Neuro-Oncology

24(6), 925–935, 2022 | https://doi.org/10.1093/neuonc/noab291 | Advance Access date 21 December 2021

Transcription factor GTF2B regulates AIP protein expression in growth hormone-secreting pituitary adenomas and influences tumor phenotypes

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Abstract

Background. Clinically, the low expression of wild-type aryl hydrocarbon receptor-interacting protein (AIP) in patients with sporadic growth hormone (GH)-secreting pituitary adenoma (GHPA) is associated with a more aggressive phenotype. However, the mechanism by which AIP expression is regulated in GHPA remains unclear. Herein, we investigated a transcription factor that regulates AIP expression and explored its role in tumor phenotypes.

Methods. General transcription factor IIB (GTF2B) was predicted by several bioinformatic tools to regulate AIP expression transcriptionally. Regulation by GTF2B was evaluated using chromatin immunoprecipitation (ChIP), reverse transcription PCR, luciferase reporter, and western blot experiments in SH-SY5Y cells. Furthermore, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, transwell invasive assay, ELISA, western blot, immunohistochemical staining, and terminal deoxynucleotidyl transferase dUTP nick end labeling were performed to investigate the effects of GTF2B and AIP on tumor cell proliferation, apoptosis, growth hormone secretion, and invasiveness in GH3 cells and mouse xenograft models. Moreover, correlations between *GTF2B* and *AIP* expression were explored in GHPA cases.

Results. ChIP and luciferase reporter studies demonstrated that the regulation of AIP expression by GTF2B was dependent on the intergenic-5′ untranslated region element of *AIP* and the initial residual S65 of GTF2B. In vitro and in vivo experiments indicated that GTF2B regulated AIP expression to impact the GHPA phenotype; this was confirmed by data from 33 GHPA cases.

Conclusions. We determined the regulation by GTF2B of *AIP* transcription in GHPA and its impact on tumor phenotype. Our findings suggest that GTF2B may be a potential therapeutic target for GHPA with low AIP expression.

Key Points

- GTF2B promotes *AIP* transcription to suppress GHPA cell proliferation, invasiveness, and GH hypersecretion.
- Transcriptional regulation of AIP by GTF2B is dependent on the intergenic-5U region of *AIP*.

Importance of the Study

This work explored the transcriptional mechanism by which GTF2B regulates the tumor suppressor gene *AIP* in GHPA. GTF2B promoted AIP expression by binding the intergenic-5′ untranslated region element of *AIP*, thus inhibiting GHPA tumor development. Our findings suggest the therapeutic potential of targeting GTF2B in GHPAs with low AIP expression, which always present as refractory.

Epidemiologically, due to severe complications (hypertension, headaches, visual disturbances, insulin resistance, cardiac hypertrophy, sleep apnea syndrome, and other types of tumors), patients with somatotropinoma or growth hormone-secreting pituitary adenoma (GHPA) face increased morbidity and mortality risks compared with the normal population over the medium- and long-term.[1] Moreover, some patients with these pathologically benign tumors may undergo tumor recurrence, as well as elevated levels of GH and/or insulin-like growth factors-1 (IGF-1) or glucose control deterioration despite conventional therapy (tumor resection by surgery, medicine such as somatostatin analogs, or radiotherapy), which results in very poor prognosis.[2,3]

The aryl hydrocarbon receptor-interacting protein (*AIP*) gene is considered a tumor suppressor in the pituitary; loss-of-function mutations in this gene predispose individuals to familial isolated pituitary adenomas or sporadic pituitary adenomas with invasive characteristics[4–6] and a relatively poor response to somatostatin analogs (SSAs).[7,8] However, GHPA cases with a familial background account for just 5% of pituitary adenomas, and most cases are sporadic.[9] Moreover, the prevalence of germline *AIP* mutations in patients with apparently sporadic pituitary adenomas is reportedly very low.[5,10,11] In addition, it has been confirmed that low wild-type AIP protein expression in patients with sporadic GHPA is associated with a more aggressive phenotype[12] and a worse response to SSAs,[13,14] which leads to a poorer prognosis.

Although several studies have investigated how AIP expression might be regulated in GHPA, the underlying mechanism remains unclear. One study revealed that miR-34a may be responsible for lower AIP expression in GHPAs with an invasive phenotype and SSA resistance,[15] which indicates that epigenetic regulation may play an important role in AIP protein expression, such as via long noncoding RNA, transcriptional factor regulation, and post-translational modifications.

Several promoter regions within a gene (which can be up to several hundred kilobases away) serve as modifiers, ensuring its accurate expression by recruiting transcription activating or silencing factors.[16] Over the last two decades, the development of multiple prediction algorithms has made it possible to determine transcription factor binding sites *in silico*.[17] In the present study, we used several bioinformatic tools to predict that a transcription factor, general transcription factor IIB (GTF2B), may regulate AIP expression transcriptionally by binding a noncoding evolutionary conserved region (ncECR) within the 5′ untranslated region (UTR) of *AIP*. Furthermore, we confirmed the transcriptional regulation effects of GTF2B on AIP expression in both clinical samples and cell lines, and investigated the influence of GTF2B on GHPA phenotypes in association with its effect on AIP expression.

Materials and Methods

Bioinformatics Analysis

The ECR Browser (http://ecrbrowser.dcode.org/) was used to generate a conservation profile by aligning the human *AIP* gene with its rat counterpart in a pair-wise fashion, and the selection parameters were established as >100 base pairs (bp) in length and >75% identity for the identification of ncECRs and the conserved SNPs in the identified ncECRs. The VISTA Browser (http://pipeline.lbl.gov/cgi-bin/gateway2) was complementary to the ECR Browser for ncECR confirmation. The UCSC Genome Browser on Human December 2013 (GRCh38/hg38) Assembly (https://genome.ucsc.edu/cgi-bin/hgGateway) was consulted for the *AIP* gene sequence (ncECR regions on Chr.11q13.1 with upstream or downstream sequences, for the cloning and luciferase experiments). The proprietary database MatInspector (Genomatix, Munich, Germany; https://www.genomatix.de) was used for all predictions of possible transcription factors and their interactions with the *AIP* promoter region.

Cell Culture

The human neuroblastoma cell line SH-SY5Y was purchased from ATCC (CCL-2266; Manassas, VA, USA). The rat somatomammotroph tumor cell line GH3 was purchased from the Cell Resource Center of the Chinese Academy of Medical Sciences (Beijing, China). Cells were seeded onto poly-L-lysine-coated plates and passaged when they reached 60–70% confluence. For the initial plating of cells in experiments, manual cell counts were performed using a hemocytometer. Culture medium details are described in the Supplementary Material.

Plasmids and Constructs

Complementary DNA (cDNA) encoding some proteins was generated by reverse transcription (RT) PCR of total RNA extracted from some cell lines. Additionally, expression vectors were created using site-directed mutagenesis or purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Details regarding the methods and conditions of transfection are available in the Supplementary Material.

Cloning and Luciferase Assays

All ncECRs identified in section *Bioinformatics Analysis* of the Methods were cloned into specific reporter vectors (pGL3 luciferase reporter vector system) and their luciferase activities were measured after being transfected into SH-SY5Y cells. Primers with specific restriction sites (e.g., KpnI, BglII, or XhoI) were designed (Supplementary Table S1). The intergenic-5′ untranslated region (IG-5U)^{AIP} elements with or without single nucleotide polymorphisms (SNPs) were also cloned into the firefly luciferase-expressing vector psiCHECK-2 (Promega, Madison, WI, USA) to confirm the binding sites in IG-5U^{AIP} elements. Further details are given in the Supplementary Materials.

Chromatin Immunoprecipitation (ChIP) and Quantitative Real-time PCR Assay

SH-SY5Y cells were transfected with wild- or mut-GTF2B, or with *AIP* siRNA overexpression vectors for the ChIP test. The ChIP real-time PCR assay was used to detect and quantify the enrichment of GTF2B at the binding site of IG-5U^{AIP} of *AIP*. All experiments are detailed in the Supplementary Material and Supplementary Table S2.

Real-time PCR

RT-PCR was then performed using the Light Cycler 2.0 (Roche, USA) and SYBR Premix Ex Taq II (Takara Bio, Shiga, Japan; DRR081A). Experimental details, including the primers and PCR conditions, are listed in the Supplementary Material and Supplementary Table S2.

Cell Proliferation Assay

Cell proliferation assays were performed using the MTT assay (Sigma-Aldrich, M5655) for various treatments at different time points after transfection (24 h, 48 h, and 72 h). The absorbance of cell supernatant aliquots at 490 nm was measured using an automatic plate analyzer (Bio-Rad Laboratories, Hercules, CA, USA).

Transwell Invasion Assay

For the invasion assay, 8 μm Transwells in a 24-well plate were coated with 150 μL of a mixture of culture medium and reconstituted Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) at a ratio of 2:1. Absorbance at OD 560 nm was then measured using a plate reader. Further details are available in the Supplementary Material.

Protein Extraction and Western Blot Analysis

The experiment included cell harvesting, protein extraction and separation, immunoblot incubation, and antibody probing. Antibody information is listed in Supplementary Table S3, and the steps of the experiment are described in the Supplementary Material.

Animal Care and Ethics Statement

Six-week-old female BALB/c nude mice were housed in laminar flow cabinets under specific pathogen-free conditions with free access to food and water. All animal handling and procedures were performed according to a protocol that was approved by the institutional animal care and use committee of Zhejiang University.

GH3 cells (1×10^8/L) with various transduction treatments were injected with Matrigel Matrix (Corning, Corning, NY. USA; No. 354262) into the right axilla of mice at random to make up various groups in the animal experiment. Next, overexpression plasmids and/or siRNA vectors (blank vectors were used as negative controls (NC)) in various treatments dissolved with diluted water were intratumorally injected into the animals (5 nmol/kg) every 3 days for 6 weeks.[18] After 12 days of injections, the tumor volumes were calculated using the following formula: volume (mm^3) = ($L \times W^2$) × 0.54; where L = the longest diameter and W = the shortest diameter. In addition, the tumor volume of each mouse was recorded once every 3 days. All mice were sacrificed upon completion of the 42-day experiment, followed by tumor excision, measuring, and refrigeration for next studies.

Immunohistochemical (IHC) Analysis

Formalin-fixed paraffin-embedded xenograft tumor sections were collected for IHC experiments, which are detailed in the Supplementary Material. Antibody information is listed in Supplementary Table S4.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Staining of GH3 Xenografts

Tissue harvested from GH3 xenografts was fixed in 4% formaldehyde and embedded in paraffin for TUNEL staining. The staining was performed using a TUNEL kit (Roche, 11684817910) as per the manufacturer's instructions. The reactivity intensity was measured and quantified using a DS-Ri2.

ELISA

The levels of GH in cell culture supernatant were measured using a rat ELISA kit (LifeSpan BioSciences, Seattle, WA, USA; LS-F27456) according to the manufacturer's protocol. Absorbance was read at 450 nm using an ELISA plate reader (Thermo Fisher Scientific). To measure GH and IGF-1 levels in mice with tumor models, blood was drawn from the retro-orbital sinus. According to the manufacturer's instructions, GH levels were measured using a rat ELISA kit (LifeSpan BioSciences, LS-F27456), while IGF-1 levels were assessed using a mouse ELISA kit (Abcam, ab108874).

Patients and Tissue Samples

All patients in the present study were confirmed to have somatotroph adenomas by IHC, according to the 2017

WHO pathological classification of pituitary adenomas. Based on the criteria by Trouillas et al.[19] and the definition suggested by Sav et al.[20] "invasion" in this study was considered to be histological/radiological signs of the tumor and/or surgical detection of the tumor in the cavernous or sphenoid sinus, while "proliferation" was defined by the presence of at least two of the following three criteria: Ki-67 ≥ 3%; mitoses: $n > 2/10$ high-power fields; and p53: >10 positive nuclei/10 high-power fields. All tumors were then classified into five grades: 1a (non-invasion), 1b (non-invasion and proliferation), 2a (invasion), 2b (invasion and proliferation), and 3 (malignant). They were then further classified into two groups: low grade (grades 1a, 1b, and 2a) and high grade (grades 2b and 3). To explore the correlations between clinical features and *GTF2B* and *AIP* mRNA expression, we enrolled 33 patients (33 samples) with somatotroph adenomas (all from the Department of Neurosurgery, The Second Affiliated Hospital of Zhejiang University, from April 2016 to December 2019). All patients were diagnosed with pituitary adenoma for the first time and did not receive any treatment before surgery. Once removed from the body, all tissue fragments of the tumor samples were immediately frozen in liquid nitrogen and stored at –80°C until molecular analysis.

This study was approved by the ethics committee of The Second Affiliated Hospital of Zhejiang University, according to the 3rd edition of the Guidelines on the Practice of Ethical Committees in Medical Research (issued by the Royal College of Physicians of London). Written informed consent was obtained from each patient in accordance with the Declaration of Helsinki.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics Version 26 (IBM Corp., Armonk, NY, USA). Data are expressed as the mean ± SD. Mann–Whitney *U* tests and two-tailed Student's *t*-tests were used for comparisons between two groups. Spearman's correlation analysis was used to analyze the correlations between two variables. A value of $P < .05$ was considered statistically significant.

Results

Prediction and Investigation of Functional ncECRs and the Corresponding Transcription Factors Within the AIP Genomic Locus

Using comparative genomics in the ECR Browser (http://ecrbrowser.dcode.org/), 13 ncECRs were identified within the *AIP* genomic region on human chromosome 11q13.1 that were conserved between humans and rats (Figure 1A). Six of these DNA sequences (U1–6) were located upstream of the *AIP* gene, four were intronic (I1–4), and one was intergenic. Both UTRs (5′UTR and 3′UTR) were also indicated as ncECRs. Because the intergenic region is very close to 5′UTR, both were merged as one ncECR (the intergenic-5U, chr11:67250220–67250629, 410bp) to be tested in the next step. All of the identified ncECRs were evaluated to investigate their activity as enhancers or silencers of *AIP* transcription using the pGL3 luciferase reporter system in the human neuroblastoma SH-SY5Y cell line. Four ncECRs (U5, I1, I3, and I4) were unable to be effectively amplified by PCR under various experimental conditions. Of the other ncECRs, intergenic-5U had the most significant increase in expression of the luciferase reporter gene ($P < .05$, vs. control group). In contrast, U6 and 3′UTR had reduced expression of the reporter gene ($P < .05$, vs. control group) (Figure 1B). These data indicate that some ncECRs in the *AIP* gene may play a regulatory role in *AIP* transcription.

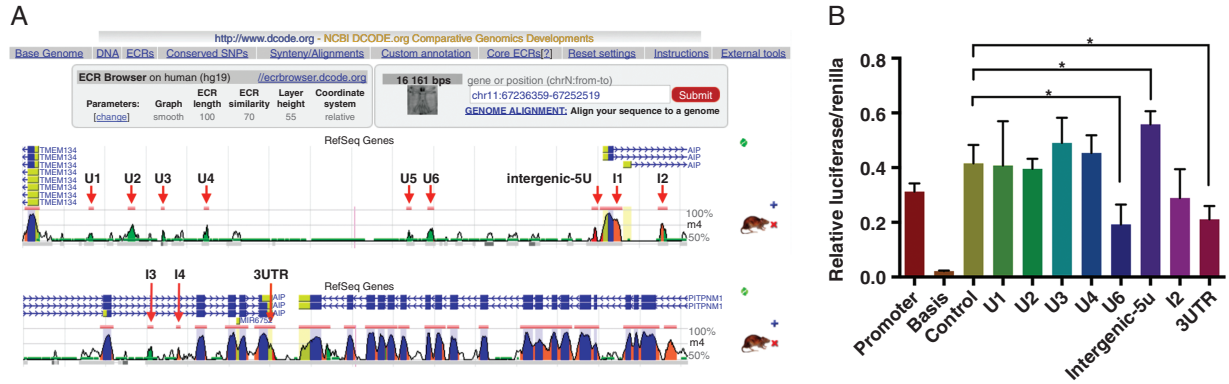


Fig. 1 Investigation functional ncECRs within the *AIP* genomic locus. A. Panel shows human–rat pair-wise comparison of Human genome (hg19) and Rat genome (rn4). Pink marked lines represent ncECRs, blue marked peaks represent coding exons, green marked peaks represent transposons and simple repeats, salmon marked peaks represent intronic regions, red marked peaks represent intergenic regions, yellow marked peaks represent the untranslated region (UTR: 5′UTR and 3′UTR) of *AIP*. U1–U6 are conserved regions upstream of AIP, I1–I4 are intragenic conserved regions, while intergenic-5U is the merged as one region by intergenic and 5′UTR sequences. B. Luciferase assay results of ncECRs of the *AIP* genomic locus. (*$P < .05$, vs. control.). The experiments were repeated three times with consistent results. The two-tailed Student's *t*-tests were used. Bar represents mean ± SD. See online supplementary material for a color version of this figure.

Next, we performed a prediction analysis of transcription factors and transcription factor binding sites (TFBSs) in the intergenic-5U element with MatInspector database that is a software tool for TFBSs description to locate matches in DNA sequences. As well as the original human–rat comparison that was used to identify ncECRs, sequences from the mouse, cow, and dog were also added in the analysis. Because the intergenic-5U element had significantly increased expression of the luciferase reporter gene, the models for screening transcription factors were designed as follows: the transcription factors needed to be enriched in the pituitary gland and be indicated as an enhancer of *AIP* transcription; TFBSs in the intergenic-5U elements needed to be present in all five species in the same orientation; and there needed to be at least three match sites for each transcription factor in one species with $P < .05$. Hence, only INRE (described as hypothetical protein B1J92_L12848g [*Candida glabrata*] by the National Center for Biotechnology Information [NCBI]) and GTF2B (also known as TF2B) conformed to these criteria. Here, GTF2B was considered an intriguing candidate for the following research because it had greater significance in the Match model (a lower *P*-value) and its protein sequence in *Homo sapiens* was available, unlike that of INRE (Supplementary Figure S1A). Moreover, the Graphical View displayed two TFBSs to GTF2B within the intergenic-5U region of *AIP* in *H. sapiens* (67250480–67250486 and 67250551–67250557), and the latter site was common to all five species (Supplementary Figure S1B). Furthermore, two conserved SNPs were identified within the intergenic-5U region by ECR Browser. One SNP (rs561050596[C/T]); 67250470) was located adjacent to one TFBS (67250480–67250486), and another SNP (rs377565228 [C/G]; 67250553) was located within another TFBS (67250551–67250557) (Supplementary Figure S1C). Alignment work using UniProt (www.uniprot.org) revealed that the protein sequences of GTF2B (or TF2B) were almost the same between humans and rats (identity: 99.684%, with one similar position, 302 amino acid [aa]) (Supplementary Figure S1D).

Evaluation of Whether GTF2B Transcription Activity on AIP Expression Depends on the Intergenic-5U Element

Here, SH-SY5Y cells were used. It has been reported that the phosphorylation of GTF2B at serine 65 is a critical event in transcription, linking the gene promoter and terminator[21] ChIP results demonstrated that GTF2B, but not GTF2B (S65A) (serine at 65 aa replaced by alanine), mediated the fold enrichment of the intergenic-5U element (chr11:67250220-67250629, 410bp) of the *AIP* promoter; this effect was decreased by *GTF2B* siRNA (Figure 2A). Furthermore, to confirm the activity that was dependent on the binding of GTF2B to the intergenic-5U element of *AIP*, a pGL3 luciferase reporter system was designed. Fluorescence intensity was strongest in the group with co-transfection of GTF2B and the intergenic-5U element of *AIP* with luciferase (GTF2B + intergenic-5U^{AIP}-Luc), while intensity was relatively weak in the group with

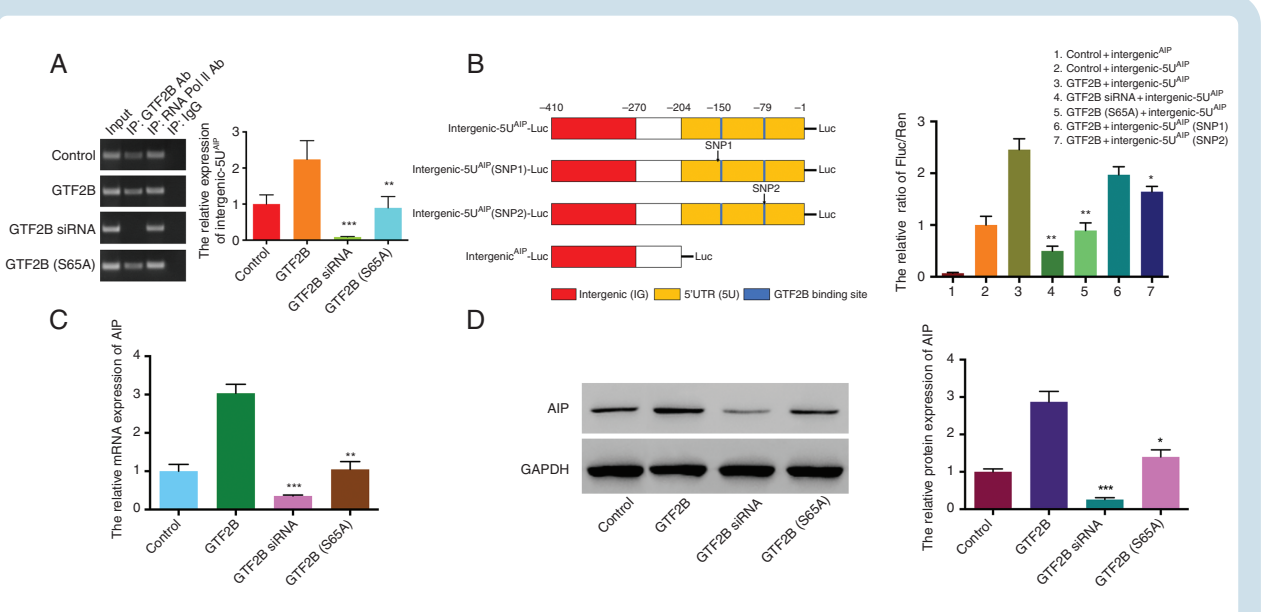


Fig. 2 The transcriptional effect of GTF2B on AIP expression in SH-SY5Y cell line. A. ChIP experiment verifies GTF2B can bind intergenic-5U element. The relative expression of intergenic-5U by RT-PCR represents difference of binding activity between various treatments (**$P < .01$ and ***$P < .001$, vs. GTF2B group). B. Binding activity dependent on TFBSs located within 5U segment. Luciferase activity normalized for Renilla luciferase activity and expressed relative to the activity of the control + intergenci-5U^{AIP} group. Different luciferase reporter constructs are design as left diagram; SNP1: rs561050596, C > T; SNP2: rs377565228 C > G; (*$P < .05$, **$P < .01$, vs. GTF2B + intergenic-5U^{AIP} group). C and D. Effects of various transfections on the expression of AIP mRNA and protein in SH-SY5Ycells, analyzed by qRT-PCR and western blotting (*$P < .05$, **$P < .01$, and ***$P < .001$, vs. GTF2B group). Three independent experiments were repeated for each result. The two-tailed Student's *t*-tests were used. Bar represents mean ± SD.

GTF2B siRNA overexpression ($P < .01$), and was not observed in the group with only the intergenic element of *AIP* (intergenic^{AIP}-Luc). Luciferase intensity in the group transfected with GTF2B (S65A) (GTF2B(S65A) + intergenic-5U^{AIP}-Luc) was also weaker than GTF2B + intergenic-5U^{AIP}-Luc ($P < .01$). Additionally, compared with GTF2B + intergenic-5U^{AIP}-Luc, the intensity was mildly decreased in the group transfected with intergenic-5U^{AIP}-Luc with SNP2 (rs377565228), but not with SNP1 (rs561050596) (Figure 2B). Finally, GTF2B overexpression in SH-SY5Y cells induced *AIP* transcription and protein expression, while *GTF2B* siRNA transfection inhibited AIP expression. Conversely, GTF2B (S65A) transfection did not seem to affect AIP expression (Figure 2C and D).

Effects of GTF2B on the AIP-Dependent Phenotypes of GH3 Cells

Because both GTF2B and AIP are highly conserved between humans and rats, the rat GH pituitary adenoma cell line GH3 was used to elucidate the influence of GTF2B and AIP on cell phenotypes through cell proliferation, invasiveness, and GH secretion and proteins expression analysis. It is known that AIP inhibits tumor growth; similarly, in the present study, *Gtf2b* also inhibited GH3 cell proliferation from the beginning (at 24 h point), which could be reversed by *Aip* siRNA treatment. However, *Gtf2b (S65A)* could restrain cell proliferation later (at 48 h point) with lower inhibitory rates, and the addition of *Aip* (Gtf2b (S65A) + Aip) could improve inhibitory rate for *Gtf2b (S65A)*(Figure 3A). Moreover, in Figure 3B and C, GH3 cells with *Gtf2b* (column 3) or –*Aip* (column 2) overexpression had a more invasive and GH-secreting phenotype than those with control (column 1) or *Gtf2b (S65A)* (column 4) transfection. Conversely, *Aip* siRNA overexpression (column 5) led to a complete reversal of the function of *Gtf2b* in tumor cells. GH3 cells with *Gtf2b (S65A)* + *Aip* overexpression (column 6) had weaker invasiveness and GH secretion than cells with *Gtf2b (S65A)*. Western blotting results displayed that the *Gtf2b* overexpression group, like *Aip* overexpression group, had less expression of Ki-67 (cell proliferation) and matrix metallopeptidase 2/9 (MMP2/9, invasiveness promotion), but more zinc-finger protein 1 (ZAC1) and E-cadherin (E-cad) (invasiveness inhibition) expression, compared to control or *Gtf2b* (*S65A*) group. The expression pattern of proteins in the *Gtf2b* overexpression group could be reversed by *Aip* siRNA, while that in the *Gtf2b* (*S65A*) group could be reversed by *Aip* overexpression. These findings indicate that the inhibition by GTF2B of GHPA cell proliferation and invasiveness and GH secretion depends on the promotion by GTF2B of AIP expression transcriptionally.

Confirmation of GTF2B Inhibition of Tumor Growth via AIP in vivo

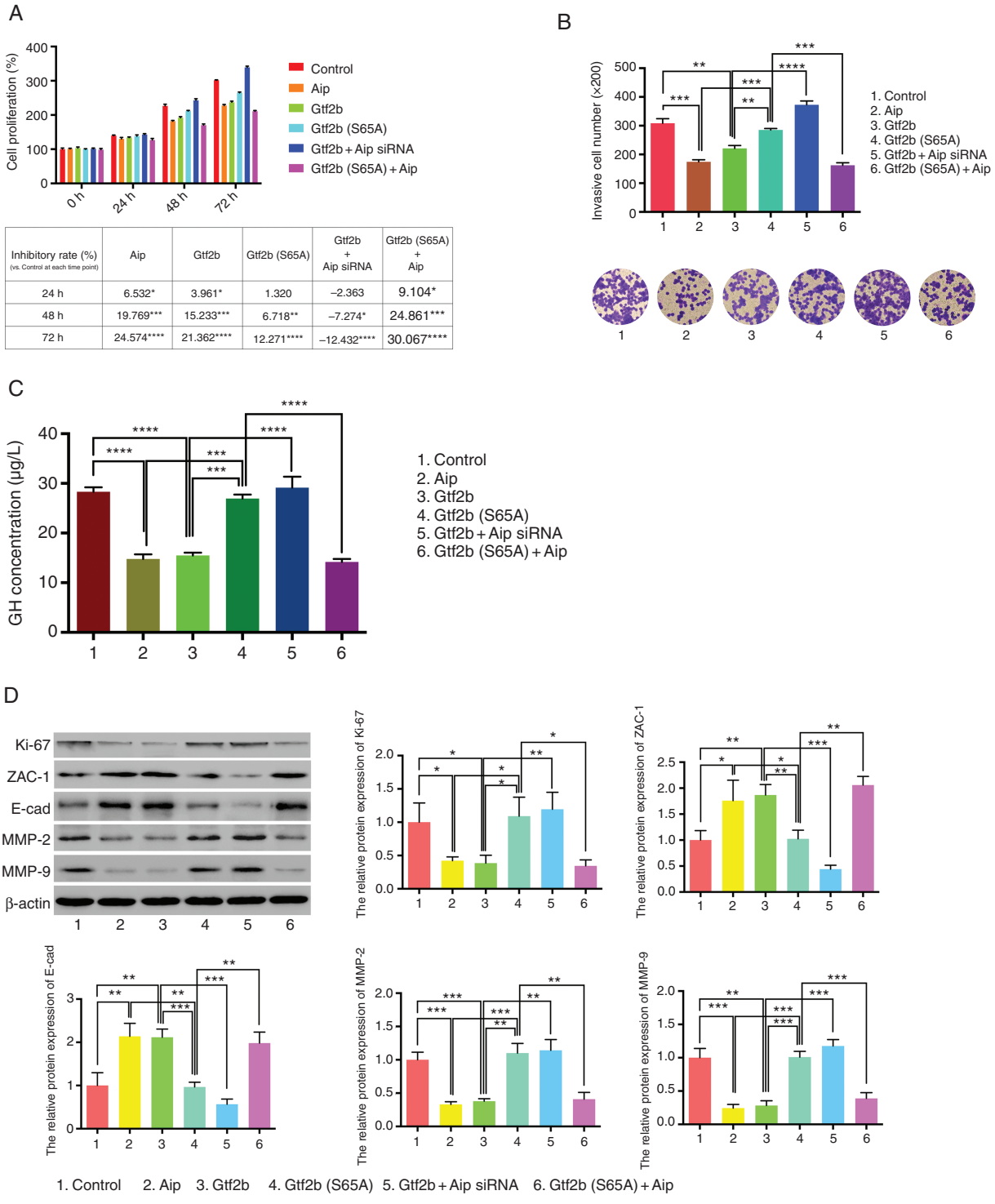
Because nude mice are immune-deficient and amenable to xenografts, GHPA models were established via transplantation of GH3 with variant transduction treatments subcutaneously into the flanks of nude mice. The tumor size and weight in the *Aip*, *Gtf2b*, and *Gtf2b (S65A)* + *Aip* groups were significantly smaller than those in the control group, while those of the *Gtf2b (S65A)* and *Gtf2b* + *Aip* siRNA groups were much larger ($P < .05$) (Figure 4A and B). To detect the effects of various treatments on GH and IGF-1 secretion in GH3 xenograft tumor-bearing mice, blood serum was examined, and both GH and IGF-1 levels in the *Aip*, *Gtf2b*, and *Gtf2b (S65A)* + *Aip* groups were much lower than in the control group (Figure 4C). Likewise, IHC and western blot results revealed that tumor samples from mice bearing GH3 with *Aip*, *Gtf2b*, and *Gtf2b (S65A)* + *Aip* had a lower Ki-67 index and lower MMP-2 and MMP-9 expression, but higher AIP, Zac-1, and E-cadherin expression (Figure 4D and Supplementary Figure S2). Furthermore, to test apoptotic rates of these groups, TUNEL staining was performed on paraffin-embedded section of GH3 xenograft tumors treated with various transduction. As shown in Supplementary Figure S3, tumor tissue from the *Aip* and *Gtf2b* groups had more TUNEL-positive cells (apoptotic cells) than the *Gtf2b (S65A). Gtf2b* + *Aip* siRNA group could decrease the number of apoptotic cells compared with *Aip* or *Gtf2b* group, while *Gtf2b (S65A)* + *Aip* led to the reversal of the lower tumor apoptotic rate in the *Gtf2b (S65A)* group. These in vivo results suggest that GTF2B impedes pituitary tumor growth via AIP regulation of tumor cell proliferation, apoptosis, invasiveness, and GH/IGF-1 secretion.

Correlations Between GTF2B and AIP Expression in GHPA Samples

To evaluate the correlations between *GTF2B* and *AIP* mRNA expression in GHPA samples, we enrolled 33 GHPA patients (male:14, female:19), and the age ranged from 24 to 57 years (mean ± SD, 40.97 ± 8.33; median, 42). No patient had *GTF2B* or *AIP* mutations or the two SNPs (rs561050596 and rs377565228) in either germline or tumor cells. Patients with high-grade GHPAs accounted for 42.42% (14/33) of the group (Table 1). There was a significant positive correlation between *GTF2B* and *AIP* mRNA expression levels in the GHPA samples (Spearman's correlation analysis; $P = .019$) (Figure 5A). In addition, in the high-grade group, *GTF2B* and *AIP* mRNA expression levels were lower than those in the low-grade group (Figure 5B). Chi-square test indicated that neither *GTF2B* nor *AIP* mRNA expression levels were related to patient age or sex; however, most of the GHPAs with low *GTF2B* or *AIP* mRNA levels were in the high-grade group or Ki-67 ≥ 3% group (Table 1).

Discussion

AIP is a tumor suppressor gene, and *AIP* gene mutations are present in approximately 20% of patients with familial isolated pituitary adenomas.[5,22] Generally, patients with *AIP* germline mutations are younger and have more aggressive tumors with worse responses to somatostatin receptor ligands (SRLs; octreotide and lanreotide).[8,22] In patients with sporadic GHPAs without *AIP* mutations, low AIP protein expression has been observed in 50% of those with SRL resistance.[13] Moreover, research by Kasuki et al. has indicated that AIP is a better marker of invasiveness



Inhibitory rate (%) (vs. Control at each time point)	Aip	Gtf2b	Gtf2b (S65A)	Gtf2b + Aip siRNA	Gtf2b (S65A) + Aip
24 h	6.532*	3.961*	1.320	−2.363	9.104*
48 h	19.769***	15.233***	6.718**	−7.274*	24.861***
72 h	24.574****	21.362****	12.271****	−12.432****	30.067****

Fig. 3 Effect of GTF2B on tumor cell phenotype of GH3 cell line. A. Proliferation analysis of tumor cell with different transfection by MTT assay. OD value of control group at 0 h was normalized as 100% and all relative cell proliferation value at different time points are shown in column diagram above; the table below displays the relative inhibitory rate vs. control group at each time point. B. Transwell array was used to assess GH3 cell invasiveness with various treatments (original magnification, ×200). C. ELISA was performed to test GH secretion of GH3 cell with various treatments. D. Western blot analysis of Ki-67, ZAC-1, E-cadherin, MMP2 and MMP9 expression in GH3 cell line with various treatments. Each condition was tested in triplicate. The two-tailed Student's *t*-tests were used. Bar represents mean ± SD, **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001.

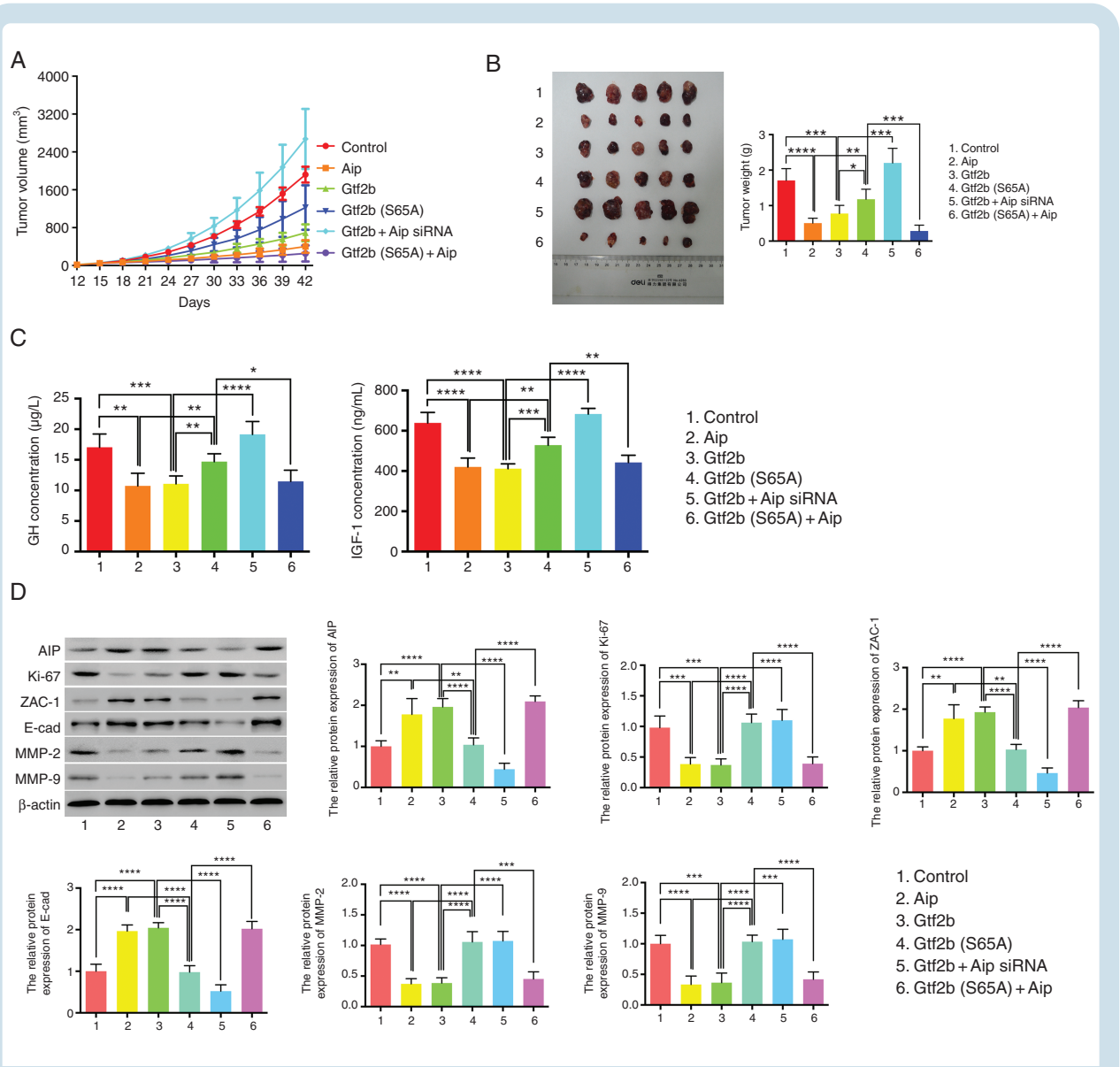


Fig. 4 The inhibition of GTF2B on tumor growth in subcutaneous GHPA models. A. Tumor volume of mice with various treatments once daily since the 12th day after tumor cell transplantation, measured for 42 days. B. Macroscopic image of tumor resected at the 42nd day (left) and the tumor weight comparison between various treatments (right). C. ELISA assays to test GH (left) and IGF-1 (right) secretion from mice with various treatments. D. Western blot analysis of Ki-67, ZAC-1, E-cadherin, MMP2 and MMP9 expression in tumor models with various treatments. Each group contained five mice, the two-tailed Student's *t*-tests were used. Bar represents mean ± SD, $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$.

in somatotropinomas than Ki-67 or p53.[12] It has recently been demonstrated that there is an altered microenvironment in AIP-mutated pituitary tumors; some tumor-derived factors interact with macrophages, resulting in infiltration, epithelial-to-mesenchymal transition, and a more aggressive phenotype.[23] These results indicate that AIP expression is important for the prognosis of the GHPA patients. Thus, the present study focused on identifying the transcription mechanism that impacts *AIP* gene expression.

It has been reported that ncECRs constitute an ordered combination of overlapping TFBSs[24] or are strongly enriched for overlapping TFBSs.[25,26] To investigate transcriptional regulation of the *AIP* region, a complementary approach was used in this study. The ECR Browser was used to identify all possible ncECRs of the *AIP* gene region and was followed by the screening of candidate regions using luciferase reporter systems. A single ncECR, intergenic-5U (the intergenic region and 5′UTR), of *AIP* was identified as a potential transcription element; this was confirmed by ChIP and luciferase assays. Furthermore, two SNPs (rs561050596 and rs377565228) within the region were designed as vectors, and experiments indicated that the intergenic-5U region is very important for the binding of the transcription factors. In fact, it has been suggested that some SNPs in these regions may modulate the transcriptional patterns of genes,[27] and that these

Table 1 The Correlation Between Clinical Features of GHPAs and the Expression of GTF2B and AIP mRNA

Clinical features	Number A (%, A/33)	Low GTF2B mRNA expression ($n = 16$) [a]		Low AIP mRNA expression ($n = 16$) [b]	
		Number B (%, B/A)	*P* value [c]	Number C (%, C/A)	*P* value [c]
Age, years					
<42	16(48.48)	8(43.75)	0.849	9(37.50)	0.387
≥42	17(51.52)	8(47.06)		7(41.18)	
Sex					
Male	14(42.42)	9(57.14)	0.247	7(50.00)	0.881
Female	19(57.58)	7(36.84)		9(47.37)	
Ki-67 (%)					
<3	21(63.64)	7(33.33)	0.021	6(28.57)	0.002
≥3	12(36.36)	9(75.00)		10(83.33)	
High grade[d]					
No	19(57.58)	5(26.32)	0.010	6(31.58)	0.024
Yes	14(42.42)	11(78.57)		10(71.43)	

[a]The median of relative GTF2B mRNA expression level is 0.033, so the number of low GTF2B mRNA expression is 16 (<0.033).
[b]The median of relative AIP mRNA expression level is 0.06, so the number of low AIP mRNA expression is 16 (<0.06).
[c]Analysis by chi-square test.
[d]The grade classification is based on the criteria by Trouillas et al.[19].

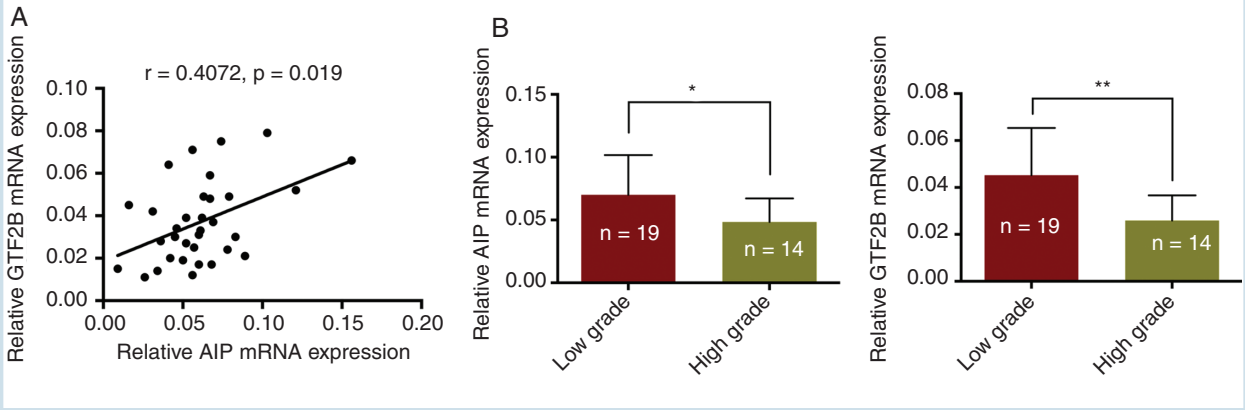


Fig. 5 The correlation between *GTF2B* and *AIP* mRNA level and their effects on tumor features in GHPA samples. A. Spearman correlation analysis was used to test the correlation between *GTF2B* and *AIP* mRNA expression level in 33 GHPAs patients. B. qRT-PCR analysis of *GTF2B* and *AIP* mRNA expression between low grade group ($n = 19$) and high-grade group ($n = 14$) of GHPA samples. The Mann–Whitney *U* test was used. Bar represents mean ± SD, $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

noncoding variants may be associated with common diseases and traits.[28] Although we confirmed that one SNP (rs377565228)in the intergenic-5U region of *AIP* was able to affect the binding of transcription factors and its subsequent transcription in vitro, none of the 33 patients in our study had either of the two SNPs. Unfortunately, there is no information about the associations between either variant and clinical presentation, or about variant frequency, in the Database of Single Nucleotide Polymorphisms of the NCBI (https://www.ncbi.nlm.nih.gov/snp/). In a study that analyzed 49 GHPA patients and 167 healthy controls, the metastasis-suppressor KiSS-1 c.-145delA (rs5780218) promoter polymorphism was reported to have a possible association with somatotropinoma incidence.[29] Furthermore, a larger study (2542 patients vs. 2788 controls) identified three new susceptibility loci associated with sporadic pituitary adenoma in a Chinese Han population cohort: 10p12.31 rs2359536, 10q21.1 rs10763170, and 13q12.13 rs17083838.[30] Thus, when considering rs561050596 and rs377565228 (or other SNPs within or near the region), more GHPA cases should be enrolled to explore the effects of both SNPs on *AIP* transcription and GHPA phenotypes in further clinical studies.

In the present study, MatInspector (Genomatix, Munich, Germany) analysis predicted that the GTF2B transcription factor was most likely to bind to the IG-5U region

with the highest fold change in the luciferase assay. This database has been widely used for genetic studies. For example, Sterling et al. reported that the synuclein alpha (*SNCA*) gene has some cis-regulatory regions for its transcription and protein expression that are associated with the Parkinson's disease process.[31] It was also predicted by Song et al. that some potential binding motifs for transcription factors exist within the promoter regions of miR-378; one such factor was a highly conserved binding site for nuclear respiratory factor 1 (NRF1) that repressed miR-378 promoter activity.[32] GTF2B, which is required for transcription initiation by RNA polymerase II, forms a complex with GTF2D and GTF2A in the nucleus for promoter sequence recognition and transcription initiation.[33] It has previously been demonstrated that GTF2B is involved in the proliferation of hepatocellular carcinoma by counteracting the transcriptional activation of hepatitis B virus X protein.[34] In addition, a new paradigm for GTF2B functionality was recently established that has potent anti-viral immunity.[35] In the current study, GTF2B affected *AIP* transcription and protein expression and influenced GHPA tumor phenotypes by binding to the ncECR intergenic-5U in *AIP*. However, the possibility cannot be excluded that other transcription factors and binding sites in these high evolutionary conserved regions, or other intronic or intergenic regions, are involved in *AIP* transcription modulation. These "parameters" may function as a regulatory network for AIP expression with their own weight coefficient, which should be studied using more clinical data in the future.

Previous studies have reported that the transcription/expression of genes is not only driven by their promoter regions; other cis-acting genomic regions also play a potential regulatory role in gene expression levels. Korbonits et al. confirmed that both miR-107[36] and miR-34a[15] negatively regulate AIP protein expression; moreover, miR-34a may respond to the low AIP expression in GHPAs with an invasive phenotype and SSA resistance.[15] Other than microRNAs (miRNAs), long noncoding RNAs have also been linked to gene regulation affecting cellular homeostasis and even malignant transformation.[37] Circular RNAs (circRNAs) are another conserved element that are deregulated in cancer via their functional role—having a sponge effect on miRNAs.[38] Recently, circTVF25 has been demonstrated to act as an miRNA sponge that sequesters miR107 and promotes proliferation and migration in bladder cancer.[39] Hence, AIP expression may be regulated through the circTVE25–miR107 pathway; this should be investigated in a future study. Furthermore, it should be considered that noncoding RNA networks may play a key molecular role in AIP expression.

Conclusion

Our research demonstrated that the transcription factor GTF2B was able to mediate AIP expression and influence GHPA phenotypes, such as tumor growth, tumor invasion, and GH hypersecretion. These effects were based on the TFBS located at the intergenic-5U region in *AIP*. It is suggested that GTF2B may be a potential therapeutic target for GHPA with low AIP expression. Moreover, other epigenetic mechanisms will also be considered in further researches.

Supplementary Material

Supplementary material is available at *Neuro-Oncology* online.

Keywords

AIP | GTF2B | somatotropinoma | transcriptional regulation

Acknowledgments

The authors thank Bronwen Gardner, Ph.D., from Liwen Bianji (Edanz) (www.liwenbianji.cn/), for editing the English text of a draft of this manuscript.

Funding

This study was supported by the National Natural Science Foundation of China (81502321 and 81870964).

Conflict of interest statement. No conflict of interest.

Authorship statement. Conception or design of the study: F.C., X.Y., and Q.W. Laboratory practice: F.C., Sha.C., Jin.Z., Y.Z., and Y.C. Drafting the article: F.C., Sha.C., Y.Z., Sha.C., H.Y., W.Y., and W.W. Subject recruitment: X.Y., W.L., She.C., Y.H., W.Y., W.W., Jin.Z., and Q.W. Data analysis and interpretation: F.C., Sha.C., Jin.Z., W.L., and Y.Z. Critical revision: F.C., Sha.C., X.Y., Jia.Z., and W.L. All authors critically reviewed the article and approved the final manuscript.

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