GNAS mutations suppress the cell invasion through regulating EMT by activating the MEG3/Wnt/β-catenin pathway in growth hormone-secreting pituitary adenoma

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Abstract

Background: 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.

Methods: 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.

Results: 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.

Conclusion: 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 [1–3]. 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 [3]. 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 [4].

It has been well-demonstrated that mutations found in the GNAS gene, encoding α subunit of the stimulatory G protein, were detected in around 40% of GHPA [5, 6]. 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 [7, 8]. The function of the GNAS mutations is thought to be involved in cell proliferation and hormone secretion [9].
Maternally expressed gene 3 (MEG3), a large non-coding RNA (lncRNA), was first identified as a tumor suppressor in the pituitary [10]. The function of MEG3 is associated with inhibition of cell invasion [11]. In addition, the cAMP response element (CRE), located at the MEG3 proximal promoter region, is critical for MEG3 expression [12]. 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 [12, 13]. 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 [14]. Knosp grade 3 and 4 were defined as invasive pituitary adenomas, and Knosp grade 0 to 2 were defined as non-invasive tumors, respectively [15]. 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) [5]. PCR amplification of codon 201 and 227 was performed using a Taq DNA-Polymerase (TTH Biotools Madrid, Spain) as previously described [5]. 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 IncRNA 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 μg/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 100μl, and the matrigel was frozen overnight at 4°C. The next day, 500 μl 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 μl 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 (length × 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 ± 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 expressed 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 correlation 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 [16, 17]. Accordingly, MEG3-overexpressed cells were treated with 20 mM of Licl [18], and MEG3-si cells were treated with 150 ng/ml Dkk1 [19]. 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 [20, 21]. 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 [22]. 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 [23–25]. 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 [26]. 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 [8]. Interestingly, MEG3 was identified as a tumor suppressor that is a downstream target of cAMP [11, 12, 27]. The previous evidence indicated that the level of MEG3 is uniquely high in GHPA, but not in NFPAs [28]. 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 [29]. Upon activation of the Wnt pathway, β-catenin accumulates in nuclei and functions as a factor [30]. 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 [31]. 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. In a variety of types of tumors, the **Wnt/β-catenin** signaling pathway is constitutively active to promote EMT [32]. 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 [33]. 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 [34]. In particular, their functions have been involved in the invasiveness of GHPA tumors [35]. 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

Funding

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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

<table>
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<th>GNAS-MUT</th>
<th>GNAS-WT</th>
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<tr>
<td>Patients(n)</td>
<td>16(36.4%)</td>
<td>28(63.6%)</td>
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<tr>
<td>Age(yr)</td>
<td>49.7±9.7</td>
<td>47.5±10.1</td>
<td>0.155</td>
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<tr>
<td>Sex(male/female)</td>
<td>10/6</td>
<td>11/17</td>
<td>0.138</td>
</tr>
<tr>
<td>Volume(mm³)</td>
<td>4901.3±4212.7</td>
<td>6861.9±3751.4</td>
<td>0.003</td>
</tr>
<tr>
<td>Knosp grade (%)</td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>0–2</td>
<td>81.3%(13/16)</td>
<td>28.6%(8/28)</td>
<td></td>
</tr>
<tr>
<td>3–4</td>
<td>18.7%(3/16)</td>
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Table 2. The primer sequences from PCR and DNA sequencing
<table>
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<th>Gene</th>
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<tr>
<td>GNAS codon 227</td>
<td>F: ATCATGGTTTCTTGACATTCCACCCC</td>
</tr>
<tr>
<td></td>
<td>R: CCACCACGAAGATGATGGCAGTC</td>
</tr>
<tr>
<td>GNAS codon 201</td>
<td>F: CAAGCAGGCTGACTATGTGCCGA</td>
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<tr>
<td></td>
<td>R: GCTGGCCACCCACGTCAAAC</td>
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<tr>
<td>MEG3</td>
<td>F: ATCATCCGTCCACCTCCTTGTCTTC</td>
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<tr>
<td></td>
<td>R: GTATGAGCATAGCAAAGGTCAAGGC</td>
</tr>
<tr>
<td>b-actin</td>
<td>F: CACCCAGCACAATGAAGATCAAGAT</td>
</tr>
<tr>
<td></td>
<td>R: CCAGTTTTAAATCCTGAGTCAAGC</td>
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**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 < 0.001) show the significances between the two groups as indicated, and “ns” shows no significances.
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.