Mutations in the GNAS Gene Prevent the Cell Invasion by Activating the MEG3/Wnt/ β -catenin Axis in Growth Hormone-secreting Pituitary Adenoma

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Abstract

Approximately 30–40% of growth hormone-secreting pituitary adenoma (GHPA) harbor somatic mutations in the *GNAS* (α subunit of the stimulatory G protein) gene. However, the latent functional role of the mutations and relative molecular mechanism in GHPA remain unknown. The *GNAS* gene mutations were detected in GHPAs using a standard PCR-sequencing procedure. The mutation-associated *MEG3* expression was measured by RT-qPCR. *MEG3* was manipulated in GH3 cells using a lentiviral expression system. Alterations in mRNA profiles in the *MEG3*-overexpressed cells were analyzed by RNA-seq. The cell invasion ability was measured using a Transwell assay, and the EMT-associated proteins were quantified by immunofluorescence and western blot. Finally, a tumor cell xenograft mouse model was applied to verify the effect of *MEG3* on tumor growth and invasiveness. The percentage of invasive tumors was significantly declined in *GNAS*-mutated GHPA tumors with the *GNAS* mutations compared to those tumors with the wild-type of *GNAS*. Consistently, the GH3 cell invasion capacity was decreased by expressing the mutant *GNAS*. *MEG3* is uniquely expressed at high levels in GHPA harboring the mutated *GNAS* gene. Accordingly, the upregulation of *MEG3* resulted in inhibiting cell invasion; and vice versa, the downregulation of *MEG3* led to enhancing cell invasion. Mechanistically, the high level of *MEG3* in mutated *GNAS* cells prevented the cell invasion via inactivation of the *Wnt/β-catenin* signaling pathway, which was further validated in vivo. The *GNAS* mutations inhibit the invasiveness of GHPA cells via inactivation of the *MEG3/Wnt/β-catenin* signaling pathway.

Introduction

Growth hormone-secreting pituitary adenoma (GHPA) accounts for 12.5% of pituitary neuroendocrine tumors, and excessive growth hormone resulted in acromegaly and systemic complications (Orme et al., 1998, Colao et al., 2004, Melmed, 2006). Acromegaly has been associated with a two-fold increase in mortality, mainly due to cardiovascular disease, which can be reversed by treatments for controlling the hormone over-production (Melmed, 2006). In addition, approximately half of patients with GHPA experienced high-risk relapse after the surgical reduction due to tumor cells infiltrating the surrounding tissues. Thus, supplementary chemotherapy still needs to control the tumor recurrence (Wilson et al., 2013).

It has been well-demonstrated that mutations found in the *GNAS* gene, encoding a subunit of the stimulatory G protein, were detected in around 40% of GHPA (Goto et al., 2014, Hayward et al., 2001). The functional studies suggested that the *GNAS* mutations lead to the constitutive activation of adenylyl cyclase (AC), thereby inducing the cyclic AMP (cAMP) signaling pathway in pituitary tumors (Lania et al., 2012, Mantovani et al., 2010). The function of the *GNAS* mutations is thought to be involved in cell proliferation and hormone secretion (Stork and Schmitt, 2002).

Maternally expressed gene 3 (*MEG3*), a large non-coding RNA (lncRNA), was first identified as a tumor suppressor in the pituitary (Zhang et al., 2003). The function of *MEG3* is associated with inhibition of cell invasion (Ma et al., 2019). In addition, the cAMP response element (CRE), located at the *MEG3* proximal

promoter region, is critical for *MEG3* expression (Zhao et al., 2006). Furthermore, cyclic adenosine monophosphate-responsive element-binding protein (CREB), as a downstream target of mutated *GNAS*, is also implicated in upregulation of *MEG3* by binding to the CRE site (Zhao et al., 2006, Yamamoto et al., 1990). Therefore, we speculate that the *GNAS* mutations may participate in the upregulation of *MEG3* expression. The present study aimed to ascertain whether the *GNAS* mutation inhibits the invasiveness of GHPA cells mainly through *MEG3*-mediated inactivation of the *Wnt/β-catenin* pathway, which may provide a new therapeutic approach for treating GHPA.

Materials And Methods

Patients and clinical characteristics.

Tumor samples were collected from forty-four patients with acromegaly who underwent endoscopic endonasal transsphenoidal surgery at the Department of Neurosurgery of Nanjing Jinling Hospital (Nanjing, China) between Nov. 2018 and Nov. 2019, including 21 males and 23 females. Additionally, 10 patients with clinically non-functioning pituitary tumors (NFPA) were included as negative controls. Approval for the study was obtained from the Ethical Committee of Nanjing Jinling Hospital (2018NZKY-008-02) and informed consents were obtained from all the patients who participated in this study. Pituitary adenomas were classified into invasive and non-invasive tumors, according to the degree of lateral extension to the cavernous sinus (CS) space by MRI scanning (Cottier et al., 2000). Knosp grade 3 and 4 were defined as invasive pituitary adenomas, and Knosp grade 0 to 2 were defined as non-invasive tumors, respectively (Knosp et al., 1993). Tumor volume was determined by (length x width x height x π)/6. The clinical characteristics of patients were described in Table 1.

Detection of mutations in the *GNAS* gene

Genomic DNA was extracted from 44 GHPA and 10 NFPA tissues using a DNA miniprep kit according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). The underlying point mutations in the *GNAS* gene have been reported in tumor specimens were CGT-to-TGT mutation at codon 201 (Arg201Cys) and CAG-to-CTG mutation at codon 227 (Gln227Leu) (Goto et al., 2014). PCR amplification of codon 201 and 227 was performed using a Taq DNA-Polymerase (TTH Biotools Madrid, Spain) as previously described (Goto et al., 2014). The PCR products were purified by a PCR purification kit (Qiagen GmbH), and then directly sequenced by an ABI3730XL analyzer (Applied Biosystems, Thermo Fisher Scientific, Inc., Carlsbad, USA). The primer sequences from PCR and DNA sequencing were listed in Table 2.

Cell culture and transduction

GH3, a rat GH-secreting pituitary tumor cell line, which produces both growth hormone and prolactin, was purchased from the Cell Culture Centre, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). GH3 cells were cultured in Ham's F12 medium supplemented with 10% FBS and 1% streptomycin and penicillin in a humidified 5% CO₂ incubator at 37°C.

A pWPT lentiviral expression vector was used to clone the wild-type or mutant-type *GNAS* gene and the generated construct was termed as follows: pWPT-*GNAS* (expressing *GNAS* wild-type), pWPT-*GNAS*-Q227L (expressing the mutated *GNAS* at Q227L), and pWPT-*GNAS*-R201C (expressing the mutated *GNAS* at R201C), respectively. GH3 cells were plated in 6-well plates at 70% confluence and then injected with lentivirus to express the wild-type or mutant-type *GNAS*.

RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

Total RNAs were isolated from tissues or cells using Trizol reagent and reverse transcribed into complementary DNA (cDNA) using the TaqMan MicroRNA Reverse Transcription Kit (TaKaRa, Dalian, China). RT-qPCR was performed using an SYBR Green PCR Master Mix (Takara, Japan) according to the manufacturer's instructions. The sequences of qPCR primers were listed in Table 2. The lncRNA *MEG3* was normalized by β -actin and the level of *MEG3* in GHPA was further normalized by its level in NFPA.

RNA-seq

Total RNA was qualified by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and the next-generation sequencing library was prepared according to the protocol provided by the manufacturer (NEBNext® Ultra™ RNA Library Prep Kit for Illumina® HiSeq system). The sequences and data analysis were performed by Genewiz, China.

Western blots

Proteins were extracted from tumor cells and tissues within RIPA buffer (Beyotime Biotech., Shanghai, China), and separated in SDS-Page gels and transferred onto polyvinylidene fluoride membranes. After blocking with 5% fat-free milk in a Tris-buffered saline with 0.1% Tween 20, the membranes were incubated at 4°C overnight with the primary antibodies against MMP-2, β -catenin, MMP-9, and β -actin, which were purchased from Cell Signaling Tech., Danvers, MA, USA). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Tech.). The images were visualized using enhanced chemiluminescence (Beyotime). Pierce ECL Western Blotting Substrate (Thermo Scientific) was used to detect the chemiluminescence signals. Densitometric analyses of the western blot bands were performed using the Bio-Rad Imaging system (Bio-Rad, Hercules, California, USA).

Transwell assay

Matrigel matrix diluent (300 μ g/mL) was used to coat the bottom of the upper chamber of the Transwell chamber and in a pre-cooled environment at 4°C. The volume ratio of serum-free F12 medium to matrigel in the upper chamber was 4:1, the total volume was 100 μ l, and the matrigel was frozen overnight at 4°C. The next day, 500 μ l of F12 medium containing 10% FBS was added into the lower chamber. Cells (1×10^5) were seeded in the upper chamber. After incubation for 48h, non-invading cells in the upper chamber were removed. Cells that invaded the bottom chamber were fixed with 4% paraformaldehyde

and stained using 0.1% crystal violet (Beyotime). Five random fields were selected for counting purposes under a microscope (magnification, ×200).

Immunofluorescence

Tumor tissue sections from surgical resection of GHPA. Tissue slides were fixed with 4% formaldehyde, blocked using 10% normal goat serum. Primary antibodies against β -catenin, E-cadherin, N-cadherin, Vimentin were diluted at 1:100 in PBS and added to the slides. All the antibodies were obtained from Cell Signaling Technology. After incubation overnight at 4°C. After removal from the incubation chamber, slides were washed thrice with PBST. Then sections were incubated for 40 minutes in the dark in a humidified chamber at room temperature with goat polyclonal secondary antibody to rabbit IgG (1:300, Abcam Biotechnology, USA) reconstituted in PBS. Sections were washed three times with PBST. After the counterstaining procedure, sections were treated with glycerol/PBS (2:1) for 10 minutes in the dark at room temperature. counted in five randomly chosen fields using an Axiovert 200 fluorescent microscope.

Subcutaneous xenografts in nude mice

The animal experiments were approved by the Animal Experimentation Ethics Committee of the Jinling Hospital of Nanjing University.

Four-week-old female athymic BALB/c nude mice were purchased from the Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China) and were housed and maintained in laminar airflow chambers under specific pathogen-free conditions. GH3 cells with different levels of *MEG3* (10^7 cells/0.1ml) were subcutaneously injected into the right back side of mice. One week after the injection. After the tumor formed, lithium chloride (60 mg/kg/d in 100 μ l saline), a β -catenin activator, was administered daily by intraperitoneal injecting in the group with the high levels of *MEG3*. The other groups were injected with saline alone as the controls. Tumor volumes were measured with a vernier caliper twice a week and calculated as $(\text{length} \times \text{width}^2)/2$. Four weeks after injection, the mice were sacrificed by cervical dislocation, and dissected tumors were weighed and processed to determine the expression levels of the relative proteins, including β -catenin, E-cadherin, N-cadherin, MMP-2, MMP-9.

Immunohistochemistry (IHC)

Tumor tissues from mice were fixed and antigens were exposed via heat-induced epitope retrieval with exposure to citric acid buffer (pH=7.0), blocked in 10% normal goat serum, and incubated with 3% hydrogen peroxide. The tissue slides were incubated with the primary antibodies against β -catenin (1:200), E-cadherin (1:200), N-cadherin (1:200), MMP-2 (1:200), MMP-9 (1:200) overnight at 4°C, and then followed by incubated with a goat anti-rabbit secondary antibody (Beyotime) for 15 min at 37°C. The number of the positive cells was subsequently counted in each section in 10 random microscope fields (magnification, x200).

Statistical analysis

Statistical data analysis was done using SPSS 19.0. The Student's unpaired T-test and Fisher's exact test were used for intergroup analysis. The results were presented as mean $\pm$ standard deviation and the correlation was analyzed using a Spearman's correlation. $P < 0.05$ was considered as statistical significance.

Results

The *GNAS* mutations are collected to GHPA

Total 44 patients with GHPA and 10 patients with NFPA were enrolled in this study. The *GNAS* mutations were scanned by directly sequencing the genomic PCR products amplified from the patient's tumor samples with the specific primer sets. The results showed 16 out of the 44 cases carry the *GNAS* mutations, including 8 cases in codon 227 but other 8 cases in codon 201. In contrast, no *GNAS* mutation was detected in all 10 NFPA patients. Subsequently, the patients were divided into two groups (GNAS-WT, $n=28$, GNAS-MUT, $n=16$). Compared to the GNAS-WT group, the tumor volumes were significantly reduced in the GNAS-MUT group. Additionally, the percentage of invasive tumors (KnoSp grade 3-4) in the GNAS-MUT tumors was also remarkably decreased (Table 1). There were no statistical differences in age or gender between the two groups.

The mutant *GNAS* leads to inhibiting the GH3 cell invasion

The results from our clinical analysis predicted that the *GNAS* mutations are associated with the invasiveness of GHPA. Accordingly, GH3 cells expressing the wild-type or the mutant-type of *GNAS* were constructed. The cells were injected with lentivirus carrying Flag-tagged Q227L (pWPT-GNAS-Q227L) or R201C (pWPT-GNAS-R201C), as well as GNAS-WT control (pWPT-GNAS) (Fig. 1A). Furthermore, the cell invasion capacity was significantly decreased in GH3-Q227L and GH3-R201C cells compared to GH3-GNAS-WT cells (Fig. 1B). In addition, the expression levels of MMP-2 and MMP-9 in GH3-Q227L and GH3-R201C cells were apparently reduced as well (Fig. 1C).

The mutant *GNAS* upregulates *MEG3* expression

It was speculated that the *GNAS* mutations may participate in the upregulation of *MEG3* expression. To verify the prediction, the expression levels of *MEG3* in NFPA and GHPA tumor tissues were quantified by RT-qPCR. As expected, the levels of *MEG3* strikingly increase in 44 GHPA tumor tissues compared to 10 NFPA tumor tissues (Fig. 2A). Intriguingly, *MEG3* expression further highly increases in the 16 GHPA tumor tissues with mutant *GNAS* compared to 28 wild-type tumors (Fig. 2B). Consistently, the high levels of *MEG3* were verified in the GHPA tumor tissues with a single mutant site at Q227L and R201C, respectively (Fig. 2C). Importantly, tumor invasiveness was significantly declined in the high level of *MEG3* group compared to the low level of the *MEG3* group (Fig. 2D), suggesting that *MEG3* may negatively correlate with tumor cell invasion in the GHPAs.

MEG3 inhibits the invasiveness of GH3 cells

To verify if *MEG3* is able to inhibit cell invasion, *MEG3* was manipulated using lentiviral expression systems, either ectopically expressed or knocked down in GH3 cells. After lentivirus infection the cells, *MEG3* was quantified by immunofluorescence and RT-qPCR (Fig. 3A). Expectedly, compared to lentiviral vector control, the overexpression of *MEG3* resulted in decreasing cell invasion. In contrast, the silence of *MEG3* led to increased cell invasion (Fig. 3B). Consistently, the protein expression levels of MMP-2 and MMP-9 were decreased in *MEG3*-overexpressed cells, while their levels increased in *MEG3*-silenced cells (Fig. 3C). These results suggest that the *GNAS* mutations inhibit the invasiveness of GHPA tumors partially through activating *MEG3*.

***MEG3* inhibits cell invasion via inactivating the *Wnt/β-catenin* signaling pathway**

To further investigate the mechanism by which *MEG3* inhibits GHPA cell invasion, *MEG3* was overexpressed in GH3 cells and the RNA expression profile was analyzed by RNA-seq. The results revealed that the *Wnt/β-catenin* signaling pathway is potential in the involvement of cell invasion regulation (Fig. 4A). Consistently, The mRNA level of β -catenin significantly decreased in GH3 cells with the *GNAS* mutations. Ectopic expression of *MEG3* in GH3 cells resulted in reducing β -catenin expression, conversely, the silence of *MEG3* in GH3 cells led to increasing the level of β -catenin (Fig. 4B and C). Likewise, the level of β -catenin in GHPA tumors carrying the *GNAS* mutations was apparently lower than that in wild-type tumors, which was associated with the high level of *MEG3* (Fig. 4D and E). The results suggested that *MEG3* negatively regulates β -catenin and promotes cell invasion, particularly in GHPA cells carrying the *GNAS* mutations.

Inactivation of β -catenin suppresses the GHPA cell invasion

Licl and Dickkopf1 (Dkk1) have been identified as an activator and a suppressor of β -catenin, respectively (Niehrs, 2006; Gupta et al., 2012). Accordingly, *MEG3*-overexpressed cells were treated with 20 mM of Licl (Zhang et al., 2019), and *MEG3*-si cells were treated with 150 ng/ml Dkk1 (Zhuang et al., 2017). As expected, β -catenin expression was increased in the Licl-treated cells but decreased in the Dkk1-treated cells (Fig. 5A). In addition, the Licl treatment enhanced the cell invasion, but the Dkk1 treatment inhibited the cell invasion (Fig. 5B). Furthermore, Licl was able to upregulate the expression levels of MMP-2 and MMP-9. In contrast, Dkk1 was capable to suppress MMP-2 and MMP-9 expression (Fig. 5C). These results suggest that *MEG3* suppresses GHPA cell invasion by inhibiting the β -catenin-activated.

The *GNAS* mutations inhibit the epithelial-to-mesenchymal transition (EMT) process

EMT is widely recognized to play a fundamental role in the promotion of cell mobility and tumor metastasis and the *Wnt/β-catenin* signaling pathway is a key mechanism underlying EMT (Li et al., 2019; Ghahhari and Babashah, 2015). To reveal the effect of the *GNAS* mutations on the EMT process, the expression of β -catenin regulated EMT-associated proteins was quantified by immunofluorescence, such E-cadherin as a cell adherent marker, N-cadherin and Vimentin as mesenchymal markers. The results showed that E-cadherin increased but N-cadherin and Vimentin decreased in GHPA tumors carrying

the *GNAS* mutations and the high constitutive levels of *MEG3*, as compared to the wild-type of GHPA tumors (Fig. 6A and B). Altogether, our finding suggests that the *GNAS* mutations inhibit the invasiveness of GHPA via downregulation of EMT, as illustrated in Fig. 6C.

***MEG3* inhibiting the cell invasion was validated in vivo**

To further verify that *MEG3* suppresses the GHPA cell invasion by inhibiting β -catenin-regulated EMT, β -catenin was manipulated by overexpressing *MEG3* in GH3 cells and then treating with Licl. The cells were subcutaneously injected into nude mice for tumor formation. Compared to the control group injected with GH3 cells, the tumor volume and weight were reduced in *MEG3*-overexpressed cells, but dramatically increased by Licl, indicating that Licl sufficiently abrogated the negative effect of *MEG3* in the regulation of β -catenin (Fig. 7A-C). Consistent with the regulated levels of β -catenin in the formed tumors, E-cadherin increased by overexpressing *MEG3*, but further decreased by treating with Licl. In contrast, the expression levels of N-cadherin, β -catenin, MMP-2 and MMP-9 decreased by elevating *MEG3* and increased by Licl-mediated induction (Fig. 7D and E). The results confirmed that *MEG3* negatively regulates EMT via downregulating β -catenin.

Discussion

GHPA is a typically benign tumor with a high incidence and large economic burden and often manifests with invasive growth (Katznelson et al., 2014). Numerous studies indicated that the presence of the paradoxical *GNAS* point mutations strongly reflects the biological characteristics of GHPAs, such as a tendency for densely granulated tumors and smaller tumor size (Landis et al., 1990, Spada et al., 1990, Spada et al., 1991). A previous study has suggested that Gsa protein encoded by the *GNAS* gene is a key for activating the cAMP-dependent pathway in pituitary cells for differentiation and proliferation (Billestrup et al., 1986). However, the potential role and explicit mechanism of *GNAS* gene mutation in the invasiveness of GHPAs remains to be fully elucidated. In this regard, the finding from this study indicated that the incidence of invasiveness was markedly reduced in GHPA tumors carrying the *GNAS* mutations, compared to the wild-type of *GNAS*. Furthermore, GH3 cells possessing Q227L or R201C mutation appeared a lower percentage of invasive cells in comparison with the corresponding wild-type control cells. Thus, it is speculated that *GNAS* gene mutations inhibit the invasiveness of GHPA cells.

The *GNAS* mutations have been proposed to involve the constitutive activation of cAMP formation, which plays a causal role in pituitary adenomas (Mantovani et al., 2010). Interestingly, *MEG3* was identified as a tumor suppressor that is a downstream target of cAMP (Zhao et al., 2006, Zhang et al., 2010, Ma et al., 2019). The previous evidence indicated that the level of *MEG3* is uniquely high in GHPA, but not in NFPAs (Gejman et al., 2008). Thus, we have speculated that the *GNAS* mutations suppress the invasiveness of GHPA mainly through activating *MEG3*. As expected, the high levels of *MEG3* were only detected in GHPA tumors carrying the *GNAS* mutations. The *MEG3* levels were also significantly increased in GH3 cells expressing *GNAS* gene mutations, compared to the cell expressing the wild-type of *GNAS* gene, suggesting that the *GNAS* mutations inhibit the GHPA cell invasion through *MEG3* activation.

Furthermore, to ascertain that the effect of *MEG3* in cell invasion, we manipulated *MEG3* in GH3 cells. Ectopic expression of *MEG3* resulted in reducing the cell invasion, and vice versa, the silence of *MEG3* led to enhancing the invasiveness. Altogether, our results suggest that *MEG3* plays an important role in the promotion of GHPA invasiveness.

The canonical *Wnt/β-catenin* signaling pathway is thought to be a key regulator in the EMT process and tumor progression (Krishnamurthy and Kurzrock, 2018). Upon activation of the Wnt pathway, β-catenin accumulates in nuclei and functions as a factor (Nusse and Clevers, 2017). In the present study, we showed that the *GNAS* mutations lead to an increase of *MEG3* but a decrease of β-catenin. Furthermore, the activation of β-catenin by LiCl enhanced the cell invasion and the inactivation of β-catenin by Dkk1 inhibited the cell invasion. Subsequently, Elevated *MEG3* leads to downregulation of β-catenin in GHPA cells. In parallel, the silence of *MEG3* upregulated the β-catenin expression, suggesting that *MEG3* inhibits the invasiveness of GHPA cells by inactivating the *Wnt/β-catenin* pathway.

Anterior pituitary with an epithelial phenotype expresses multiple cadherin proteins like E-cadherin that functions for cell attachment (Fougner et al., 2010). EMT is a vital mechanism underlying tumor cell invasion and metastasis. It has been well documented that the loss of E-cadherin and/or the increase of N-cadherin are hallmarks of EMT (27, 28). In a variety of types of tumors, the *Wnt/β-catenin* signaling pathway is constitutively active to promote EMT (Liang et al., 2017). The present study uncovered that β-catenin and EMT-related functional proteins are altered by *MEG3* in GHPA tumors carrying the *GNAS* mutations. Accordingly, enforcedly elevated *MEG3* in GHPA cells led to the upregulation of E-cadherin but downregulation of N-cadherin, and Vimentin via altering β-catenin transcriptional regulation.

In addition, MMPs, the important proteolytic enzymes in the degradation of extracellular matrix and basement membrane, are crucial for tumor cell invasion (Di Nezza et al., 2002). Among of MMPs, MMP-2 and MMP-9 have been demonstrated to play vital roles in tumor invasion due to their potent ability to degrade collagen types IV (Scheau et al., 2019). In particular, their functions have been involved in the invasiveness of GHPA tumors (Yang and Li, 2019). Similar to other EMT-related proteins, our study further showed the levels of MMP-2 and MMP-9 decrease in GHPA tumors carrying the *GNAS* mutations, as well as the increase of *MEG3* expression in GHPA cells resulted in decreasing the expression of MMP-2 and MMP-9, confirming the previous finding that MMPs participate in the progression of GHPA. Furthermore, how *MEG3* regulates β-catenin-mediated transcriptional activation is being currently investigated.

Conclusions

In summary, this study revealed that *GNAS* mutations inhibit the invasiveness of GHPA tumors by increasing the level of *MEG3*. The upregulation of *MEG3* in GHPA cells suppresses the cell invasion capacity through inhibiting the *Wnt/β-catenin* signaling pathway. The silence of *MEG3* upregulates β-catenin and enhances EMT. The finding suggests that *MEG3* may serve as a biomarker for the detection of GHPA phenotype and inhibition of the *Wnt/β-catenin* signaling pathway may provide a useful therapeutic approach in the intervention of GHPA.

Abbreviations

GHPA: Growth hormone-secreting pituitary adenoma, *GNAS*: α subunit of the stimulatory G protein, *MEG3*: maternally expressed 3, EMT: epithelial-to-mesenchymal transition, FACS: fluorescence-activated cell sorter, CCK-8: Cell Counting Kit-8 (tradename), MMP: matrix metalloproteinase, siRNA: small interfering RNA or short interfering RNA, WT: wild type.

Declarations

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Competing of interests

The authors declare that they have no competing of interests.

Availability of data and materials

The datasets during and analysed during the current study available from the corresponding author on reasonable request.

Code availability

Not applicable

Authors' contributions

Chiyuan Ma conceived and designed the study. Chao Tang, Chunyu Zhong, Junhao Zhu, Jin Yang and Xiangming Cai performed the experiments. Chao Tang, Chunyu Zhong, Junhao Zhu interpreted and analyzed the data. Chao Tang, Chunyu Zhong and Yong Xu wrote and revised the manuscript. All authors have read and agreed the content of the manuscript.

Ethics approval and consent to participate

The animal experiments protocols were approved by the Institutional Animal Committee of Jinling Hospital.

Consent for publication

Not applicable

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Tables

Table 1 Comparison of clinical characteristics of patients with and without GNAS mutations

	GNAS-MUT	GNAS-WT	P value
Patients(n)	16(36.4%)	28(63.6%)	
Age(yr)	49.7±9.7	47.5±10.1	0.155
Sex(male/female)	10/6	11/17	0.138
Volume(mm ³)	4901.3±4212.7	6861.9±3751.4	0.003
Knosp grade (%)			0.001
0–2	81.3%(13/16)	28.6%(8/28)	
3–4	18.7%(3/16)	71.4%(20/28)	

Table 2: The primer sequences of PCR and DNA sequencing

Gene	Primer sequences
<i>GNAS</i> codon 227	F: ATCATGGTTTCTTGACATTCACCCC
	R: CCACCACGAAGATGATGGCAGTC
<i>GNAS</i> codon 201	F: CAAGCAGGCTGACTATGTGCCGA
	R: GCTGGCCACCCACGTCAAAC
<i>MEG3</i>	F: ATCATCCGTCCACCTCCTTGTCTTC
	R: GTATGAGCATAGCAAAGGTCAGGGC
<i>b-actin</i>	F: CACCCAGCACAAATGAAGATCAAGAT
	R: CCAGTTTTTTAAATCCTGAGTCAAGC

Figures

Figure 1

The *GNAS* mutations inhibit the GH3 cells invasion. A: The wild-type and mutant *GNAS* gene were expressed in GH3 cells by transduction with lentiviral vectors: pWPT-*GNAS*-Q227L, pWPT-*GNAS*-R201C and pWPT-*GNAS*. B: The cell invasion capacity was measured using a Transwell assay. C: The levels of MMP-2 and MMP-9 were quantified by Western blot. **($p < 0.01$), ***($p < 0.001$) show the significances between the two groups as indicated, and “ns” shows no significances.

Figure 2

The *GNAS* mutations upregulate *MEG3* expression. A: The expression of *MEG3* in GHPA and NFPA was quantified by RT-qPCR analysis. B and C: The correlation between *MEG3* expression and the *GNAS* mutations were determined by RT-qPCR analysis. D: The correlation between *MEG3* expression and the percentage of invasive tumors was analyzed. **($p < 0.01$), ***($p < 0.001$) show the significances between the two groups as indicated.

Figure 3

***MEG3* inhibits the invasiveness of GHPA cells.** A: *MEG3* was overexpressed or knocked down in GH3 cells. The relative levels of *MEG3* were quantified by immunofluorescence assay and confirmed by RT-qPCR. B: The cell invasion was analyzed using a Transwell assay. C: The expression levels of MMP-2 and MMP-9 in *MEG3*-overexpressed cells were measured by Western blot. *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$) show the significances between the two groups as indicated, and “ns” shows no significances.

Figure 4

MEG3 inhibits the invasiveness of GHPA with the *GNAS* mutations by inactivating the *Wnt/β-catenin* signaling pathway.

A: RNA-seq was used to examine the gene expression profiles in MEG3-overexpressed GH3 cells compared to vector-only control. The altered mRNA expression profile was analyzed using KEGG pathway

enrichment. B: The reduction of β -catenin mRNA expression in GHPA tumors with the *GNAS* mutations or GH3 cells with a high level of *MEG3* was confirmed by RT-qPCR. C: The relative β -catenin protein levels were measured by Western blot. D and E: The correlation between *MEG3* and the β -catenin protein levels was analyzed RT-qPCR and IHC. *($p < 0.05$), **($p < 0.01$) show the significances between the two groups as indicated.

Figure 5

MEG3-mediated downregulated β -catenin inhibits the invasion of GH3 cells.

A: *MEG3* was manipulated by expressing or silencing *MEG3* in GH3 cells. In addition, the expression of β -catenin was induced by LiCl or repressed by Dkk1. The level of β -catenin in the *MEG3*-manipulated GH3 cells was measured using Western blot. B: The relative cell invasion was quantified by Transwell assay. C: The expression of MMP-2 and MMP-9 was also measured using Western blot. *($p < 0.05$), **($p < 0.01$) show the significances between the two groups as indicated.

Figure 6

The *GNAS* mutations inhibit EMT in GHPA tumors. A and B The expression of EMT-related proteins in GHPAs tumor tissues were quantified by immunofluorescence (magnification, x200). C: Depiction of the

suggested mechanism underlying the *GNAS* mutations inhibiting the invasiveness of GHPA.

Figure 7

***MEG3* inhibits the GHPA cell invasion *in vivo*.**

A-C: GH3 cell lines with different levels of *MEG3* were subcutaneously injected into null mice and followed by Licl treatment. The excised tumors were photographed and the tumor volume and weight in each group were measured. D: The levels of β -catenin, E-cadherin, N-cadherin, MMP-2, and MMP-9 in

tumor tissues were quantified by IHC (magnification, x200). *(p < 0.05), **(p < 0.01), ***(p < 0.001) show the significances between the two groups as indicated.