Original Article
Pit-1 mRNA and protein expression in human pituitary adenomas

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Abstract: Aims: To investigate the role of Pit-1 in the functional differentiation of pituitary adenoma cells. Methods and results: All 104 eligible cases were acquired from the archives of the Pathology Department, the Fifth People’s Hospital of Shanghai, Fudan University, China. Patient age ranged from 16 to 82 years (median 45.6 years). Pit-1 protein was detected via immunohistochemistry (IHC). Positive Pit-1 expression was observed in 46.2% (48/104) of the cases. Of the 48 positive cases, 16 (33.3%) were classified as 1+; 24 (50.0%) were 2+; and 8 (16.7%) showed 3+. The rates of positivity for the Pit-1 protein in thyroid-stimulating hormone (TSH) adenomas (100%, 4/4) and growth hormone (GH)/prolactin (PRL) adenomas (88.0%, 22/25) were significantly higher than those in other types of adenomas (P<0.05). Among the 104 cases, fresh specimens were collected from 22 patients to detect Pit-1 mRNA levels via real-time reverse transcription polymerase chain reaction (RT-PCR). Pit-1 mRNA was detected in 36.4% (8/22) of the pituitary adenomas. Among these 8 positive cases, 7 were GH/PRL or TSH adenomas, and the remaining case was an FSH/LH adenoma. Pit-1 mRNA levels were significantly increased in GH/PRL and TSH adenomas compared with other types of pituitary adenomas and pituitary tissues (P<0.05). No significant difference was found between the detection of Pit-1 protein and mRNA (P>0.05). Conclusions: Pit-1 plays an important role in the development of pituitary adenomas. However, some additional transcriptional factors or enhancers may be required, and further investigation is necessary to clearly determine the role of Pit-1.

Keywords: Pit-1, pituitary adenoma, immunohistochemistry, RT-PCR

Introduction
The anterior pituitary regulates a broad spectrum of physiological functions including growth, reproduction, lactation, and general metabolism. The endocrine functions of the pituitary gland are accomplished by six cell types, which are defined by the hormones that they produce and excrete: somatotrophs (secrete growth hormone [GH]), lactotrophs (produce prolactin [PRL]), thyrotrophs (secrete thyroid-stimulating hormone [TSH]), corticotrophs (produce adrenocorticotropic hormone [ACTH]), gonadotrophs (secrete luteinizing hormone [LH] or follicle-stimulating hormone [FSH]) and IL-specific melanotrophs (secrete melanocyte-stimulating hormone) [1].

Pituitary adenomas are relatively uncommon neoplasms, accounting for about 10% of all intracranial tumors and 90% of intrasellar lesions, and have an annual incidence of about 25 per million population: more than two-thirds are characterized by excess hormone secretion, which manifests as a clinical syndrome [2]. Most pituitary adenomas are histologically benign and grow slowly, causing neither endocrine dysfunction nor local mass effects. However, a percentage undergo parasellar invasion, postoperative recurrence and, on rare occasions, craniospinal and/or systemic metastasis [3].

Although they are monoclonal in nature, pituitary adenomas exhibit functional diversity and behavioral unpredictability. Therefore, much effort has focused on evaluating tumorigenesis-inducing molecular factors and their correlation with clinicopathological studies. However, the precise pathogenesis of pituitary tumor formation is currently far from established. Some insight into pituitary pathogenesis may be
derived from events that occur during normal embryonic pituitary development. Human anterior pituitary development begins with invagination of the oropharyngeal epithelium (stomodeum) to form Rathke's pouch in the fourth week of gestation and is complete by week 12. The signalling pathways and transcription factors involved in pituitary gland development are continually being characterized. Several pituitary transcription factors and cofactors have been reported to be active in all or a subset of pituitary cell lineages during a specific developmental period. Numerous growth factors (TGF-α and TGF-β, EGF, and FGFs), growth factor receptors (EGF-R and FGF-R4), cyclin-dependent kinases (CDKs), and other cell cycle regulators (e.g., PTTG, a member of the securin family), have been implicated in tumorigenesis, but convincing evidence regarding their primary roles remains lacking. However, there has been increasing interest in transcription factors that are involved in pituitary development (e.g., Pit-1, FGF, and BMP) as important mediators of adenoma formation [2]. Of particular importance, and currently best characterized, are the transcription factors Pit-1 and Prop-1. In this article, we focus on the potential role of Pit-1 (pituitary-specific transcription factor-1), which is responsible for the expression of GH, PRL and TSH in somatotropic, lactotrophic, and thyrotrophic cells, respectively.

Pit-1 is a member of the POU-domain family, which is characterized by a conserved 60-amino acid homeodomain (POU-homeodomain) and a second 75-amino acid region near the N terminus of the homeodomain (POU-specific domain), Pit-1 is a nuclear protein of 291 amino acids. It has been reported that the pituitaries of dwarf mice in which the Pit-1 gene is deleted lack thyrotropic, lactotrophic and somatotropic cells, indicating the importance of the Pit-1 gene in the maintenance of these three cell lineages.

Pit-1 is a nuclear transcription factor that is involved in the differentiation and growth of somatotrophs, lactotrophs, and thyrotrophs as well as in the production of their respective hormones in the normal pituitary. However, whether Pit-1 is associated with abnormal cell proliferation in pituitary adenomas development is controversial. In general, Pit-1 protein has been highly correlated with tumor immunohistochemistry (IHC) staining for GH, PRL and TSH. Several investigators have reported Pit-1 gene expression in human pituitary adenomas. Sanno N et al. [4] identified Pit-1 mRNA in various cell types of human pituitary adenomas, including clinically non-functioning adenomas; however, additional transcriptional factors or enhancers may also be required. In some studies, Pit-1 expression has been detected in over 70% of non-functioning tumors. For example, Yamada et al. [5] demonstrated that Pit-1 mRNA was expressed not only in GH, PRL and TSH cell adenomas, but also in other types of adenomas, and further studies will be necessary to elucidate the role of Pit-1 transcripts in the three types of non-functioning adenomas without GH, PRL and/or TSH-beta mRNA expression.

However, previous investigations addressing the relationship between Pit-1 mRNA and hormone production in human pituitary adenomas have been limited. In this study, we investigated Pit-1 protein and mRNA levels in individual adenoma cells using IHC and real-time reverse transcription polymerase chain reaction (RT-PCR) to further clarify its role in the functional differentiation of adenoma cells.

Materials and methods

Tissue samples

Samples were obtained from formalin-fixed, paraffin-embedded blocks of pituitary adenoma specimens from 104 patients accessioned at the Department of Pathology, the Fifth People's Hospital of Shanghai, Fudan University (Shanghai, China), between 2010 and 2012. Patient age ranged between 16 and 82 years (median 45.6 years). The clinical diagnoses included 9 GH-secreting adenomas, 12 prolactinomas, 4 GH-PRL-producing adenomas, 32 gonadotropin (FSH/LH)-producing adenomas, 10 ACTH-secreting adenomas, 3 TSH-producing adenomas, 5 null cell adenomas, and 25 plurihormonal adenomas. For the control group, 4 non-tumorous normal pituitaries were collected at autopsy from adult patients with no evidence of an endocrine abnormality. Representative paraffin blocks from routinely fixed and processed tissues were available for review and immunohistochemical analysis in all cases. The histopathological features were reviewed by two pathologists (Li XJ and Liu XP). Representative images of hematoxylin and eosin
Pit-1 expression in human pituitary adenomas

(H&E) staining of pituitary adenomas are shown in Figure 1 [images were viewed under a light microscope (BX45, Olympus, Tokyo, Japan)]. Among the 104 cases, fresh specimens were collected from 22 patients in order to analyze Pit-1 mRNA expression by real-time RT-PCR, including 3 cases of GH adenoma, 6 of PRL adenoma, 2 of GH-PRL adenoma, 1 of TSH adenoma, 4 of FSH/LH adenoma, 2 of ACTH adenoma and 2 of null cell adenoma; normal pituitaries were collected in 2 cases. The study was approved by the ethics committee of the Fifth People’s Hospital of Shanghai, Fudan University (Shanghai, China). Written informed consent was obtained from the patients’ families.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on the most representative 4-μm-thick sections of formalin-fixed, paraffin-embedded tissues using a Leica automated immunostainer (Leica, BOND-MAX, Solms, Germany) via the standard EnVision method. The ChemMate™ EnVision™ detection kit (DakoCytomation, Glostrup, Denmark) was employed. Primary antibodies against human GH, PRL, TSH, ACTH, LH, FSH, and Pit-1 (Table 1) were applied overnight at 4°C. Pit-1 immunohistochemical results were classified into four grades according to the percentage of immunoreactive cells: -, no positive cells; 1+, <10% positive cells; 2+, between 10% and 50% positive cells; and 3+, >50% positive cells. For GH, PRL, TSH, ACTH, LH, and FSH, any positive cytoplasmic staining in >10% of pituitary adenoma cells was regarded as positive. Heat-induced epitope retrieval was performed using a steamer. Appropriate positive and negative controls were included for each antibody.

RNA isolation and RT-PCR analysis

Total RNA was isolated from tissues using RNAiso Plus according to the manufacturer’s instructions and then reverse transcribed to cDNA using PrimeScript® RT Master Mix and the following program: 37°C for 15 min, 85°C for 5 s, and 4°C. qPCR for Pit-1 and GAPDH was

Table 1. Antibodies

<table>
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<th>Antibody</th>
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<th>Dilution</th>
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</tr>
<tr>
<td>PRL</td>
<td>PRL-02</td>
<td>Shanghai Changdao Biotech Co., Ltd., Shanghai, China</td>
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<tr>
<td>ACTH</td>
<td>AH26</td>
<td>Shanghai Changdao Biotech Co., Ltd., Shanghai, China</td>
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<tr>
<td>LH</td>
<td>LH01</td>
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<tr>
<td>FSH</td>
<td>FS02</td>
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<td>1:100</td>
</tr>
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<td>Pit-1</td>
<td>Polyclonal</td>
<td>Santa Cruz Biotechnology (Shanghai) Co., Ltd, Shanghai, China</td>
<td>1:100</td>
</tr>
</tbody>
</table>

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**Table 2.** Kits used for RNA isolation and RT-PCR analysis

<table>
<thead>
<tr>
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<th>Catalog</th>
<th>Function</th>
<th>Source</th>
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<tbody>
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<td>TaKaRa (Dalian, China)</td>
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<tr>
<td>PrimeScript® RT Master Mix</td>
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<td>Reverse transcription</td>
<td>TaKaRa (Dalian, China)</td>
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<tr>
<td>SYBR® Premix Ex Taq™</td>
<td>RR420A</td>
<td>Real-time PCR</td>
<td>TaKaRa (Shiga, Japan)</td>
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</table>

**Table 3.** Expression of the Pit-1 protein in pituitary adenomas

<table>
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<tr>
<th>Pit-1</th>
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<th>PRL</th>
<th>GH-PRL</th>
<th>ACTH</th>
<th>TSH</th>
<th>FSH/LH</th>
<th>Plurihormonal</th>
<th>Null</th>
<th>Pituitaries</th>
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<td>2</td>
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<td>0</td>
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<td>0</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
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<td>1</td>
<td>3</td>
<td>6</td>
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<td>1</td>
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<td>24</td>
</tr>
<tr>
<td>3+</td>
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<td>3</td>
<td>2</td>
<td>0</td>
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<td>32</td>
<td>25</td>
<td>5</td>
<td>4</td>
<td>104</td>
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</table>

performed in a 10-μL reaction volume using SYBR® Premix Ex Taq™ and an ABI7900HT Real-Time PCR System (Life Technologies, Singapore). The thermal cycling conditions consisted of one cycle at 95°C for 30 s and 40 cycles of amplification at 95°C for 5 s and annealing/elongation at 60°C for 30 s. Pit-1 mRNA expression was normalized to the geometric mean mRNA expression of the housekeeping gene (GAPDH) and was calculated using the formula $2^{-\Delta C_t}$ [$\Delta C_t = C_t$(Pit-1) - $C_t$(GAPDH)], where $C_t$ represents the threshold cycle for each transcript. The kit that we used is shown in Table 2. The PCR primer sequences for Pit-1 were as follows: forward primer 5’-GT-GTCTACCAGTCTCCAACC-3’, corresponding to nucleotides 570-589 in exon 1 of Pit-1 cDNA; and reverse, (5’-ACTTTCCTCACGTGTTCC-3’), an antisense 20-mer corresponding to nucleotides 269-288 in exon 3 of Pit-1 cDNA. The obtained PCR product was 247 bp, which was previously described by Gil-Puig C et al [6].

**Statistical analysis**

Statistical analyses were performed using a Chi-squared test or Fisher’s exact test in SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). Statistical significance was indicated by $P<0.05$.

**Results**

**Pit-1 protein expression determined via immunohistochemical staining**

The Pit-1 IHC results are summarized in Table 3. Pit-1 was localized in the cytoplasm of pituitary adenoma cells. Positive Pit-1 protein expression was observed in 46.2% (48/104) of the cases. The negative controls showed no cytoplasmic staining. Of the 48 positive cases, 16 (33.3%) showed 1+ positivity, 24 (50.0%) showed 2+ positivity, and 8 (16.7%) showed 3+ positivity.

Among the 32 cases scored as 2+ or 3+ for Pit-1, 20 were GH/PRL/TSH adenomas, and 4 were plurihormonal adenomas. All 4 TSH adenomas were immunohistochemically positive for Pit-1 (i.e., 3 were pit-1 2+, and 1 was Pit-1 3+). Representative IHC images of Pit-1 in pituitary adenomas are shown in Figure 2.

**Correlation between Pit-1 protein expression and pituitary adenoma typing**

The rates of positivity for Pit-1 protein in pituitary GH, PRL, GH-PRL, ACTH, TSH, FSH/LH, plurihormonal and null cell adenomas and in normal pituitaries were 88.9% (8/9), 90.9% (10/11), 80.0% (4/5), 22.2% (2/9), 100.0% (4/4), 31.3% (10/32), 24.0% (6/25), and 50.0% (2/4), respectively. TSH adenomas showed the highest rate of positivity, and ACTH adenomas had the lowest rate.

The rates of positivity for Pit-1 protein in TSH adenomas (100%, 4/4) and GH/PRL adenomas (88.0%, 22/25) were significantly higher than those in other types of adenomas (including ACTH, FSH/LH, plurihormonal and null cell adenomas, which showed an overall positive rate of 40.0%; $P<0.05$), but were not significantly different from that in normal pituitaries ($P=0.062$). No significant differences in the
rate of positivity were detected between any type of pituitary adenoma and normal pituitary tissue ($P>0.05$).

**Pit-1 mRNA levels detected via real-time RT-PCR**

PCR amplification of cDNA prepared from pituitary adenomas and normal pituitary glands produced a 247-bp PCR product corresponding to the human Pit-1 gene. We evaluated Pit-1 mRNA levels by real-time RT-PCR. The amplification plot for Pit-1 mRNA is shown in **Figure 3**, and the qualification data for Pit-1 mRNA are shown in **Figure 4**. Pit-1 mRNA was detected in 36.4% (8/22) pituitary adenomas. Among the 8 positive cases, 7 were classified as GH/PRL or TSH adenomas, and the remaining case was an FSH/LH adenoma. The mean Pit-1 mRNA levels in the positive cases ranged from $1.6728E-05$ to $4.9886E-05$, with an overall mean of $2.9841E-05$. Pit-1 mRNA was not detected in the other types of adenomas or in normal pituitaries. Pit-1 mRNA levels were significantly higher in GH/PRL or TSH adenomas compared with the other types of pituitary adenomas and normal pituitaries ($P<0.05$).

**Correlation between Pit-1 protein expression determined via IHC and Pit-1 mRNA levels determined via real-time RT-PCR**

The Pit-1 protein and mRNA expression levels in the fresh specimens from 22 patients are shown in **Table 4**. Positive Pit-1 protein expression was observed in 59.1% (13/22) of the
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Figure 3. Amplification plot of pit-1 mRNA obtained via real-time RT-PCR. Pit-1 mRNA was detected in 36.4% (8/22) of the pituitary adenomas. Among the 8 cases, 7 were GH/PRL or TSH adenomas, and the remaining case was an FSH/LH adenoma.

Figure 4. The qualification data for Pit-1 mRNA are shown in Figure 4. The expression level of Pit-1 mRNA was normalized to the geometric mean of the mRNA expression of the conserved gene GAPDH to control the variability in expression levels and was obtained using the calculation $2^{-\Delta \Delta Ct} = 2^{-(Ct_{Pit-1} - Ct_{GAPDH})}$, where the Ct value represents the threshold cycle for each transcript. The mean Pit-1 mRNA level in positive cases ranged from 1.6728E-05 to 4.9886E-05, with an overall mean value of 2.9841E-05.

Discussion

The mechanisms of human pituitary tumorigenesis have been only partly elucidated. With the remarkable recent progress in molecular biology techniques, the processes involved in pituitary cell differentiation and growth as well as the regulation of hormone production are being clarified. Molecular biology and immunohistochemical studies have revealed several aspects of pituitary tumorigenesis. According to Mayai et al., [7] pituitary cell types are classified into the following three lineages based on the combination of expressed transcription factors and co-factors: (1) GH-PRL-TSH cells, which express Pit-1; (2) pro-opiomelanocortin (POMC) cells, which express neurogenic differentiation 1 (NeuroD1)/pituitary cell-restricted T-box factor (Tpit); and FSH/LH cells, which express steroidogenic factor 1 (SF-1)/GATA-binding protein 2. Pit-1 is a nuclear transcription factor that is required for the differentiation and growth of somatotrophs, lactotrophs, and thyrotrophs as well as the production of the respective hormones in the normal pituitary gland [8].

In the present study, Pit-1 protein was found to be expressed in the cytoplasm of adenoma cases, and Pit-1 mRNA was detected in 36.4% (8/22) of the cases. Among the 13 cases with positive Pit-1 immunostaining, 10 were GH/PRL or TSH adenomas. Among the 8 cases in which Pit-1 mRNA was detected via real-time RT-PCR, 7 were GH/PRL or TSH adenomas. No significant difference was found between the detection of Pit-1 protein and mRNA in pituitary adenomas or normal pituitaries ($P>0.05$).
adenomas. We therefore suggest that the pathogenesis of pituitary adenomas, especially GH/PRL or TSH adenomas, is associated with the Pit-1 gene. Analyses of Pit-1 expression in human pituitary adenomas using different methods have produced inconsistent results. Asa et al., [10] utilized Northern blotting to show that Pit-1 localized to adenomas that expressed GH, PRL, or TSH but was not detected in other types of adenomas. In contrast, Lloyd et al. [11] showed that Pit-1 was expressed in all types of adenomas.

Various studies support a critical role for Pit-1 in the development of human pituitary adenomas [4, 5, 9], but its mechanism of action in pituitary tumor development remains unknown. Takahashi [12] showed that Pit-1 is an essential transcription factor that plays a role in the differentiation and maintenance of GH-, PRL-, and TSH-producing cells, and Pit-1 gene mutations result in a specific defect in the production and/or secretion of these hormones. Julline N et al. [13] indicated that under normal conditions, Pit-1 is important for the maintenance of cell proliferation; however, over-expression of Pit-1 induces cell death, and through this dual action, Pit-1 may play a role in the expansion/regression cycles of the pituitary lactotroph population during and after lactation. Nevertheless, some investigators have suggested that Pit-1 was not associated with the pathogenesis of pituitary adenomas. Pellegrini-Bouiller I et al. [14] reported that pituitary tumorigenesis does not appear to be associated with a gross alteration in Pit-1 gene expression in humans. Pit-1 might activate genes required for the proliferation or survival of three of the pituitary cell types, and these genes may directly or indirectly regulate somatotrophs and lactotrophs by activating the receptors for GRF and SRIF or dopamine. Kazuya et al. [15] suggested that regulation hormone production might not be the major role of the Pit-1 gene in pituitary adenomas.

We used IHC and RT-PCR methods to detect Pit-1 protein and mRNA, respectively. We detected Pit-1 protein not only in GH/PRL and TSH adenomas but also in all other types of adenomas and in normal pituitaries. However, in the 22 adenoma cases in which we examined Pit-1 mRNA expression, 7 of the 8 positive cases were GH/PRL/TSH adenomas, and Pit-1 mRNA was detected in only 1 FSH/LH adenoma; these data suggest that Pit-1 plays an important role in the pathogenesis of pituitary GH/PRL/TSH adenomas. Pit-1 expression in various other types of adenomas may indicate the involvement of unidentified transcription factors or specific mediators. Additionally, we found that the rate of Pit-1 positivity was significantly higher in GH/PRL or TSH adenomas than in other adenomas; however, the rate was not significantly different from that in normal pituitary gland tissue. We therefore suggest that high Pit-1 expression levels represent a constant feature of human pituitary GH, PRL and TSH adenomas. However, why Pit-1 products are detected in other adenomas and in normal pituitary glands remains to be undetermined. We propose that some stem cells may enter pituitary adenomas or the pituitary gland during the differentiation process, leading to detectable Pit-1 protein expression in other types of adenomas or in normal pituitaries. We also observed no significant difference between detecting Pit-1 protein and mRNA. We hypothesize that the limited number of cases in which we detected Pit-1 mRNA was responsible for the lack of a significant difference which may have been observed if the quantity of specimens was sufficiently large.

We extracted RNA from thin sections instead of from frozen tissue masses to avoid detecting false signals generated by non-tumor cell contamination. PCR is an acceptable method for quantitating gene expression, and the results can be normalized through simultaneous amplification of a control gene. We used PCR to quantitate Pit-1 mRNA levels, and we normalized our results to the geometric mean of GAPDH mRNA expression [16]. With PCR, it is necessary to quantitate and normalize gene expression when the reactions are in the expo-

<table>
<thead>
<tr>
<th>Pit-1</th>
<th>GH</th>
<th>PRL</th>
<th>GH-PRL</th>
<th>ACTH</th>
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<td>4</td>
<td>2</td>
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</tr>
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</table>

Table 4. Summary of Pit-1 protein and mRNA expression in fresh specimens from 22 cases
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According to Palmieri D et al., [17] HMGA proteins bind both Pit-1 and Pit-1-responsive DNA elements, thus positively modulating Pit-1 promoter activity. Moreover, Pit-1 overexpression greatly enhances pituitary cell proliferation by inducing c-Fos expression in gonadotropic cells or by inhibiting Mia expression in GH/PRL secreting cells. In fact, microfection of Pit-1 antisense sequences blocks the growth of a GC somatotropic cell line and dominant-negative Pit-1 mutants, reduce apoptosis via a caspase-independent pathway [18, 19]. Additionally, Pit-1 upregulates the expression of genes such as Ghihr [20], which is involved in cell proliferation. Interestingly, recent studies have also identified increased Pit-1 expression in breast carcinoma [21-23], suggesting a potential role for Pit-1 in the proliferation of other cell types.

In conclusion, we suggest that Pit-1 plays an important role in the development of pituitary adenomas, especially GH/PRL or TSH adenomas. However, some additional transcription factors or enhancers may be required. Pit-1 is required for pituitary cell proliferation during physiological development. Nevertheless, further investigation is necessary to clearly determine the role of Pit-1.

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Disclosure of conflict of interest

None.

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