Expression of Cyclin E/Cdk2/$p27^{Kip1}$ in Growth Hormone Adenomas

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■ OBJECTIVE: **The present study was conducted to evaluate the levels of Cdk2, cyclin E, p21, and p27 in growth hormone adenomas (GHPAs) and analyze their association with clinicopathologic features.**

■ METHODS: **We collected 46 GHPA specimens and clinical materials from March 2012 to December 2015 at Beijing Tiantan Hospital. We analyzed the expression of Cdk2, cyclin E, p21, and p27 using immunohistochemistry, quantitative real-time polymerase chain reaction, Western blot, and methylation-specific polymerase chain reaction and sequencing.**

■ RESULTS: **The levels of cyclin E and Cdk2 were much greater in the invasive group than in the noninvasive group ($P < 0.05$). Significant differences were found between cyclin E and p21 and tumor size ($P < 0.05$) and between cyclin E expression and invasion ($P < 0.05$). Tumors were more likely to require whole resection in patients with low cyclin E expression ($P < 0.05$). The high-level p27 group had better progression-free survival than did the low-level p27 group ($P < 0.01$). Quantitative real-time polymerase chain reaction and Western blot analysis revealed a similar trend for Cdk2, cyclin E, p21, and p27 protein levels in growth hormone specimens ($P < 0.01$). An average of 33 CpG sites per sample were analyzed using matrix-assisted laser desorption ionization time-of-flight mass array, and in 7 of the 33 CpG sites, the methylation levels of p27 were >50%. Statistically significant differences were found in 4 CpG sites between the invasive and noninvasive specimens ($P < 0.01$).**

■ CONCLUSIONS: **Overexpression of cyclin E/Cdk2 and loss of p27 appears to be associated with a poor prognosis and might play a role in the treatment of GHPAs in the future.**

INTRODUCTION

Acromegaly mainly results from excessive secretion of growth hormone (GH) and insulin-like growth factor 1, which is associated with gigantism, cardiovascular and cerebrovascular diseases caused by pituitary adenomas in >98% of cases.[1] Therapeutic options include 2 classes of pharmacologic agents: central-acting inhibitors of GH secretion and GH receptor blocking agents, which normalize the GH and insulin-like growth factor 1 levels and shrink the tumor size.[2] Lower GH levels predict better responses to dopamine agonists or somatostatin analogues and high GH levels predict poor treatment outcomes.[3] Surgery is the first-line of treatment for growth hormone adenomas (GHPAs), and a cure is not, at present, available for invasive GHPAs.[4] Multimodal therapy is needed and has been shown to control the disease in 50% the patients with aggressive adenomas (102 patients total).[5] At present, no recurrent somatic mutations have been observed, except in the

Key words
- Cdk2
- Cyclin E
- Growth hormone adenomas
- Invasion
- Methylation
- $p27^{Kip1}$

Abbreviations and Acronyms
cAMP: Cyclic adenosine $3'$:$5'$ monophosphate
CDK: Cyclin-dependent kinase
CDKI: Cdk inhibitor
CT: Cycle threshold
DNA: Deoxyribonucleic acid
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GH: Growth hormone
GHPA: Growth hormone adenoma
mRNA: Messenger RNA
O6: O6-cyclohexylmethylguanine
PCR: Polymerase chain reaction
RT: Real-time
SSTR: Somatostatin receptor

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Table 1. The Clinicopathologic Data in 46 Patients with Growth Hormone Adenoma

	GHPAs		
Category	**Invasive**	**Noninvasive**	***P* Value**
Patients	13	33	
Age (years)			
Range	17—66	21—75	>0.05
Mean ± SD	35.7 ± 12.4	38.4 ± 15.2	
Sex			
Males	8	16	0.375
Females	5	17	
GH			
(ng/mL)	38.69 ± 12.25	16.23 ± 12.15	<0.05
Tumor size			
$\leq$2.583 cm^3	3	19	0.027
>2.583 cm^3	10	13	
Resection			
All	5	26	0.009
Partial	8	7	
Recurrence			
Yes	5	4	0.043
No	8	29	

GHPA, growth hormone adenoma; SD, standard deviation; GH, growth hormone.

guanine nucleotide-binding protein alpha-stimulating activity polypeptide gene. Interestingly, several nonrecurrent alterations have been shown to affect other genes involved in cyclic adenosine 3′:5′ monophosphate (cAMP) signaling.[6,7] The G protein α subunit activates the cAMP-dependent pathway by stimulating production of cAMP, and participates in diverse cell processes that cause various pathophysiologic disorders, including tumorigenesis.[8] Altered cell cycle genes, selective oncoprotein activation, or loss of suppressor factors may be associated with aberrant growth factor signaling in pituitary adenomas.[9]

Regulation of cell cycle signal transduction pathways is attracting attention. During normal pituitary development, exit from the progenitor cell cycle is controlled by p57(Kip2), followed by p27(Kip1) in differentiated cells.[10] Some studies[11,12] have revealed that human pituitary tumors are a critical target of cell cycle deregulation, and at least one of the cell cycle regulators may change during pituitary tumorigenesis, as evidenced by deregulation of cyclin-dependent kinase (CDK) and Cdk inhibitor (CDKI). Cyclins bind to the corresponding CDKs and activate them to regulate cell cycle phase transition, which is essential for cell cycle regulation. Cell cycle phase transition is tightly regulated by CDKIs to ensure a suitable cell progression for normal homeostasis.[13-15] As 1 of the 2 families of cell cycle inhibitors, the Cip/Kip family of proteins, p21^{Cip1} and p27^{Kip1} are able to bind to Cdk2-cyclin E/Cdk2-cyclin A complexes and interfere with their activity.[13] The excessive activity of cyclin E/Cdk2 complex drives cells to prematurely replicate their deoxyribonucleic acid (DNA), resulting in genome instability and tumorigenesis.[16-18] Dysfunction of p27 has been frequently observed in many human cancers, including lung, breast, prostate, and colorectal cancers, and it often results from p27 protein degradation and cytoplasmic mislocalization, which are highly regulated by the phosphorylation status of p27.[19] There is evidence that overexpression of cyclin E and/or deficiency of p21 and p27 has been associated with pituitary tumorigenesis and progression of pituitary adenoma.[20,21]

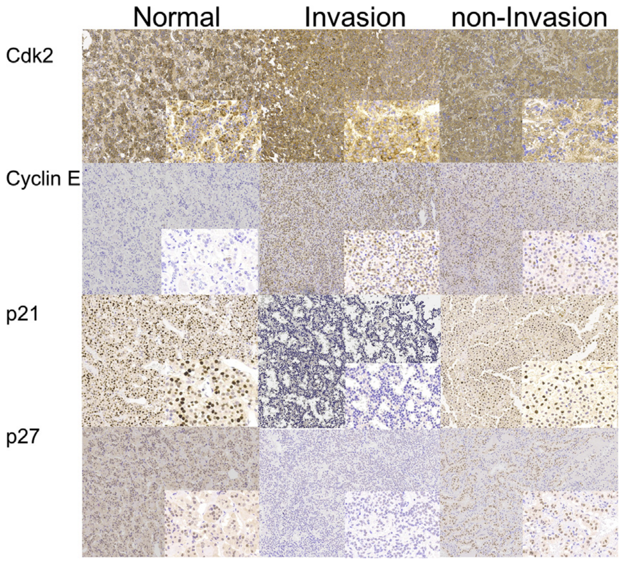

Figure 1. CDK2, cyclin E, p21, and p27 in growth hormone adenoma (GHPA) specimens through tissue microassay. Normal, healthy pituitary; Invasion, invasive GHPA specimens; non-Invasion, non-invasive GHPA specimens. Bar, 60 μm.

Epigenetic activation and inactivation of genes play critical roles in many human diseases, particularly cancer. The objective of the present study was to investigate whether gene expression of cyclin E/Cdk2/p21/p27 was correlated with GH levels and invasion of GHPAs using immunohistochemistry, Western blot, and real-time-polymerase chain reaction (RT-PCR). We also identified the promoter methylation level of p27 in GHPAs and studied potential associations between these cell cycle regulators and clinical findings, such as prognostic and diagnostic value, and a novel therapeutic method.

METHODS

Patients and Specimens

GHPAs were obtained from 46 operative patients who underwent either endoscopic transsphenoidal surgery or craniotomy between March 2012 and December 2015 at Beijing Tiantan Hospital. Patients who had received previous radiation therapy or had plurihormonal GH tumors were excluded from this study. GHPA was diagnosed according to clinical symptoms, hormone levels, magnetic resonance imaging information, histopathologic analysis, and electron microscopic examination. We adopted the diagnostic criteria of 2 invasive pituitary adenomas that contain Hardy-Wilson grade IV and/or Knosp grades III and IV.[22,23] There were 13 invasive GHPAs and 33 noninvasive GHPAs. The study protocol was approved by the Ethics Committee of Beijing Tiantan Hospital, and written informed consent was obtained from all patients for surgery and research purposes. The patient characteristics are summarized in **Table 1**. Five specimens from healthy pituitary glands were obtained from a donation program (age range, 21–45 years). None of the donors had a history of pituitary disease.

Immunohistochemistry

Tissue from healthy pituitary glands and GHPAs was fixed in 10% formalin and embedded in paraffin. Paraffin-embedded tissues were cut into 5-μm serial sections, then transferred onto adhesive slides, and dried at 65°C for 30 minutes. The sections were deparaffinized in xylene and rehydrated through immersion in graded ethanols. Endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution for 30 minutes, and the sections were microwaved for 10 minutes at 100°C in citrate buffer (0.01 mol/L, pH 6.0) for antigen retrieval. After washing 3 times with phosphate-buffered saline for 5 minutes per wash, the sections were incubated in 5% normal goat serum to prevent nonspecific binding. Then, the sections were incubated with primary antibodies, anti-Cdk2 (1:600; Abcam, USA), anti-cyclin E (1:300; Abcam, USA), anti-p27 (1:100; Abcam, USA) and anti-SSTR2 (1:100, Abcam, USA), at 4°C overnight, followed by immunodetection using a 2-step polymer detection system (Polink-2 plus Kit; GBI Labs, Manchester, England) and visualization with 3,3′-diaminobenzidine. The slides were counterstained with Mayer's hematoxylin, dehydrated in graded alcohol, and mounted with a neutral resin. For a negative control, the protocol was repeated with the primary antibody replaced with phosphate-buffered saline.

Two pathologists who were blinded to the patients' clinical records examined and scored all sections. Then, we randomly selected 5 fields for analysis at ×400 magnification. Staining scores for cyclin E, Cdk2, SSTR2, p21, and p27 were identified by a semiquantitative system based on the percentage of positive cells,

Table 2. Relationship Between CDK2, Cyclin E, p21, p27, and Clinical Parameters in 46 Patients

	Cdk2			Cyclin E			p21			p27		
Category	**High**	**Low**	***P* Value**	**High**	**Low**	***P* Value**	**High**	**Low**	***P* Value**	**High**	**Low**	***P* Value**
Sex			0.238			1.0			0.238			0.555
Male	14	10		12	12		10	14		11	13	
Female	9	13		11	11		13	9		12	10	
Age (years)			0.768			0.768			0.376			0.140
≤41	12	11		12	11		10	13		9	14	
>41	11	12		11	12		13	10		14	9	
GH (ng/mL)			0.140			0.376			0.140			0.039
≤22.57	9	14		10	13		14	9		15	8	
>22.57	14	9		13	10		9	14		8	15	
Tumor size (cm^3)			0.140			0.039			0.039			0.140
≤2.583	9	14		8	15		15	8		14	9	
>2.583	14	9		15	8		8	15		9	14	
Invasion			0.022			0.003			0.140			0.039
Yes	10	3		11	2		14	9		8	15	
No	13	20		12	21		9	14		15	8	
Resection			0.116			0.028			0.116			0.116
All	13	18		12	19		13	18		13	18	
Partial	10	5		11	4		10	5		10	5	
Recurrence			0.265			0.063			0.265			0.063
Yes	6	3		7	2		6	3		7	2	
No	17	20		16	21		17	20		16	21	

GH, growth hormone.

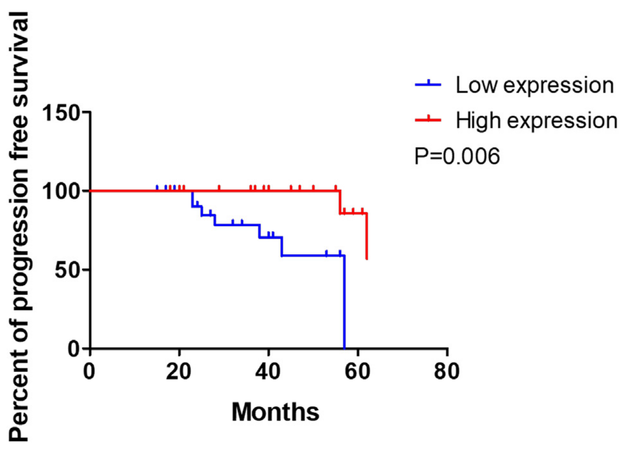

Figure 2. The average progression-free survival with p27 levels in 46 cases.

and the staining intensity was stratified on a scale of 0–3 (0 for no staining, 1 for weak, 2 for moderate, and 3 for strong). H-scores were acquired by multiplying the intensity of a stain by a constant, thus adjusting the mean for the strongest staining [score = 1.0 × (%weak) + 2.0 × (%medium) + 3.0 × (%strong)].

DNA Extraction, Purification, and Bisulfite Modification

Genomic DNA from frozen tissue was extracted using a DNeasy kit (Qiagen, German). All extracted genomic DNA was treated with sodium bisulfite (Sigma, USA). Briefly, 2 µg of genomic DNA was denatured with 5.5 µL of 3 moles fresh NaOH (final concentration 0.3 mol/L) for 10 minutes at 37°C. Then, 30 µL of 10 mmol/L hydroquinone (Sigma) and 520 µL of 3 mol/L sodium bisulfite (pH 5.0) were added, and the solution was protected from light. The mixture was inverted, added to 200 µL liquid paraffin to prevent water evaporation and reagent oxidation, and incubated at 50°C for 16 hours. The modified DNA was purified using the Wizard DNA clean-up system (Promega, USA). The purified DNA was treated again with $NaHSO_3$ and precipitated. DNA was redissolved in 20 µL of Tris-acetate-EDTA, 2 µL of which was subjected to PCR amplification.

Methylation-specific PCR and Sequencing

Three CpG islands were found using the EMBOSS Cpgplot http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/. Methylation-specific primers were designed to cover 33 CpG dinucleotides, 5000 sites upstream of the transcription start site G (forward) and 1000 sites upstream of the transcription start site G according to Sequenom EpiDesigner (#23). Primers specific for the methylated DNA (forward: GATGGTAGAAATTTTTGGGTTAAGG; reverse: CCAACAAACCTACTCTAACTAACCTC) were added to the reaction and expected to generate 403 bp-long products. The PCR protocol included 45 cycles of denaturation at 94°C for 20 seconds, annealing at 60°C for 30 seconds, 72°C for 1 minute. Then, sequenced PCR products with Sequenom mass array and the specimens' methylation were generated using EpiTYPER software v1.0 (Sequenom).

Real-Time Quantitative PCR

The samples of GHPAs and healthy anterior pituitaries were preserved in liquid nitrogen. Total RNA was recovered from the frozen tumor samples and healthy anterior pituitaries using Trizol (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The refinement and concentration of RNA was resolved using a NanoDrop 1000 instrument (Thermo Scientific, Wilmington, Delaware, USA). First-strand cDNA synthesis was generated according to the manufacturer's instructions (Invitrogen). Quantative RT-PCR was conducted on an ABI 7500 Fast system (Applied Biosystems, Foster City, California, USA) by the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). The conditions for amplification were 50°C for 2 minutes, 95°C for 2 minutes, and 40 cycles at 95°C for 15 seconds, and 60°C for 30 seconds. As the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase(GAPDH) was used as an internal control. Relative messenger RNA (mRNA) levels were computed according to the cycle threshold (CT) values, which were corrected for GAPDH expression according to the following equation: $2^{\ -\Delta\Delta CT}$ ($\Delta CT = CT$ gene of interest $-$ CT GAPDH). Relative quantification of gene expression was determined by the $2^{-\Delta\Delta CT}$ method.[24] The specificity of quantitative RT-PCR products was verified by constructing dissociation reaction plots, all of which were repeated in triplicate. The sequence of primers used for RT-PCR are as follows: Cdk2 (forward, GACCAGCTCTTCCGGATCTT; reverse, ACAAGCTCCGTCCATCTTCA), cyclin E (forward, GTGTCCTGGATGTTGACTGC; reverse, CTGCAGTGAAGACATGTGGG), p21 (forward, GGATGTCCGTCAGAACCCAT; reverse, GTGGGAAGGTAGAGCTTGGG), p27 (forward, AGGCCGACTACGAACAGATG; reverse, CTTTTGCGTTCTCGTTGTGA), GAPDH (forward, ACCACAGTCCATGCCATCACT; reverse, GTCCACCACCCTGTTGCTGTA).

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis and Western Blot Analyses

GHPA specimens and healthy pituitaries were lysed to extract their total protein in Tris-NACL-EDT buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid; all from Sigma-Aldrich) containing 1% nonidet p-40 (Calbiochem, German) with protease and phosphatase inhibitor cocktails (Roche, Swiss). The total extracted protein from GHPAs or healthy pituitaries was centrifuged at 12,000 × *g* for 30 minutes at 4°C, and the protein concentration of the supernatant was determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, USA). For Western blot analysis, 40 µg of lysate per lane was loaded into 4%–12% bis-Tris sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, separated electrophoretically, and blotted onto polyvinylidene fluoride membranes (PVDF, California, USA). Each membrane was blocked with 5% bovine serum albumin in Tris-buffered saline, incubated with antibodies against cyclin E, Cdk2, p21, p27, GAPDH, and tubulin, and then incubated with horseradish peroxidase-tagged secondary antibodies (Santa Cruz Biotechnology, Texas, USA). Finally, membranes were visualized by enhanced chemiluminescence, and densitometry was performed using a Versadoc XL imaging apparatus (Amersham Imager 600, GE, USA). Tubulin levels were analyzed as a loading control.

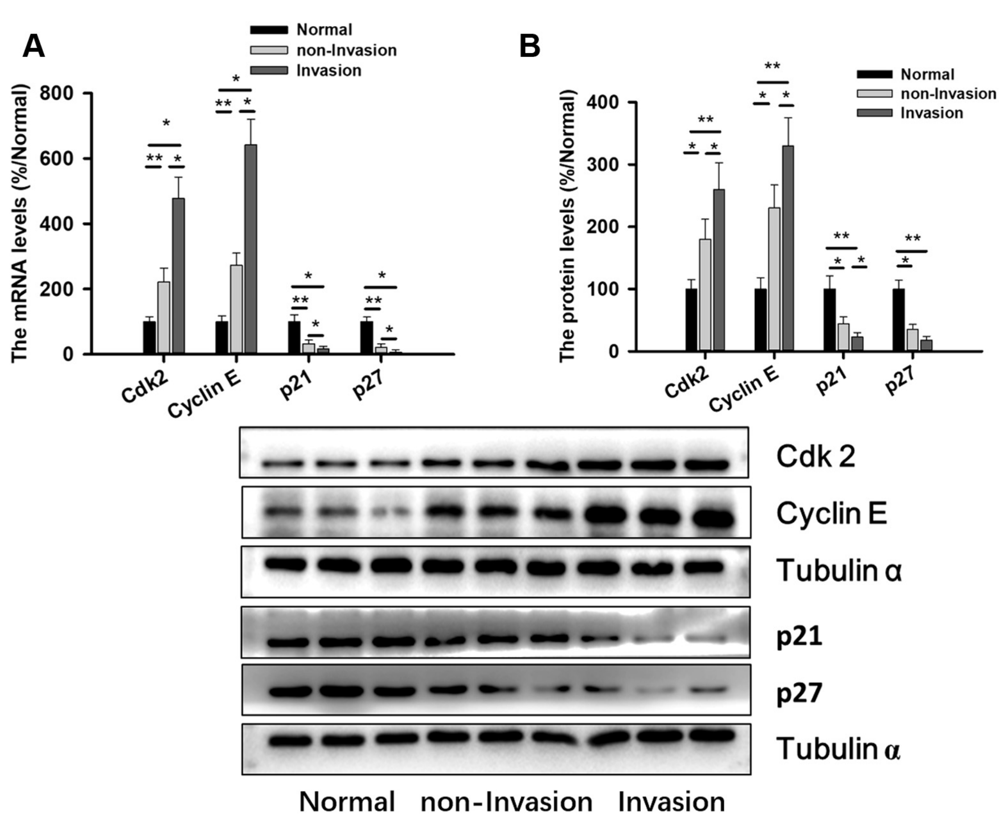

Figure 3. The messenger RNA (mRNA) and protein levels of CDK2, cyclin E, p21, and p27 in growth hormone adenoma specimens. (**A**) mRNA levels (GAPDH was used as an internal control). (**B**) Protein levels. (Tubulin was used as an internal control). n = 6; *P*< 0.01.

Cell Culture, Cell Proliferation, and Cell Migration

GH3 cells were purchased from American Type Culture Collection (ATCC; Manassas, Virginia, USA) and were cultured in nutrient mixture F12 (F12K; ATCC) supplemented with 2.5% fetal bovine serum (GIBCO, Carlsbad, California, USA) and 15% horse serum (GIBCO).

GH3 cells were plated into 96-well dishes with 10,000 cells and 100 μL of medium in every well and incubated overnight. Various concentrations (10, 30, or 100 nM) of rat-microRNA-423-5p and negative control mimics were transfected into each well after 24 hours using 5 μL Opti-MEM (GIBCO) and 0.3 μL lipofectamine 3000 (Invitrogen) according to the mimic instructions. Then, 20 μL of 3-(4,5-diethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) solution was added to each well and further incubated for 4 hours. The absorbance at 490 nm of each well was measured using an enzyme-linked immunosorbent assay plate reader (Thermo Fisher, Massachusetts, USA).

Cell migration and invasion were measured using fibronectin- and Matrigel-coated polycarbonate filters, respectively, and modified Transwell chambers (Corning, New York, USA). GH3 cells (5 × 104 cells) were added into the upper chambers. Migrating cells that adhered to the lower membrane were fixed in 4% paraformaldehyde and stained using hematoxylin (Zhongshan Company, Guangzhou, People's Republic of China). Experiments were performed in triplicate.

Statistical Analysis

SPSS software (version 21.0; IBM Corp., Armonk, New York, USA) was used for statistical analysis. Data are expressed as the mean ± standard deviation. Differences between subgroups were analyzed by independent 2-sample Student's *t*-test. The χ^2 test was used to determine the categorical variables. Variables that showed an association with aggressiveness and tumor size of GHPAs were subjected to univariate and multivariate analyses. The recurrent curves were generated by the Kaplan-Meier method. Two-sided P values of <0.05 were considered statistically significant.

RESULTS

Clinical and Pathologic Features

On the basis of the Knosp/Hardy-Wilson classification, we classified 46 casesas invasive (13 patients) and noninvasive (33 patients). There were 24 men and 22 women in this group, and the median age was 37.1 years (range, 17–75 years). Tissue from 46 specimens was used for standard hematoxylin and eosin staining and immunohistochemistry with anticyclin E, anti-cdk2, anti-p21, and anti-p27 antibodies. The patients who had symptoms of headaches and visual deficits were more common in the invasive group than in the noninvasive group, and the preoperative serum GH level was 38.69 ± 12.25 ng/mL more in invasive GHPAs versus noninvasive GHPAs (16.23 ± 12.15 ng/mL; $P < 0.05$). The tumor size was 1.32–21.54 cm^3 (median, 2.32 cm^3). Follow-up data showed that gross total resection was necessary in 5 of 13 cases (38.4%) in the invasive group and 26 of 33 cases (81.8%) in the noninvasive group ($\chi^2 = 6.901$, $P = 0.009$). Furthermore, recurrence in the invasive group occurred in 5 of 13 cases (38.4%) and in 4 of 33 cases (12.1%) in the noninvasive group ($\chi^2 = 4.111$, $P = 0.043$) (**Table 1**).

Immunohistochemistry Analysis

Immunoreactivity was observed primarily in the nucleus of tumor cells, and immunohistochemistry staining for Cdk2, cyclin E, and p27 in representative samples of pituitary adenomas tissues is shown in **Figure 1** and **Table 2**. There was a significant up-regulation of Cdk2 (H-score, 140 ± 31.7 vs. 87.8 ± 26.7) and cyclin E (H-score, 138.2 ± 34.4 vs. 68 ± 21.7) levels, and a significant down-regulation of p21 (H-score, 152.3.1 ± 35.2 vs. 215.7 ± 41.2) and p27 (H-score, 118.1 ± 28.4 vs. 173.7 ± 34.3) levels in the invasive group compared with the noninvasive group ($P < 0.05$). Nevertheless, we found that protein levels of Cdk2 and cyclin E were significantly higher in the invasive group than in the noninvasive group ($P = 0.022$, $P = 0.003$). Low p27 was found in 65.2% of cases (15/23 cases) in the invasive group and in 34.8% of cases (8/23 cases) in the noninvasive group ($\chi^2 = 4.261$, $P = 0.039$).

The median expression level was used as the cutoff. By univariate analysis, we found that Cdk2, cyclin E, and p21 expression have no relationship to age, gender, and preoperative serum GH levels; p27 expression has no relationship to age and gender, but a difference was found between p27 expression and preoperative serum GH levels. Overall, a significant association was found between cyclin E levels and tumor size ($\chi^2 = 4.261$, $P = 0.039$), as well as between p21 levels and tumor size ($\chi^2 = 4.161$, $P = 0.039$). Furthermore, tumors were more likely to require whole resection in patients with low cyclin E levels ($\chi^2 = 4.847$, $P = 0.028$).

Kaplan-Meier analysis showed that the average progression-free survival time in the high p27 group (2/23 cases) was longer than that in the low cyclin E group (7/23 cases) ($P = 0.006$) (**Figure 2**).

Quantitative PCR and Western Blot Analysis

Quantitative RT-PCR revealed a significant up-regulation of cyclin E and Cdk2 mRNAlevels in invasive and noninvasive GHPAs, compared with those in the healthy pituitary ($P < 0.01$). In addition, cyclin E and Cdk2 mRNA levels were significantly elevated in invasive GHPAs compared with noninvasive GHPAs (**Figure 3A**). In contrast, the p21 and p27 mRNA levels were significantly lower in invasive GHPAs and noninvasive GHPAs compared with those in the healthy pituitary. Furthermore, the p21 and p27 mRNA levels were significantly lower in invasive GHPAs compared with noninvasive GHPAs (**Figure 3A**; $P < 0.01$).

Western blot analysis showed that the cyclin E and Cdk2 protein levels were more elevated in invasive and noninvasive GHPAs than in the healthy pituitary, and their levels were also significantly more in invasive GHPAs versus noninvasive GHPAs ($P < 0.01$). In

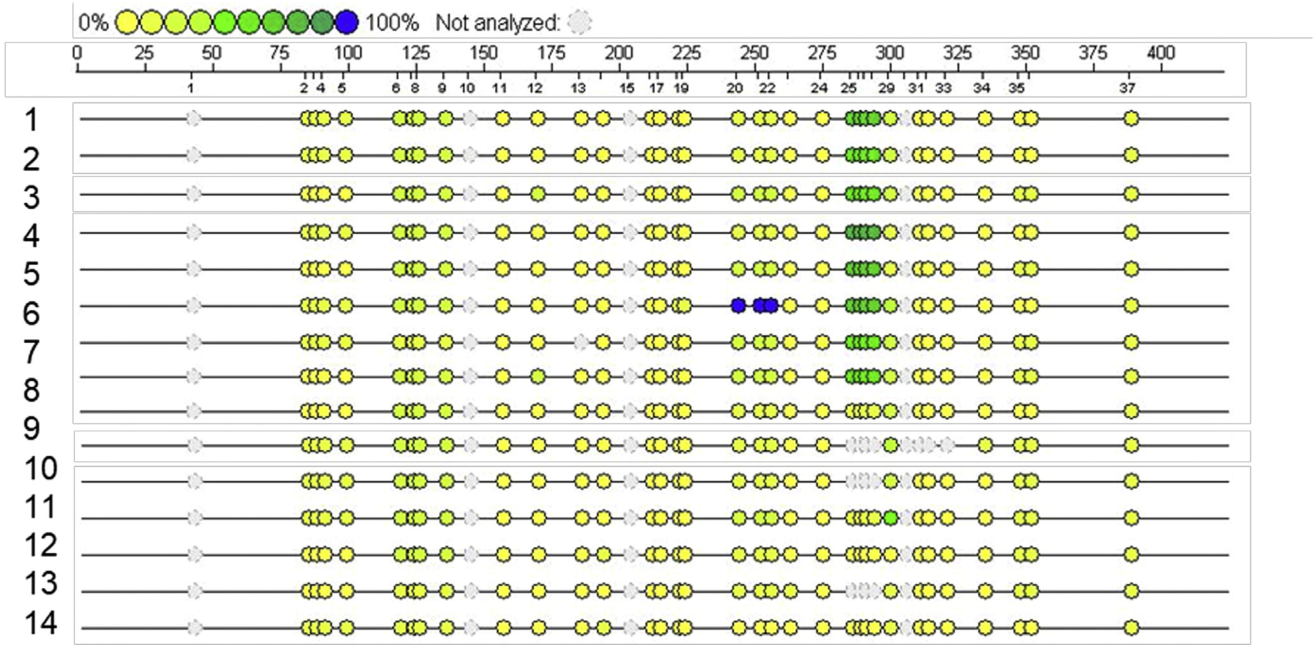

Figure 4. The methylation of p21 and p27 in patients with growth hormone adenoma. The methylation CpG sites of the p27 promoter (1–7, invasive; 8–14, noninvasive). *Yellow clusters* indicate 0 methylated, *blue clusters* indicate 100% methylated, *color gradient between yellow and blue* indicates methylation ranging from 0–100%.

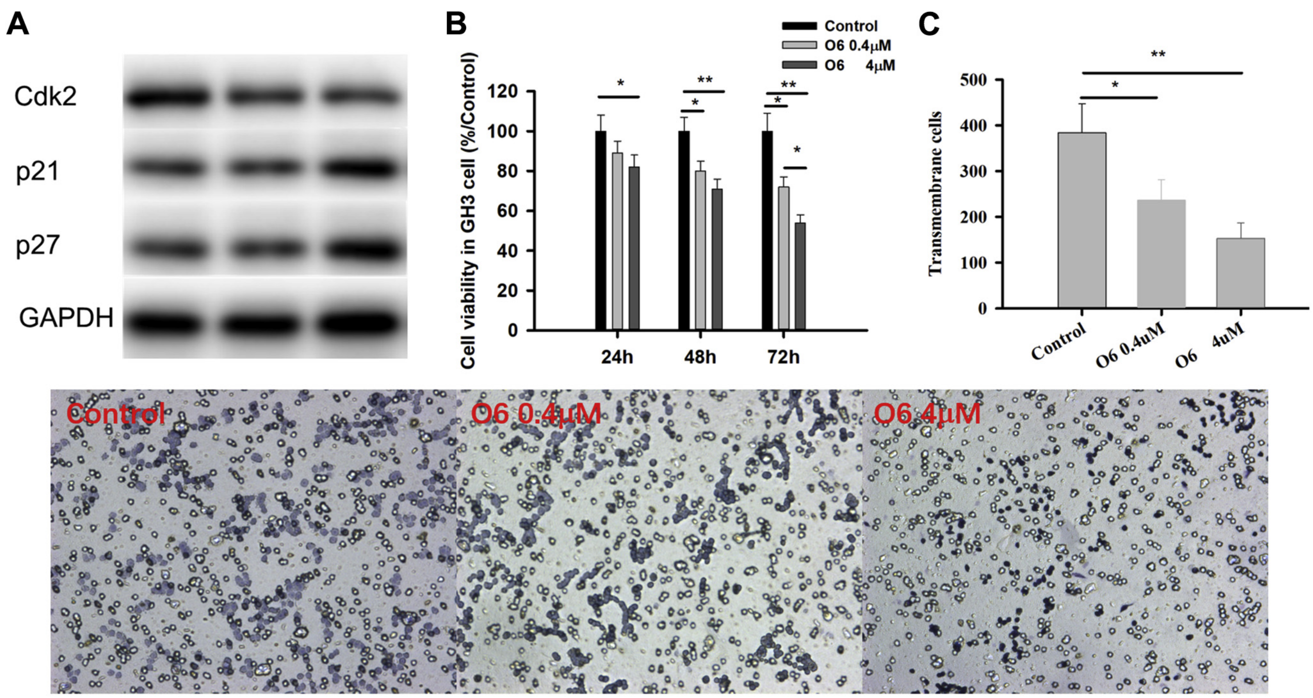

Figure 5. The effect of Cdk2 inhibitor on GH3 cell line. (**A**) The protein levels of p21 and p27 were up-regulated after Cdk2 inhibitor, O6-cyclohexylmethylguanine, treatment for 24 hours. (**B**) O6-cyclohexylmethylguanine could inhibit the cell viability after 24, 48, and 72 hours of treatment in a dose and time manner. (**C**) Transwell experiment proved that O6-cyclohexylmethylguanine reduced the transmembrane cells after 24 hours of treatment. O6, O6-cyclohexylmethylguanine; $n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$.

addition, the p21 and p27 protein levels were lower in invasive and noninvasive GHPAs than in healthy controls. In addition, the p21 and p27 protein levels were even more significantly reduced in invasive GHPAs compared with noninvasive GHPAs (Figure 3B; $P < 0.01$).

Methylation Analysis of p27 Promoter Region. The pathomechanism of pituitary adenomas includes alterations in cell cycle regulation and growth factor signaling, which are primarily caused by epigenetic changes; somatic mutations, especially germline mutations, occur more rarely.[25] Methylation analysis was performed to investigate the contribution of hypermethylation to the loss of p27 expression with the Sequenom EpiTYPER assay. An average of 33 CpG sites of p27 expression per sample were analyzed using matrix-assisted laser desorption/ionization time of flight mass array, and in 7 of the 33 CpG sites, methylation levels of p27 were >50% of the baseline value. In 4 CpG sites, significant differences were found between invasive and noninvasive specimens (Figure 4; $P < 0.01$).

The Effect of Cdk2 Inhibitor on GH3 Cell Line. To test the effect of Cdk2 in GHPAs, we used different concentrations of the Cdk2 inhibitor, O6-cyclohexylmethylguanine (O6) on the GH3 cell line. The protein level of p21 and p27 in 4 μM O6 group were 1.8 ± 0.23 and 2.3 ± 0.35 folds of those in control group (Figure 5A; $P < 0.05$). Significant inhibition of cell proliferation was observed in the 0.4 μM O6 group compared with the control group in an MTS experiment (Figure 5B), reduced to 82% ± 6%, 71% ± 5%, and 54% ± 5% after 24, 48. and 72 hours of treatment (Figure 5B; $P < 0.01$). A transmembrane invasion assay revealed that O6 significantly suppressed the invasion of GH3 cells in a Transwell experiment (Figure 5C; $P < 0.01$). Average cells in high field were 237 ± 44 in the 0.4-μM group, 153 ± 34 group in the 4-μM O6 group compared with 384 ± 63 in the control group (Figure 5C).

DISCUSSION

Cyclins are activated by binding to CDKs to form active cyclin-CDK complexes, which are inhibited by CDKIs.[13-15] In particular, cyclin E-CDK2 plays an important role in the control of G1-S transition for cell cycle regulation and serves as a mediator of antimutagenic signals of checkpoint responses, which may be prevalent in many types of tumors after their dysregulation.[11,26] In our study, we found up-regulation of cyclin E-CDK2 and down-regulation of p21 and p27 in GHPA specimens, as well as hypermethylation of the promoter region of p21 and p27.

The cyclin E-CDK2 complex mainly regulates cell cycle progression at the G1-S phase transition, which is primarily inhibited by the CDKI p27.[13,27] In contrast, the complex can phosphorylate p27 and ultimately lead to its degradation.[28] In addition, some studies have found an association between the overexpression of cyclin E and the progression of some cancers, such as leukemia, breast carcinomas, lymphomas, and corticotroph adenomas that contribute to tumor development.[29-31] In the healthy pituitary, the cell cycle progression is slower than in skin or digestive tract cells, and cyclin E expression levels are almost undetectable, whereas the expression of p27 is relatively more.[32] In addition, overexpression of cyclin E has been correlated with more elevated stage of breast tumors and more invasive hepatocellular carcinoma. These results are consistent with our finding of high levels of cyclin E-CDK2 in invasive GHPAs compared with those in noninvasive GHPAs. We found that cyclin E may be associated with the invasion of GHPAs by cooperation with p21 and p27, which was found in lower levels in invasive GHP, compared with those in noninvasive GHPAs. In an in vitro experiment, the Cdk2 inhibitor, O6, could increase the protein level of p21 and p27 in GH3 cells. The cell proliferation and invasion of GH3 cells was inhibited in dose and time manner.

As members of the Cip-Kip family of proteins, p21 and p27 have a conserved N-terminal domain that binds to Cdk2-cyclin E, Cdk2-cyclin A, or Cdk4/6-cyclin D complexes and interferes with their functioning.[33] p21 appears to be an effector of p53-mediated cell arrest, and p27 is a negative regulator during normal cell cycle progression.[13,34] Overexpression of p21 and p27 can induce stagnation of the tumor cell cycle, and lower levels of p21 and 27 often correlate with increased tumor invasiveness and poor prognosis.[35-38] Furthermore, mouse models of cyclin E overexpression that are crossed with p27 knockouts may have a high incidence of pituitary adenomas, suggesting a synergistic effect between cyclin E and p27.[29] In our study, we also found that levels of p21 and p27 were lower in invasive GHPAs than in noninvasive GHPAs. There was a significant difference between invasive and noninvasive specimens in 4 CpG sites of the p27 promotor, but no difference in the p21 promotor. Methylation of the p27 promoter should account for the low p27 level in GHPA specimens.

Previous studies have reported that all somatostatin receptors (SSTRs) may be involved in the inhibition of cell proliferation and that the effects of somatostatin analogues may be partly mediated by p27, which can induce inhibition of the cell cycle.[39] It has been confirmed that octreotide (a somatostatin analogue) can increase p27 expression in human adenomas and decrease cyclin E protein levels according to the distribution of SSTRs in pituitary adenomas.[40] We hypothesize that a synergistic effect between somatostatin/SSTR (especially SSTR2) and cyclin E/cdk2/p27 may play an important role in curing GHPAs, particularly for patients who are resistant to surgery or have tumors that cannot be completely resected, especially in invasive GHPAs.

CONCLUSIONS

In the present study, we have found that cyclin E/cdk2/p27 may be useful biomarkers for the diagnosis of invasive GHPAs. Our study indicates that these cell cycle regulators may be used as significant biomarkers for identifying invasive GHPAs, assessing prognostic value, and developing new types of targeted drugs for pituitary adenomas. However, because of the small sample size in our study, there may be some selection bias, and we believe that it is necessary to confirm our conclusion with a larger sample size.

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