) mRNA levels in the invasive GHPAs compared with the non-invasive group, as shown in Table 3 and Figure 2A.

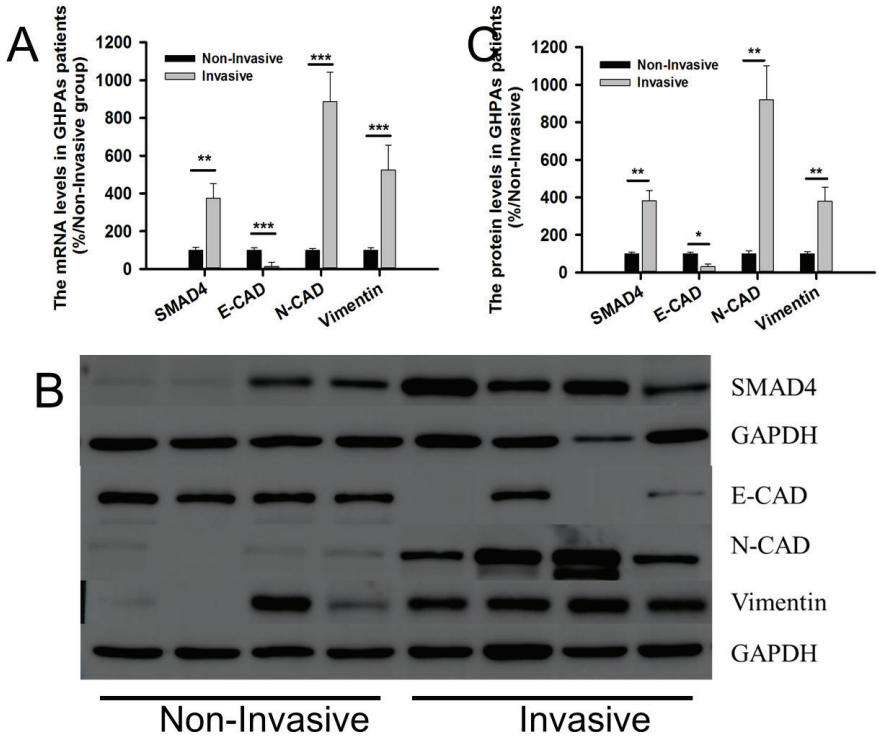

Figure 2: PCR and western blot analysis of SMAD4 and the genes related to EMT in the GHPA specimens. A: The mRNA levels of SMAD4, E-cadherin (E-CAD), N-cadherin (N-CAD), and Vimentin in the 61 GHPA specimens. B: The protein levels of SMAD4, E-CAD, N-CAD, and Vimentin in the 61 GHPA specimens. C: Statistical analysis of the western blot data.

Western blot analysis was used to detect SMAD4 expression in the 19 invasive specimens and 42 non-invasive specimens. The protein levels of SMAD4, E-cadherin, N-cadherin, and vimentin in the invasive group were 3.83-fold, 0.31-fold, 9.2-fold, and 3.8-fold those of the non-invasive group, respectively, as shown in Figure 2B,C. According to the median mRNA levels, the PFS in the high SMAD4 group was lower than that in the low SMAD4 group (p=0.026), as shown in Figure 3.

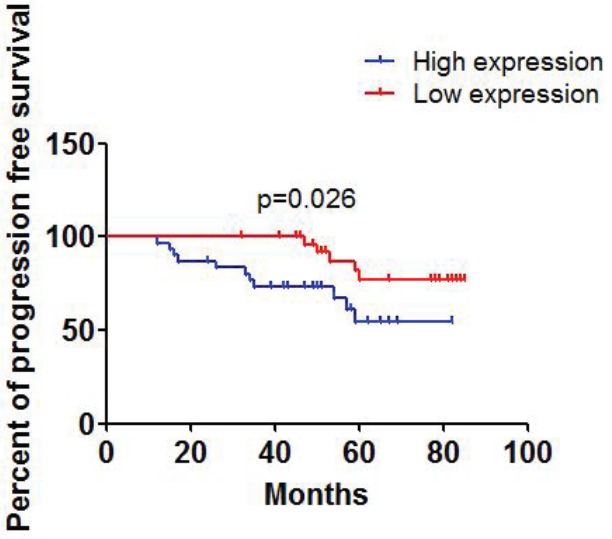

Figure 3: PSF of 61 GHPAs according to the SMAD4 levels.

3.4 Overexpression of SMAD4 regulated the genes related to EMT in GH3 cells

The efficiency of overexpression of SMAD4 was first measured in GH3 cells. The mRNA levels of SMAD4 were 2.77, 5.69, 60.55, and 90.61-fold that of the control group at 24, 48, 72, and 96 h after transfection, respectively (Figure 4A). Western blot analysis showed that the SMAD4 protein levels in GH3 cells were 1.1, 1.5, 3.8, and 4.1-fold that of the control group at 24, 48, 72, and 96 h after transfection (Figures 4B and 4C). EMT in tumor cells was characterized by decreased expression of the epithelial marker E-cadherin and increased expression of the mesenchymal markers N-cadherin and vimentin. Our findings showed significant downregulation of E-cadherin mRNA levels and upregulation of N-cadherin and vimentin mRNA levels after overexpression of SMAD4 compared with the control group (p<0.001). Furthermore,

Table 3: The mRNA levels of SMAD4, E-CAD, N-CAD and vimentin in 61 GHPAs patients.

Number	Category	SMAD4 (ΔCT)	E-CAD (ΔCT)	N-CAD (ΔCT)	Vimentin (ΔCT)
1	Invasive	-1.293	-0.61079	-1.81642	-1.56792
2	Invasive	-1.92385	-0.42722	-2.19278	0.372415
3	Invasive	-1.58734	1.188364	-1.80819	-0.14489
4	Invasive	1.719958	0.571421	3.37209	-0.88194
5	Invasive	-2.12263	3.668694	-1.36751	1.388776
6	Invasive	1.88306	4.390532	2.943225	0.968368
7	Invasive	1.730274	4.234172	2.472812	-0.17352
8	Invasive	-3.23187	-0.238261	-2.436719	0.287138
9	Invasive	2.06882	2.683476	1.287182	2.381739
10	Invasive	-2.1192	3.628347	0.287192	1.562841
11	Invasive	1.66823	2.36882	-2.382191	-0.382126
12	Invasive	1.49382	1.283021	-1.648736	-1.283276
13	Invasive	-2.892245	4.281293	-0.281362	-2.038263
14	Invasive	-0.287225	1.937266	-2.182351	-0.273164
15	Invasive	-3.82382	-1.223184	-2.628711	-0.382134
16	Invasive	-2.332688	2.326684	4.286371	-1.382142
17	Invasive	-1.882132	3.281163	-0.273618	3.281724
18	Invasive	2.018455	3.826783	2.832813	-2.028172
19	Invasive	1.663298	4.287122	1.271386	-1.76831
20	Non-Invasive	1.774347	-1.61018	2.617881	3.229507
21	Non-Invasive	2.015728	-0.41521	3.798973	3.372608
22	Non-Invasive	2.089746	-1.63087	6.494172	2.04606
23	Non-Invasive	3.134202	-1.11013	6.053079	3.166369
24	Non-Invasive	2.548817	-1.02282	4.660718	2.442292
25	Non-Invasive	2.700965	-0.54788	3.569956	1.762701
26	Non-Invasive	-1.229832	2.118293	5.428319	-0.128321
27	Non-Invasive	3.028701	3.629364	4.812938	3.381529
28	Non-Invasive	1.832922	-2.128236	-1.281276	2.896134
29	Non-Invasive	2.028813	-1.112837	5.287391	3.729716
30	Non-Invasive	-2.228612	-2.182263	4.289162	-1.273821
31	Non-Invasive	4.132422	-3.623864	-1.721082	4.012371
32	Non-Invasive	2.632133	-0.282217	4.398264	-0.274812
33	Non-Invasive	2.882612	3.627822	2.382196	3.278516
34	Non-Invasive	3.118227	-0.182293	4.187392	3.193725
35	Non-Invasive	4.22821	-1.283376	-2.018274	3.726351
36	Non-Invasive	2.182231	0.267381	1.829722	4.283714
37	Non-Invasive	3.016322	0.287134	3.826716	0.372871

Continued **Table 3:** The mRNA levels of SMAD4, E-CAD, N-CAD and vimentin in 61 GHPAs patients.

Number	Category	SMAD4 (ΔCT)	E-CAD (ΔCT)	N-CAD (ΔCT)	Vimentin (ΔCT)
38	Non-Invasive	-1.022386	-0.278391	4.762894	3.298417
39	Non-Invasive	-1.339821	-1.282733	3.827391	2.384612
40	Non-Invasive	3.208835	-2.378216	3.628611	3.748219
41	Non-Invasive	-2.776231	4.282671	-1.628942	0.382182
42	Non-Invasive	4.129833	-1.483255	-0.281126	-1.328376
43	Non-Invasive	3.102132	-3.275128	5.729812	3.642813
44	Non-Invasive	1.228324	0.238719	2.6127356	2.0274325
45	Non-Invasive	3.287634	2.217392	6.328712	3.172831
46	Non-Invasive	-0.238712	-2.117269	4.287326	4.482136
47	Non-Invasive	3.228766	-1.281827	6.1236716	2.09152
48	Non-Invasive	4.286624	-0.281164	4.528719	2.347619
49	Non-Invasive	0.441029	-0.821284	-0.1702812	3.291723
50	Non-Invasive	0.310719	-3.182923	-1.283298	-0.128362
51	Non-Invasive	1.458597	-2.182936	3.738721	-1.392134
52	Non-Invasive	0.361096	-1.298731	4.627684	3.681472
53	Non-Invasive	0.135615	0.281129	5.201724	4.0126329
54	Non-Invasive	-0.5524	-0.21293	3.728721	3.729172
55	Non-Invasive	0.855096	-0.821927	4.038217	2.738124
56	Non-Invasive	0.654499	-0.284621	2.483901	-0.281462
57	Non-Invasive	0.208053	2.371254	3.782143	-1.283581
58	Non-Invasive	1.294783	-3.820187	-0.23817	3.672193
59	Non-Invasive	-0.52388	-2.321821	1.283471	4.012834
60	Non-Invasive	-0.21548	-0.273491	0.263871	1.293744
61	Non-Invasive	-0.97771	-1.283927	6.016239	3.569214

the data showed significant downregulation of E-cadherin and upregulation of N-cadherin and vimentin compared with the negative control transfection group according to western blot analysis, as shown in Figure 4 (p<0.001). The following experiments were performed 72 h after transfection in GH3 cells.

3.5 Overexpression of SMAD4 promoted the invasion of GH3 cells

Consistent with our observations in the GHPA specimen assays, we measured cell invasion and migration by performing Transwell assays *in vitro* and found that overexpression of SMAD4 expression greatly increased the migratory and invasive behaviors of GH3 cells, along with elevated N-cadherin and vimentin levels. The number of positive cells was 761±159 after 72 h transfection in GH3 cells, and 184±57 in the control group (Figure 5). The transmembrane invasion assay revealed that overexpression of SMAD4 significantly increased the invasion of GH3 cells, which positively correlated with SMAD4 levels.

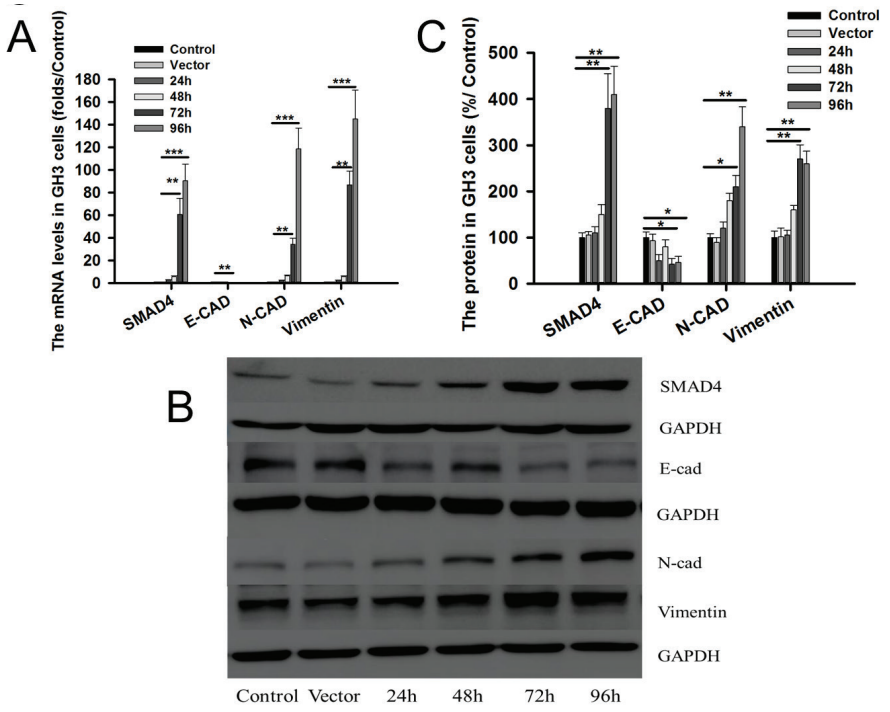

Figure 4: PCR and western blot analysis of the expression of SMAD4 and genes related to EMT in GH3 cells. A: The mRNA levels of SMAD4 and genes related to EMT closely correlated with transfection time. B: The protein levels of SMAD4, E-CAD, N-CAD, and Vimentin in GH3 cells after overexpression of SMAD4. The levels of SMAD4 and Vimentin peaked after 72 h of transfection. C: Statistical analysis of western blot data.

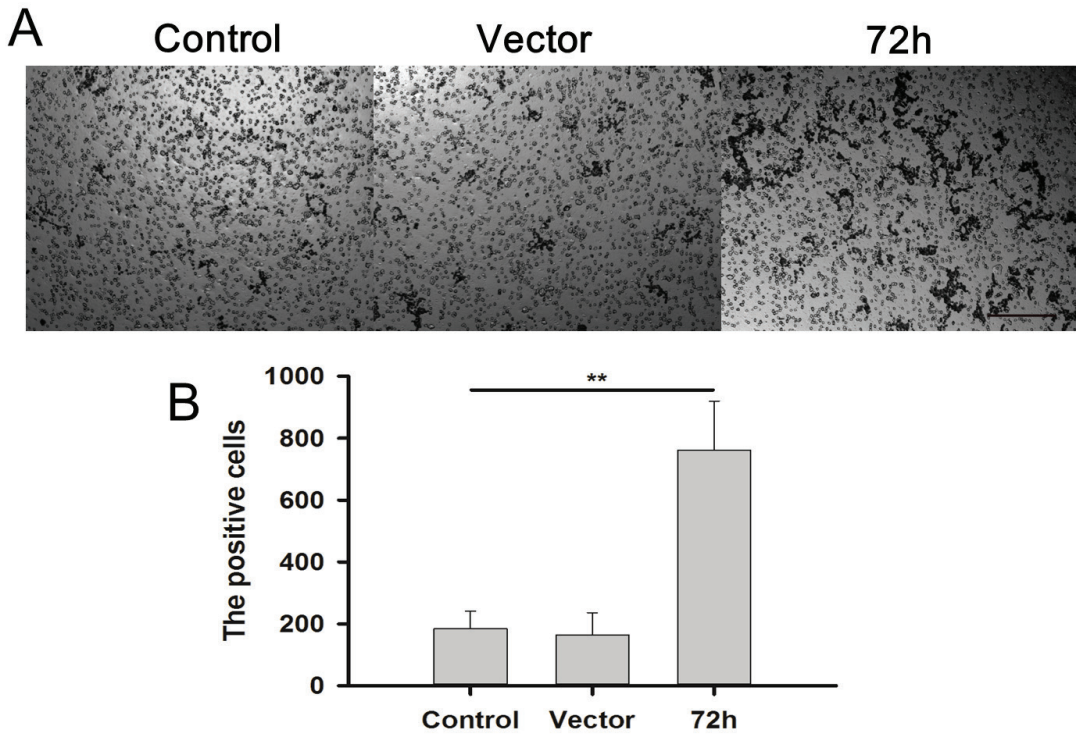

Figure 5: Overexpression of SMAD4 promoted the invasion of GH3 cells. A: Positive cells in the Transwell test. bar=60μm B: The statistical results of the Transwell test. The number of positive cells overexpressing SMAD4 was nearly 4-fold that of the control group.

4 Discussion

The aim of this study was to determine whether there is a relationship between the expression of SMAD4 and clinicopathological features in patients with GHPAs. In tumor specimens, we found a significant upregulation of SMAD4 levels in invasive GHPAs compared with noninvasive GHPAs by TMAs. This finding was confirmed by RT-PCR and western blot analysis. To the best of our knowledge, this is the first study to demonstrate a correlation between SMAD4 expression and GHPAs.

Our results indicate that upregulation of SMAD4 may promote the malignant potential of GHPAs. This discovery was unexpected in light of the dogma that SMAD4 is a potent tumor suppressor. The SMAD4 gene (also known as DPC4), which was initially identified as a candidate tumor suppressor in various human tumors and is deleted in pancreatic carcinoma, is located at locus 4 on chromosome 18q21.1 [35]. Mutations resulting in loss of SMAD4 expression or weak SMAD4 expression strongly are correlated with poor prognosis [36–38]. However, SMAD4 exerts a tumor-promoting role in hepatocellular carcinoma and breast cancer [39, 40]. Therefore, the role of SMAD4 is likely more complex than simply a tumor suppressor. SMAD4 may have various functions in different tumor types and at different stages of tumor development, which is consistent with the dichotomous function of TGF-β signaling.

Various steps are involved in the progression of tumor cells, including invasion, which requires EMT. E-cadherin is typically expressed in epithelial cells and provides a physical link between adjacent cells and intracellular cytoskeleton [14]. Downregulated expression of E-cadherin destroys the intracellular junction and thus epithelial cells acquire the ability to migrate, which facilitates tumor invasion and metastasis [41]. Tumor cells that express mesenchymal markers such as N-cadherin and vimentin have a greater tendency to be invasive and metastasize than tumor cells only expressing epithelial markers [42, 43]. In this study, we found that mRNA and protein expression levels of E-cadherin were significantly reduced in invasive GHPAs compared with non-invasive GHPAs. This finding is in agreement with previous studies [44, 45]. We also found that the mRNA and protein expression levels of N-cadherin and vimentin are significantly upregulated in invasive GHPAs compared with non-invasive GHPAs. Therefore, our study confirmed previous suggestions that EMT plays an important role in pituitary adenoma progression [46, 47]. Our analysis of the changes in EMT biomarkers in invasive GHPAs strengthens this conclusion.

TGF-β/SMAD signaling has been identified to play a significant role during the activation of EMT in various cancers [48]. SMAD4 is indispensable for TGF-β-induced EMT [49, 50]. It has previously been shown that ectopic SMAD4 expression levels could affect the activation of EMT. Yang and colleagues reported that SMAD4 mRNA levels positively correlate with N-cadherin mRNA levels in human NSCLC tissue [51]. *In vitro* studies showed that overexpression of SMAD4 enhance TGF-β-induced EMT in NMuMG cells [52] and PC3 cells [31]. To further investigate the mechanism, we analyzed the invasion of GH3 cells in an *in vitro* model by altering the levels of SMAD4 using transfection technology, and assessed whether EMT was induced using EMT biomarkers. Firstly, western blot analysis revealed a low level of SMAD4 expression in wild-type GH3 cells. Following transfection of the SMAD4 gene into GH3 cells, a Transwell assay revealed that upregulation of SMAD4 significantly promoted the invasion of GH3 cells, indicating that invasion was positively correlated with SMAD4 expression. Further studies showed that the overexpression of SMAD4 downregulated the level of E-cadherin, and upregulated the levels of N-cadherin and vimentin. These results suggested that the overexpression of SMAD4 promoted the invasion of GH3 cells by inducing EMT. An increasing number of studies have shown that tumor cell invasion is suppressed when EMT is inhibited by targeting SAMD4 [31, 53–55]. Our study demonstrated the relationship between the expression of SMAD4 and the induction of EMT in tumor progression, revealing the mechanism underlying the role of SMAD4 in the oncogenic response. Taken together, our findings provide new insight into the potential role of SMAD4 in promoting the invasion of GHPAs and suggest that SMAD4 is a potential molecular target for developing new therapeutic strategies to combat invasive GHPAs via the suppression of EMT.

A previous study showed that SMAD4 overexpression in hepatocellular carcinoma was strongly associated with overexpression of TGF-β receptors [56], indicating activation or dysregulation of the TGF-β signaling pathway during tumorigenesis. Inducing EMT in tumors may contribute to the switch in TGF-β signaling from a tumor-suppressive to a tumor-promoting pathway. The expression of other upstream markers of the TGF-β pathway or downstream markers regulated by SMAD4 that affect EMT in GHPAs should be investigated in future studies.

In summary, our study demonstrated that the overexpression of SMAD4 is positively linked to the invasion and recurrence of GHPAs. Furthermore, the mechanism of action of SMAD4 may be associated with induction of EMT. Our *in vitro* studies indicated that

upregulation of SMAD4 promote invasion of GH3 cells by inducing EMT. Therefore, SMAD4 may be an effective biomarker for predicting the invasion and recurrence of GHPAs, and a potential therapeutic target for patients with invasive GHPAs.

Acknowledgements: This study was supported by the National High-tech Research and Development Program of China (863 Program, 2015AA020504), National Natural Science Foundation of China (81601205), Beijing Natural Science Foundation of China (7162035), and the Beijing High-level Talent Plan (2015-3-040).

Conflict of Interest: Authors declare no conflict of interest.

References

[1] Ezzat S., Asa S.L., Couldwell W.T., Barr C.E., Dodge W.E., Vance M.L., et al., A systematic review, Cancer., 2004, 101, 613-619.
[2] Aghakhani K., Kadivar M., Kazemi-Esfeh S., Zamani N., Moradi M., Sanaei-Zadeh H., Prevalence of pituitary incidentaloma in the Iranian cadavers, Indian. J. Pathol. Microbiol., 2011, 54, 692-694.
[3] Asa S.L., Ezzat S., The pathogenesis of pituitary tumors, Annu. Rev. Pathol., 2009, 4, 97-126.
[4] Ribeiro-Oliveira A. Jr., Barkan A., The changing face of acromegaly--advances in diagnosis and treatment, Nat. Rev. Endocrinol.,2012, 8(10), 605-611.
[5] Giustina A., Chanson P., Kleinberg D., Bronstein M.D., Clemmons D.R., Klibanski A., et al., Expert consensus document: A consensus on the medical treatment of acromegaly, Nat. Rev. Endocrinol., 2014, 10(4), 243-248.
[6] Marquez Y., Tuchman A., Zada G., Surgery and radiosurgery for acromegaly: a review of indications, operative techniques, outcomes, and complications, Int. J. Endocrinol., 2012, 386-401.
[7] Shimon I., Jallad R.S., Fleseriu M., Yedinak C.G., Greenman Y., Bronstein MD., Giant GH-secreting pituitary adenomas: management of rare and aggressive pituitary tumors, Eur. J. Endocrinol., 2015, 172(6), 707-713.
[8] Starke R.M., Raper D.M.S., Payne S.C., Vance M.L., Oldfield E.H., Jane J.J., Endoscopic vs microsurgical transsphenoidal surgery for acromegaly: outcomes in a concurrent series of patients using modern criteria for remission, J. Clin. Endocrinol. Metab., 2013, 98(8), 3190-3198.
[9] Jane J.J., Starke R.M., Elzoghby M.A., Reames D.L., Payne S.C., Thorner M.O., et al., Endoscopic transsphenoidal surgery for acromegaly: remission using modern criteria, complications, and predictors of outcome, J. Clin. Endocrinol. Metab., 2011, 96(6), 2732-2740.
[10] Shirvani M., Motiei-Langroudi R., Transsphenoidal surgery for growth hormone-secreting pituitary adenomas in 130 patients, World. Neurosurg., 2014, 81(1), 125-130.
[11] Meta O., Ezzat S., Asa SL., Biomarkers of aggressive pituitary adenomas, J. Mol. Endocrinol., 2012, 49, R69-78.
[12] Sav A., Rotondo F., Syro L.V., Scheithauer B.W., Kovacs K., Biomarkers of pituitary neoplasms, Anticancer. Res., 2012, 32, 4639-4654.
[13] Su Y., Li J., Shi C., Radice GL., N-cadherin functions as a growth suppressor in a model of K-ras-induced PanIN, Oncogene, 2016, 35(25), 3335-3341.
[14] Chauvet N., Romanò N., Meunier A.C., Galibert E., Fontanaud P., Mathieu M.N., Combining Cadherin Expression with Molecular Markers Discriminates Invasiveness in Growth Hormone and Prolactin Pituitary Adenomas, J Neuroendocrinol, 2016, 28(2),12352.
[15] Roberts A.B., Wakefield LM., The two faces of transforming growth factor β in carcinogenesis, PNAS, 2003, 100, 8621-8623.
[16] Saitoh M., Epithelial-mesenchymal transition is regulated at post-transcriptional levels by transforming growth factor-β signaling during tumor progression, Cancer. Sci., 2015, 106(5), 481-488.
[17] Mizuno T., Cloyd J.M., Vicente D., Omichi K., Chun Y.S., Kopetz S.E., et al., SMAD4 gene mutation predicts poor prognosis in patients undergoing resection for colorectal liver metastases, Eur. J. Surg. Oncol., 2018,44(5),684-692.
[18] Nikolic A., Ristanovic M., Zivaljevic V., Rankov A.D., Radojkovic D., Paunovic I., SMAD4 gene promoter mutations in patients with thyroid tumors, Exp. Mol. Pathol., 2015,99(1),100-103.
[19] Grover P., Nath S., Nye M.D., Zhou R., Ahmad M., Mukherjee P., SMAD4-independent activation of TGF-β signaling by MUC1 in a human pancreatic cancer cell line, Oncotarget, 2018, 9(6), 6897-6910.
[20] Coates R.F., Gardner J.A., Gao Y., Cortright V.M., Mitchell J.M., Ashikaga T., et al., Significance of positive and inhibitory regulators in the TGF-β signaling pathway in colorectal cancers, Hum. Pathol., 2017, 66,34-39.
[21] Yamamoto T., Kawada K., Itatani Y., Inamoto S., Okamura R., Iwamoto M., et al., Loss of SMAD4 Promotes Lung Metastasis of Colorectal Cancer by Accumulation of CCR1+ Tumor-Associated Neutrophils through CCL15-CCR1 Axis, Clin. Cancer. Res., 2017,23(3),833-844.
[22] Izumi S., Nakamura S., Mano S., Akaki S., Well differentiation and intact Smad4 expression are specific features of groove pancreatic ductal adenocarcinomas, Pancreas, 2015 ,44(3),394-400.
[23] Ding Z.Y., Jin G.N., Wang W., Chen W.X., Wu Y.H., Ai X., et al., Reduced expression of transcriptional intermediary factor 1 gamma promotes metastasis and indicates poor prognosis of hepatocellular carcinoma, Hepatology., 2014,60(5),1620-1636.
[24] Valcourt U., Kowanetz M., Niimi H., Heldin C.H., Moustakas A., TGF-beta and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition, Mol. Biol. Cell., 2005, 16(4), 1987-2002.
[25] Medrano EE., Repression of TGF-beta signaling by the oncogenic protein SKI in human melanomas: consequences for proliferation, survival, and metastasis, Oncogene., 2003, 19,22(20), 3123-3129.
[26] Tetteh P.W., Kretzschmar K., Begthel H., van den Born M., Korving J., Morsink F., et al., Generation of an inducible colon-specific Cre enzyme mouse line for colon cancer research, Proc. Natl. Acad. Sci. USA., 2016,113(42),11859-11864.

[27] Zhao S., Wang Y., Cao L., Ouellette M.M., Freeman J.W., Expression of oncogenic K-ras and loss of Smad4 cooperate to induce the expression of EGFR and to promote invasion of immortalized human pancreas ductal cells, Int. J. Cancer., 2010, 127(9), 2076-2087.
[28] Hesling C., Fattet L., Teyre G., Jury D., Gonzalo P., Lopez J., et al., Antagonistic regulation of EMT by TIF1 gamma and Smad4 in mammary epithelial cells, EMBO. Rep., 2011, 12(7), 665-672.
[29] Deckers M., van Dinther M., Buijs J., Que I., Löwik C., van der Pluijm G., et al., The tumor suppressor Smad4 is required for transforming growth factor beta-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells, Cancer. Res., 2006, 66(4), 2202-2209.
[30] Kang Y., Ling J., Suzuki R., Roife D., Chopin-Laly X., Truty M.J., et al., SMAD4 regulates cell motility through transcription of N-cadherin in human pancreatic ductal epithelium, PLoS. One., 2014, 9(9), e107948.
[31] Lan X., Lu G., Yuan C., Mao S., Jiang W., Chen Y., et al., Valproic acid (VPA) inhibits the epithelial-mesenchymal transition in prostate carcinoma via the dual suppression of SMAD4, J. Cancer. Res. Clin. Oncol., 2016, 142(1), 177-185.
[32] Zhenye L., Chuzhong L., Youtu W., Xiaolei L., Lei C., Lichuan H.,et al., The expression of TGF-β1, Smad3, phospho-Smad3 and Smad7 is correlated with the development and invasion of nonfunctioning pituitary adenomas, J. Transl. Med., 2014,2,71.
[33] Roncaroli F., Scheithauer B.W., Papillary tumor of the pineal region and spindle cell oncocytoma of the pituitary: new tumor entities in the 2007 WHO Classification, Brain. Pathol., 2007, 17(3), 314-318.
[34] Gao H., Xue Y., Cao L., Liu Q., Liu C., Shan X., et al., ESR1 and its antagonist fulvestrant in pituitary adenomas, Mol. Cell. Endocrinol.,2017, 443, 32-41.
[35] Hahn S.A., Schutte M., Hoque A.T., Moskaluk C.A., da Costa L.T., Rozenblum E., DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1, Science,1996, 271(5247), 350-353.
[36] Liu N., Yu C., Shi Y., Jiang J., Liu Y., SMAD4 expression in breast ductal carcinoma correlates with prognosis, Oncol. Lett., 2015, 10(3), 1709-1715.
[37] Yan P., Klingbiel D., Saridaki Z., Ceppa P., Curto M., McKee T.A., et al., Reduced Expression of SMAD4 Is Associated with Poor Survival in Colon Cancer, Clin. Cancer. Res., 2016, 22(12), 3037-3047.
[38] Singhi A.D., Foxwell T.J., Nason K., Cressman K.L., McGrath K.M., Sun W., et al., Smad4 loss in esophageal adenocarcinoma is associated with an increased propensity for disease recurrence and poor survival., Am. J. Surg. Pathol., 2015, 39(4), 487-495.
[39] Hernanda P.Y., Chen K., Das A.M., Sideras K., Wang W., Li J., et al., SMAD4 exerts a tumor-promoting role in hepatocellular carcinoma, Oncogene, 2015, 34(39), 5055-5068.
[40] Tram E., Ibrahim-Zada I., briollais L., Knight J.A., Andrulis I.L., Ozcelik H., Identification of germline alterations of the mad homology 2 domain of SMAD3 and SMAD4 from the Ontario site of the breast cancer family registry (CFR), Breast. Cancer. Res., 2011, 13(4), R77.
[41] Takeichi M., Cadherin cell adhesion receptors as a morphogenetic regulator, Science,1991, 251(5000), 1451-1455.
[42] Scanlon C.S., Van Tubergen E.A., Inglehart R.C., D'Silva NJ,. Biomarkers of epithelial-mesenchymal transition in squamous cell carcinoma, J. Dent Res., 2013, 92(2), 114-121.
[43] Shih W., Yamada S., N-cadherin-mediated cell-cell adhesion promotes cell migration in a three-dimensional matrix, J. Cell. Sci., 2012, 125(Pt 15), 3661-3670.
[44] Zhou K., Jin H., Luo Y., Expression and significance of E-cadherin and β-catenins in pituitary adenoma, Int. J. Surg. Pathol., 2013, 21(4), 363-367.
[45] Fougner S.L., Lekva T., Borota O.C., Hald J.K., Bollerslev J., Berg J.P., The expression of E-cadherin in somatotroph pituitary adenomas is related to tumor size, invasiveness, and somatostatin analog response, J. Clin. Endocrinol. Metab., 2010, 95(5), 2334-2342.
[46] Jia W., Zhu J., Martin T.A., Jiang A., Sanders A.J., Jiang W.G., Epithelial-mesenchymal Transition (EMT) Markers in Human Pituitary Adenomas Indicate a Clinical Course, Anticancer. Res., 2015, 35(5), 2635-2643.
[47] Lekva T., Berg J.P., Fougner S.L., Olstad O.K., Ueland T., Bollerslev J., Gene expression profiling identifies ESRP1 as a potential regulator of epithelial mesenchymal transition in somatotroph adenomas from a large cohort of patients with acromegaly, J. Clin. Endocrinol. Metab., 2012, 97(8), E1506-1514.
[48] Massague J., TGF-beta in cancer, Cell., 2008, 134, 215-230.
[49] . Xie F., Zhang Z., van Dam H., Zhang L., Zhou F., Regulation of TGF-β Superfamily Signaling by SMAD Mono-Ubiquitination, Cells., 2014, 3(4), 981-993.
[50] Schwarte-Waldhoff I., Schmiegel W., Smad4 transcriptional pathways and angiogenesis, Int. J. Gastrointest. Cancer., 2002, 31(1-3), 47-59.
[51] Yang H., Wang L., Zhao J., Chen Y., Lei Z., Liu X., et al., TGF-β-activated SMAD3/4 complex transcriptionally upregulates N-cadherin expression in non-small cell lung cancer, Lung. Cancer., 2015, 87(3), 249-257.
[52] Guo J., Song B., Li X., Hε C., Yang G., Yang X., et al., Downregulation of gangliotetraosylceramide and β1,3-galactosyltransferase-4 gene expression by Smads during transforming growth factor β-induced epithelial-mesenchymal transition, Mol. Med. Rep., 2015, 11(3), 2241-2247.
[53] Liu Y., Li Y., Wang R., Qin S., Liu J., Su F., et al., MiR-130a-3p regulates cell migration and invasion via inhibition of Smad4 in gemcitabine resistant hepatoma cells, J. Exp. Clin. Cancer. Res., 2016, 35, 19.
[54] Qiao P., Li G., Bi W., Yang L., Yao L., Wu D., microRNA-34a inhibits epithelial mesenchymal transition in human cholangiocarcinoma by targeting Smad4 through transforming growth factor-beta/Smad pathway, BMC. Cancer., 2015, 15, 469.
[55] Lee C.C., Yang W.H., Li C.H., Cheng Y.W., Tsai C.H., Kang J.J., Ligand independent aryl hydrocarbon receptor inhibits lung cancer cell invasion by degradation of Smad4, Cancer. Lett., 2016, 376(2), 211-217.
[56] Torbenson M., Marinopoulos S., Dang D.T., Choti M., Ashfaq R., Maitra A., et al., Smad4 overexpression in hepatocellular carcinoma is strongly associated with transforming growth factor beta II receptor immunolabeling, Hum. Pathol., 2002, 33(9), 871-876.