

RETRACTED ARTICLE: LncRNA XIST depletion prevents cancer progression in invasive pituitary neuroendocrine tumor by inhibiting bFGF via upregulation of microRNA-424-5p

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Kai Zhou
Shaoshan Li
Guojia Du
Yandong Fan
Pengfei Wu
Hongjie Sun
Tingrong Zhang

Department of Neurosurgery, The First
Affiliated Hospital of Xinjiang Medical
University, Urumqi 830054, People's
Republic of China

Background: Long noncoding RNAs (lncRNAs) are vital regulators in human cancers including pituitary neuroendocrine tumor (PitNET) and could function as competing endogenous RNAs (ceRNAs) of microRNAs (miRNAs). The main objective of this study is to identify effect of lncRNA X-inactive specific transcript (XIST) and microRNA-424-5p (miR-424-5p) on PitNET.

Methods: Microarray analysis was employed to identify the PitNET-related differentially expressed lncRNAs. PitNET tissues, including both invasive and non-invasive subtypes in parallel with normal pituitary tissues were collected for the determination of the expression of XIST, miR-424-5p and basic fibroblast growth factor (bFGF) and the interaction among them. Subsequently, the expression of XIST, miR-424-5p and bFGF in PitNET cells was altered to elucidate their biological significance in the aspects of proliferation, migration, invasion, and the apoptosis.

Results: Both XIST and bFGF exhibited high expression, but miR-424-5p had a low expression in invasive PitNET tissues as compared to non-invasive PitNET normal pituitary tissues. Additionally, XIST competitively bound to miR-424-5p to elevate the expression of bFGF. Furthermore, depleted XIST or bFGF, or elevated miR-424-5p was revealed to suppress the proliferation, migration, invasion, and promote cell cycle arrest and apoptosis of invasive PitNET cells. miR-424-5p repressed the proliferation, migration, invasion of invasive PitNET cells by targeting bFGF.

Conclusion: In conclusion, the fundamental findings of the present study suggested that the functional suppression of XIST downregulated bFGF to inhibit the development of PitNET by increasing miR-424-5p expression, proposing XIST as a novel therapeutic target for PitNET.

Keywords: invasive pituitary neuroendocrine tumor, lncRNA XIST, microRNA-424-5p, bFGF, invasion, migration

Correspondence: Tingrong Zhang
Department of Neurosurgery, The First
Affiliated Hospital of Xinjiang Medical
University, No. 137, Liyushan South Road,
Urumqi 830054, Xinjiang Uygur
Autonomous Region, People's Republic of
China
Tel +86 1 869 088 1506
Email xjzk2012@sina.cn

Introduction

A part of pituitary neuroendocrine tumor (PitNET) cases are defined as invasive based on the signs of tumor invasion and proliferation.¹ Invasive PitNET are considered as tumors originated from adenohypophysis representing biologically middle forms between the benign tumors and the metastasizing pituitary carcinomas.² The dural invasion by PitNET is mainly associated with the persistence of tumor tissue following transsphenoidal surgery.³ This proposes the

treatment of invasive PitNET as a challenging clinical problem which endocrinologist and neurosurgeons commonly face.⁴ Therefore, it is in urgent need to identify more effective modalities for invasive PitNET in order to improve its treatment outcome and increase the survival rate. Fortunately, the understanding of subcellular mechanisms with the involvement of pituitary tumorigenesis contributes to the identification of tumor aggression markers and novel targets.⁵

Long non-coding RNAs (lncRNAs) represent the families of non-coding RNAs containing nucleotides with a length of >200 that play significant roles in many cancers and tumors.⁶ LncRNA X-inactive specific transcript (XIST) refers to a product of the XIST gene, which mainly regulates transcriptional silencing of X chromosome in mammals and generally has elevated expression levels that appear in multiple cancers.⁷ It has been demonstrated that XIST participates in the various cellular biological processes including differentiation, genome maintenance and proliferation.⁸ The expression of lncRNA XIST was widely reported to be correlated to cancers and tumors, such as glioma and ovarian cancer.⁹ Moreover, it was previously disclosed that this lncRNA could serve as a competing endogenous RNA (ceRNA) of microRNAs (miRNAs) to affect its post-transcriptional regulation.¹⁰ For example, XIST behaves as a miR-194-5p sponge with the motive to increase the expression of MAPK1 in hepatocellular carcinoma.¹¹ It was demonstrated that microRNA-424 (miR-424) is a miRNA regulating diverse cellular biological processes which include cell proliferation, cell cycle, and differentiation.¹² Furthermore, it has been suggested that miR-424-5p plays a critical role in cancers including pancreatic cancer and hepatic cancer.¹³ MiR-424 was previously reported to repress the development of infantile skin hemangioma by inhibiting basic fibroblast growth factor (FGF) and its receptor FGFR1.¹⁴ bFGF, also known as FGF2, is considered a kind of polypeptide growth factor participating in many processes in tumors, such as angiogenesis and tumorigenesis.¹⁵ As an angiogenic factor, bFGF has been revealed to be an indicating marker of the invasiveness of PitNET.¹⁶ Notably, lncRNA PVT1 increases the expression of bFGF by acting as a ceRNA of miR-152 in gastric cancer cells.¹⁷ In addition, based on our prediction using online software DIANA TOOLS available at <http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/page&view=software>, there are potential binding sites between hsa-miR-424-5p and XIST and between hsa-

miR-424-5p and bFGF. Based on these findings, the hypothesis of this study is that the interplay between lncRNA XIST, miR-424-5p and bFGF were involved in the development of invasive PitNET. Therefore, this present study was conducted to confirm this hypothesis.

Methods and materials

Ethical statements

This study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University. All the patients involved in this study signed the informed consent.

Microarray-based gene expression analysis

The Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>) was utilized to screen out PitNET-related expression dataset GSE51618, which was then followed by background correction and standard pre-treatment with the use of affy package of R Language Programming. Differentially expressed genes (DEGs) were exhibited using the Limma package with $|\log \text{foldchange (FC)}| > 2$ and $P < 0.05$ as the threshold. A heat map of the DEGs was plotted using the “pheatmap” package (<https://cran.r-project.org/web/packages/pheatmap/index.html>).

Study subjects

A total of 23 normal pituitary tissues were obtained from autopsy of adults without pituitary dysfunction aging between the ages of 28 to 60 years with an average age of (38.83±8.84) years. This group included 9 males and 14 females. Specimens were gathered from 86 cases of PitNET via transsphenoidal resection, and all cases were enrolled in the First Affiliated Hospital of Xinjiang Medical University from January 2017 to December 2017. The case included 39 males and 47 females aged from 24 to 68 years, with an average age of (39.47±9.51) years. The size and invasiveness of the tumors were determined through preoperative radiological and surgical examination combined with the modified Hardy classification. The cases were classified into non-invasive tumors that included 9 grade I cases (microadenoma, <10 mm in diameter) and 26 grade II cases (capsular macroadenoma with or without suprasellar extension, ≥10 mm in diameter) and invasive tumors that included 32 grade III cases (local infiltration of sphenoid and/or cavernous sinuses) and 19 grade IV cases (with central nervous

system/extracranial spread with or without metastasis). Cases (51) in grade III (n=32) and grade IV (n=19) were all selected as invasive PitNET samples. The diagnostic criteria of invasive PitNET were as follows: (1) grade III-V or stage C, D or E in accordance with Hardy Wilson guidelines; (2) imaging implication prior to operation, such as destruction of cavernous sinus, parasellar or hypothalamus; (3) tumor infiltration in sellar diaphragm or sellar floor bones was observed through pathological examination; and (4) perforation of cavernous sinus inner wall appeared during endoscopic surgery. The diagnostic criteria of non-invasive PitNET included focal tumors in the sellar region without compression of peripheral structures during imaging and surgery. The exclusion criteria were that primary diagnosis through initially clinical and imaging data displayed other tumors, such as canalis pharyngeal canal carcinoma, sellar nodular meningioma or Rathke's cyst.^{18,19}

Invasive PitNET cell isolation

Invasive PitNET tissues were initially preserved in sterile saline and three times washed with Dulbecco's modified Eagle medium (DMEM) and F12 (DF12; Gibco, Grand., NY, USA). Tissues were then stored in a bottle of 100 mL penicillin and cut into small blocks. After that, tissue blocks were detached with 2.5 g/L trypsin (Sigma-Aldrich, St. Louis, MO, USA), 6.7 mg/L collagenase (Sigma-Aldrich, St. Louis, MO, USA) and 0.3 mg/L DNAse (Sigma-Aldrich, St. Louis, MO, USA) in a water bath at 37 °C for 25 mins. After the addition of fetal bovine serum (FBS; Gibco, Grand., NY, USA), the tissue blocks were filtered with a 200-mesh filter and centrifuged for 5 mins at 179× g. After the addition of DF12 culture medium and centrifugation again, the cells were added with DF12 containing 100 mL/L FBS to adjust the cell concentration. The cells were then inoculated in the culture dishes for culturing at 37 °C with 50 mL/L CO₂ and saturated humidity.

Immunohistochemistry

Invasive PitNET tissues were fixed in Bouin's solution for 4 hrs (normal pituitary tissues were conventionally fixed by polyformaldehyde), dehydrated with gradient alcohol, cleared by xylene, embedded in paraffin and finally sliced into 5 µm sections. After being deparaffinized by xylene, the sections were dehydrated with gradient alcohol and treated with 100% methanol containing 0.3% H₂O₂ for 10 mins to eradicate endogenous peroxidase activity. After the non-specific antigen was blocked with 10%

bovine serum albumin (BSA), the sections were incubated with 0.5 µg/mL rabbit anti-human antibody to bFGF (ab126861, Abcam, Cambridge, MA, UK) at 4 °C for 48 hrs. Moreover, the sections were stained using the Avidin-Biotin Complex (ABC) technique, colored by diaminobenzidine (DAB), dehydrated, cleared and sealed using neutral balsam. Under the use of a microscope, the cells with cytoplasm stained in brown-yellow were viewed as positive cells. The primary antibodies were replaced by phosphate-buffered saline (PBS) and normal goat serum in the control group.

Plasmid construction and cell transfection

Human invasive PitNET cells in the logarithmic phase were detached with trypsin and inoculated in a six-well plate at a density of 1×10⁵ cells/well. Once the cell confluence reached 60~80%, cells were diluted in Opti-minimal essential medium (MEM; Gibco, Grand., NY, USA) for transfection. LipofectamineTM 2000 reagent (Invitrogen, Carlsbad, CA, USA) was mixed with diluted plasmids, allowed to stand at room temperature for 20 mins, and then added to the six-well culture plate for transfection. The invasive PitNET cells were transfected with blank plasmid, negative control scramble siRNA (si-NC), siRNA targeting XIST (si-XIST), mimic NC plasmid, miR-424-5p mimic, si-bFGF and the plasmid overexpressing bFGF (oe-bFGF) respectively. After 4 hrs, culture medium was renewed with DMEM containing 10% FBS. After 48 hrs, cells were observed under an inverted microscope.

Fluorescence in-situ hybridization (FISH) test

The FISH test was used in order to identify the subcellular localization of XIST in cells in accordance with the instructions of RiboTM IncRNA FISH Probe Mix (Red) (Ribobio, Guangzhou, Guangdong, China). Cells were inoculated into a 24-well plate at a density of 6×10⁴ cells/well. When cell confluence was about 80%, cells were fixed with 1 mL 4% paraformaldehyde at room temperature, treated with 2 µg/mL protease K (Sigma-Aldrich, SF, CA, USA), glycine (YZ-140,689, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and ethylphthalide reagent, and incubated in 250 µL pre-hybridization solution at 42 °C for 1 h. Then, the cells were incubated overnight with the addition of 250 µL hybridization solution (300 ng/mL) containing XIST-specific probe at 42 °C. After three phosphate-buffered saline-tween20 (PBST) washes, 4',6-diamidino-2-phenylindole (DAPI;

ab104139, 1: 100, Abcam, Cambridge, MA, UK) diluted with PBST was added into the 24-well culture plate for 5 mins nucleus staining. Afterwards, the cells were mounted with anti-fluorescence quenching agent and observed and photographed under a fluorescence microscope (Olympus, Tokyo, Japan) with 5 different visual fields selected.

Dual-luciferase reporter gene assay

Bioinformatics prediction website was used to recognize the interaction among XIST, bFGF and miR-424-5p, and the binding site analysis of XIST, miR-424-5p and bFGF was performed. The full-length sequence of XIST and the 3'untranslated region (3'UTR) sequence of bFGF containing the respective miR-424-5p binding sites as well as the sequences obtained after site-directed mutation of the miR-424-5p binding site were cloned into the downstream psiCheck2 plasmid. The recombinant vectors namely bFGF-wild type (WT), bFGF-mutant type (MUT), XIST-WT and XIST-MUT were obtained. The recombinant vectors were then co-transfected with mimic NC and miR-424-5p mimic into cells respectively. The dual-luciferase assay kit (Promega, Madison, WI, USA) was applied to measure the luciferase activity. After incubation for 48 hrs, cells were lysed in 1× passive lysis buffer, and then the firefly luciferase activity was detected using Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) with the renilla luciferase activity set as the internal control of the experiment. The experiment was repeated three times.

RNA-pull down

The invasive PitNET cells were transfected with 50 nM biotinylated Wt XIST (Wt-bio-XIST) and Mut XIST (Mut-bio-XIST). After 48 hrs, the cells were incubated in the specific lysis buffer (Ambion, Austin, TX, USA) for 10 mins. Then, the lysate was incubated with M-280 streptavidin magnetic beads (23-032, Sigma-Aldrich, St. Louis, MO, USA) pre-coated with RNase-free BSA and yeast tRNA (P-2300, Sigma-Aldrich, St. Louis, MO, USA). The beads were incubated at 4 °C for 3 hrs, washed two times with pre-cooled lysis buffer, three times with low-salt buffer and one time with high-salt buffer. The binding RNA was purified by Trizol, and miR-424-5p enrichment was detected by reverse transcription quantitative polymerase chain reaction (RT-qPCR).

RNA immunoprecipitation (RIP) assay

The binding between XIST and Argonaute 2 (AGO2) was detected with accordance to the instructions of the Magna

RIP RNA-Binding Protein Immunoprecipitation kit (Millipore, Billerica, MA, USA). Cells were ice bathed with radioimmunoprecipitation (RIPA) lysis buffer (P0013B, Beyotime Institute of Biotechnology, Shanghai, China) for 5 mins, and centrifuged at 35,068× g for 4 mins at 4 °C, followed by collection of the supernatant. A portion of the cell lysate was taken as Input and the other portion was incubated with the antibodies for co-precipitation. The 50 µL magnetic beads taken from each co-precipitation reaction system were resuspended in 100 µL RIP Wash Buffer (EHJ-BV08102, Xiamen Jiahui Biotechnology Co., Ltd., Xiamen, Fujian, China) and then incubated with 5 µg antibody. Then, the magnetic bead-antibody complex resuspended in 900 µL RIP Wash Buffer was incubated with 100 µL cell lysate overnight at 4 °C. Subsequently, samples were placed on the magnetic base to collect the magnetic bead-protein complex. Samples and Input were detected with protease K, and then RNA was extracted for subsequent RT-qPCR. The antibody used in RIP assay was AGO2 (ab32381, 1: 50, Abcam, Cambridge, MA, UK) and immunoglobulin G (IgG) (ab109489, 1: 100, Abcam, UK) as the negative control (NC). The bound RNA was purified by Trizol and enrichment of miR-424-5p and XIST was determined by RT-qPCR.

RNA isolation and quantitation

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA), and the RNA was reversely transcribed into complementary DNA (cDNA) in accordance with the instructions of the PrimeScript Reverse Transcription kit (TaKaRa, Tokyo, Japan). Fluorescence quantitative PCR was carried out with reactions prepared using the SYBR® Premix Ex Taq™ II kit (TaKaRa, Dalian, Liaoning, China) in the ABI7500 fluorescence quantitative PCR system (7500, ABI Company, Oyster Bay, NY, USA). The primer sequences are shown in Table 1. U6 was used as the internal reference of miR-424-5p and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for others.

Western blot analysis

Total proteins were extracted from tissues and cells using RIPA lysis buffer (R0010, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) containing phenylmethanesulfonyl fluoride (PMSF), incubated on the ice for 30 mins, centrifuged at 25,764× g for 10 mins at 4 °C to collect the supernatant. The protein concentration was evaluated

Table 1 Primer sequences for reverse transcription quantitative polymerase chain reaction

Gene	Primer sequence
XIST	F: 5'-GACACAAGGCCAACGACCTA-3' R: 5'-TCGCTTGGGTCTCTATCCA-3'
miR-424-5p	F: 5'-CAGCAGCAATTCATGT-3' R: 5'-TGGTGTCGTGGAGTCG-3'
bFGF	F: 5'-ATGGCAGCCGGGAGCATCACC-3' R: 5'-CACACACTCCTTGATAGACACAA-3'
MMP-2	F: 5'-GTGCTGAAGGACACACTAAAGAAGA-3' R: 5'-TTGCCATCCTTCTCAAAGTTGTAGG-3'
MMP-9	F: 5'-CCCGGACCAAGGATACAG-3' R: 5'-GGCTTCTCTCGGTACTG-3'
Bax	F: 5'-GGTTTCATCCAGGATCGAGACGG-3' R: 5'-ACAAAGATGGTCACGGTCTGCC-3'
Bcl-2	F: 5'-ATGTGTGTGGAGAGCGTCAACC-3' R: 5'-TGAGCAGAGTCTTCAGAGACAGCC-3'
GAPDH	F: 5'-CCTGGCCAAGGTCATCCATG-3' R: 5'-GGAAGGCCATGCCAGTGAGC-3'
U6	F: 5'-TGCTCGCTTCGGCAGC-3' R: 5'-AAAATATGGAACGCTTCACG-3'

Abbreviations: F, forward; R, reverse; XIST, X inactive specific transcript; miR-424-5p, microRNA-424-5p; bFGF, basic fibroblast growth factor; MMP, matrix metalloproteinase; Bax, Bcl-2-associated X protein; Bcl-2, B-cell CLL/Lymphoma 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

using a bicinchoninic acid (BCA) kit (23,22; Pierce and Warriner, Rockford, IL, USA) and adjusted with deionized water. A total of 50 µg protein samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel at 80 V for 2 hrs and transferred onto the polyvinylidene fluoride (PVDF) membrane (ISEQ00010, Millipore, Billerica, MA, USA) using the wet transfer method. Later, the membrane was blocked with tris buffered saline with tween (TBS-T) buffer containing 5% skim milk powder for 2 hrs and incubated overnight at 4 °C with the following primary antibody (Abcam, Cambridge, UK), rabbit anti-human antibody to bFGF (0.5 ng/lane, ab126861), matrix metalloproteinase (MMP)-2 (ab37150, 1: 200), MMP-9 (ab38898, 1: 1000), Bcl-2-Associated X (Bax; ab32503, 1: 1000) and B-cell lymphoma-2 (Bcl-2; ab32124, 1: 1000). Following that, the membrane was then washed 3 times with TBST (10 mins for each time), incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody to IgG (1: 5000, Beijing Zhongshan Biotechnology Co., Ltd., Beijing, China). Subsequently, the proteins were visualized using electrochemiluminescence (ECL) solution (BB-3501, Ameshame

biosciences, Ameshame, UK) in the dark for imaging. Afterward, the images were obtained using the BIO-Rad image analysis system (BIO-RAD, Hercules, CA, USA). The relative protein expression was analyzed using the Quantity One v4.6.2 software, and expressed as the gray value ratio of the corresponding protein to the GAPDH protein. The experiment was repeated three times.

Scratch test

The transfected invasive PitNET cells were inoculated on the six-well plates at a density of 5×10^4 cells/well. A 2 mm scratch in the monolayer was created across the center of the well. At 0 h and 24 h after scratching, the migration distance of cells in the scratch area was observed by the inverted microscope (Olympus CX23, Olympus, Tokyo, Japan), and multiple visual fields were randomly selected for photography. The Image Pro Plus software was used in order to measure the precise area and height of scratches at 0 h and 24 h to calculate cell migration rate.

Transwell assay

The Transwell chambers (Millipore, Bedford, MA, USA) were pre-washed and seeded into the 24-well plate. A total of 0.5 mL culture medium was added into the apical and basolateral chambers respectively and hydrated in the incubator for 2 hrs. After cells were detached, the cell suspension (5×10^4 cells/mL) was prepared. After the addition of 0.5 mL complete medium, the hydration chamber was transferred to the 24-well plate and added with 0.1 mL diluted cell suspension. After incubation for 24 hrs, the cells on the membrane were removed using a cotton swab, followed by PBS washing. The cells were fixed with pre-cooled paraformaldehyde for approximately 30 mins, stained with 1% crystal violet for 10 mins, and observed under the inverted microscope (Olympus, Tokyo, Japan).

5-ethynyl-2'-deoxyuridine (EdU) assay

Human invasive PitNET cells in the logarithmic growth phase were inoculated into 96-well plates at the density of $4 \times 10^3 \sim 1 \times 10^5$ cells/well, and then cultured to normal growth stage. EdU solution was diluted with cell culture medium at a ratio of 1: 1000 for the preparation of an appropriate medium. Cells in each well were then incubated with 100 µL medium for 2 hrs, fixed with 50 µL fixative solution for 30 mins, treated with 50 µL glycine (2 mg/mL) for 5 mins and 100 µL penetrating agent for 10 mins. Following this, the cells in each well were

incubated with 100 μ L 1 \times Apollo[®] staining solution for 30 mins at room temperature. The cells were then washed 2~3 times with 100 μ L penetrating agent (10 mins for each time), 1~2 times with 100 μ L methanol (5 mins for each time) and with PBS for 5 mins. Afterwards, the cells in each well were incubated with 100 μ L 1 \times Hoechst 33,342 solution diluted with deionized water at a ratio of 1: 100 at room temperature for 30 mins under conditions void of light.

Flow cytometry

Human invasive PitNET cells were fixed by frozen anhydrous ethanol overnight at 4 °C and then centrifuged at 7 \times g with the supernatant discarded. Each sample was thoroughly mixed with 500 μ L 1 \times fluorescent activated cell sorting (FACS) buffer containing 0.1% bovine serum albumin, PBS and 0.01% NaN₃, and 2.5 mL ribonuclease A (10 mg/mL) and preserved at room temperature for about 15 mins. The sample was then incubated with 25 μ L propidium iodide (PI; 1 mg/mL; Shanghai Beyotime Biotechnology Co., Ltd., Shanghai, China) in the dark for 15 min. Cell cycle was detected by a flow cytometer (FACSCantoII; Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

After cell transfection for 48 hrs, cells were treated with ethylene diamine tetraacetic acid (EDTA)-free 0.25% trypsin. Then Annexin-V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining solution was prepared by Annexin-V-FITC, PI and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at the ratio of 1: 2: 50 based on the instructions of the Annexin-V-FITC cell apoptosis detection kit (554547, Shanghai Shuoqia Biological Technology Co., Ltd., Shanghai, China). Subsequently, 1 \times 10⁶ cells were resuspended with 100 μ L staining solution. The cells were then incubated for 15 mins at room temperature, followed by addition of 1 mL HEPES buffer. The fluorescence was measured at 515 nm for FITC and at 620 nm for PI with excitation at 488 nm to measure cell apoptosis.

Statistical analysis

All data was analyzed with a Statistic Package for Social Science (SPSS) 21.0 statistical software (IBM Corp., Armonk, NY, USA). Measurement data were expressed as mean \pm standard deviation. All data were subjected to normal distribution and homogeneity of variance tests. When conforming to normal distribution and homogeneity of variance, the data between two groups were compared using the statistical unpaired *t*-test, data among multiple

groups were analyzed using one-way analysis of variance (ANOVA) with post hoc test. Data in skewed distribution or without homogeneity of variance were calculated using the rank-sum test. A *p*-value <0.05 that was calculated from three tests demonstrated statistical significance.

Results

XIST might be involved in invasive PitNET by regulating bFGF via miR-424-5p

At first, microarray analysis was used to screen the invasive PitNET-related lncRNAs. From the invasive PitNET-related lncRNA expression dataset GSE51118, lncRNA XIST with high expression at a large FC in invasive PitNET was exhibited as the candidate lncRNA (Figure 1A). In addition, a prior study revealed that siRNA-mediated bFGF gene silencing has the potential to inhibit the proliferation, migration and invasion of human PitNET cells.²⁰ However, it is not clear whether XIST is involved in the regulation of bFGF in invasive PitNET. XIST has been revealed to serve as a ceRNA of miRNAs to regulate the expression of oncogene ZEB2.²¹ Besides, the DIANA TOOLS (<http://diana.imis.athena-innovation.gr/DianaTools/index.php?R=site/page&view=software>) with score ≥ 0.5 and RAID v2.0 (<http://www.ma-society.org/rain/index.html>) with score ≥ 0.5 databases were employed to explore whether XIST could act as a ceRNA of miRNA to regulate bFGF, finding that both XIST and bFGF had hsa-miR-424-5p binding sites (Figure 1B–D). MiR-424 was found to inhibit the progression of cervical cancer.¹² Therefore, we speculate that XIST may act as a ceRNA of miR-424-5p to regulate the expression of bFGF, thus influencing cell proliferation and invasion in invasive PitNET.

XIST silencing inhibits proliferation, migration and invasion, and accelerates cell cycle arrest and apoptosis of invasive PitNET cells

For the purpose of exploring whether XIST was dysregulated in invasive PitNET, RT-qPCR was performed to determine the expression of XIST in normal pituitary tissues, non-invasive PitNET tissues (grade I, II), and invasive PitNET tissues (grade III, IV). It was found that the normal pituitary tissues and non-invasive PitNET tissues exhibited no significant change in the XIST expression; In comparison with the normal pituitary tissues and non-invasive PitNET tissues, the invasive PitNET tissues displayed markedly upregulated expression of XIST (Figure 2A). In order to further study

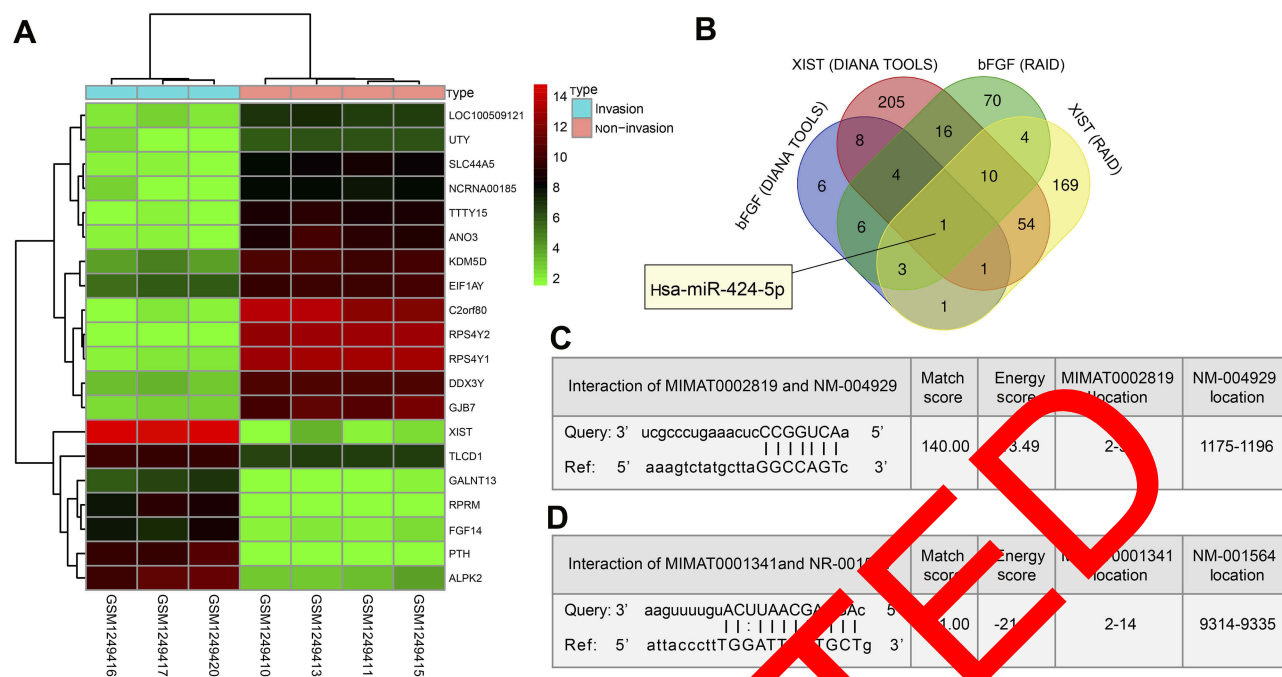


Figure 1 The potential interaction between XIST and miR-424-5p, bFGF and miR-424-5p is involved in invasive PitNET. **(A)** A heat map of differentially expressed lncRNAs in invasive and non-invasive PitNET tissues from dataset GSE51618, in which the abscissa indicates the lncRNA type, the ordinate indicates the lncRNA expression clustering, each small square indicates the expression of one lncRNA in one sample, and the histogram in the right upper is color gradation. **(B)** Prediction of miRNAs binding to both XIST and bFGF. **(C)** The putative hsa-miR-424-5p binding sites on bFGF. **(D)** The putative hsa-miR-424-5p binding site on XIST.

Abbreviations: XIST, X inactive specific transcript; miR-424-5p, microRNA-424-5p; bFGF, basic fibroblast growth factor; IPA, invasive pituitary adenoma.

whether XIST correlates with the gender of patients with invasive PitNET, the expression of XIST in invasive PitNET tissues from 51 patients (22 males and 29 females) was determined and the results revealed no gender-related difference in the expression of XIST (Figure 3A). Moreover, it was revealed that the transfection of si-XIST led to down-regulated expression of XIST (Figure 2B). Wound healing assay, scratch test and Transwell assay were carried out to detect the effect of XIST on invasive PitNET cell proliferation (Figure 2C), migration (Figure 2D) and invasion (Figure 2E) respectively. Results suggested that significantly reduced EdU positive cells and suppressed cell migration and invasion abilities were demonstrated in the cells transfected with si-XIST. Additionally, flow cytometric data displayed that XIST gene silencing arrested cells at G1 phase with a decrease in S phase (Figure 2F) and increased the apoptosis rate of cells (Figure 2G), suggesting si-XIST promoted apoptosis of invasive PitNET cells in vitro. The Western blot analysis results showed that the protein expression of MMP-2, MMP-9 and Bcl-2 was decreased, while that of Bax was increased after XIST silencing (Figure 2H; $p < 0.05$). All these results demonstrated that XIST silencing suppresses IPA cell proliferation, invasion and migration, but promotes apoptosis.

XIST up-regulates bFGF expression by competitively binding to miR-424-5p

Following the results demonstrating that XIST silencing could inhibit the development of invasive PitNET, the focus of the experiment was altered to focus on the interaction among XIST, miR-424-5p and bFGF. As the FISH results are illustrated in Figure 3A, the blue area represented the nucleus, while the green area represented XIST, indicating that XIST was mainly expressed in the cytoplasm. There existed binding sites between miR-424-5p and bFGF, miR-424-5p and XIST predicted by RAID v2.0, which was then verified by dual-luciferase reporter gene assay. The results indicated that the luciferase activity was attenuated in cells co-transfected with miR-424-5p mimic and pisCHECK2-based XIST-WT vectors containing the miR-424-5p binding sites, while the luciferase activity did not differ in cells transfected with miR-424-5p mimic and pisCHECK2-based XIST-MUT vectors with mutated miR-424-5p binding sites (Figure 3B), which meant that XIST competitively bound to miR-424-5p. The inhibited luciferase activity was also found in cells co-transfected with miR-424-5p mimic and pisCHECK2-based bFGF-WT vectors containing the bFGF 3'-UTR with recognizing sites, while no difference in terms of luciferase activity

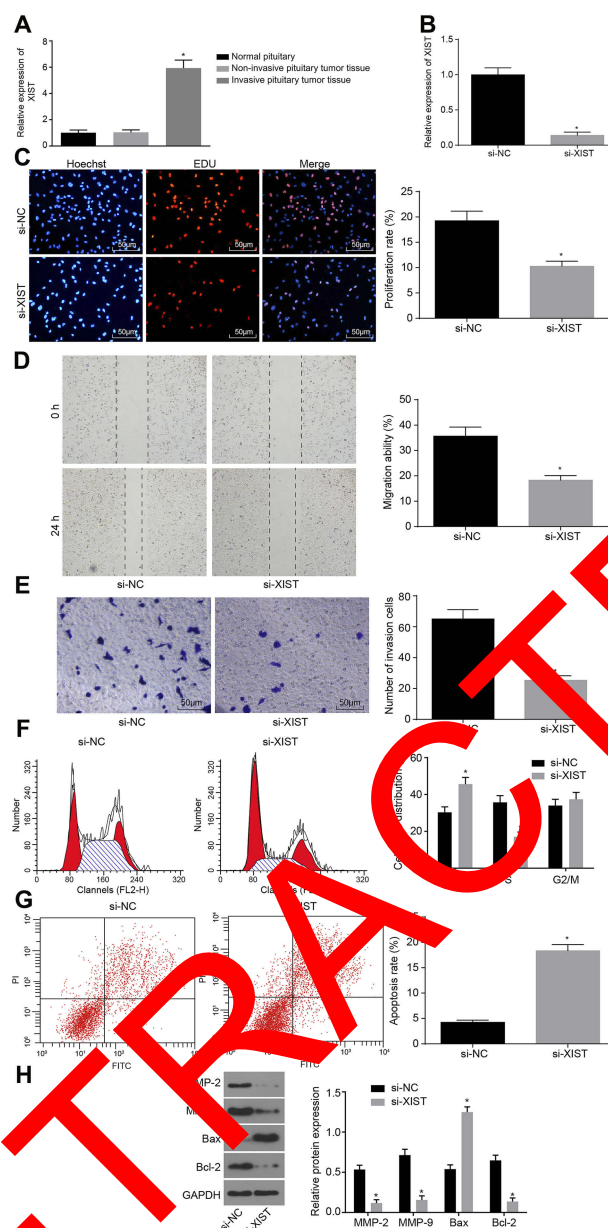


Figure 2 SiRNA-mediated depletion of XIST contributes to suppressed proliferation, migration and invasion, and enhanced cell cycle arrest and apoptosis of invasive PitNET cells. **(A)** Expression of XIST in invasive PitNET tissues (n=51), normal pituitary tissues (n=23) and non-invasive PitNET tissues (n=35) determined by RT-qPCR. Invasive PitNET cells were transfected with si-XIST or si-NC as control. **(B)** Expression of XIST determined by RT-qPCR. **(C)** Cell proliferation detected by EdU assay ($\times 200$). **(D)** Cell migration detected by scratch test ($\times 40$). **(E)** Cells invasion detected by Transwell test ($\times 200$). **(F)** Flow cytometry analysis of cell cycle distribution. **(G)** cell apoptosis rate measured by flow cytometry. **(H)** Protein expression of MMP-2, MMP-9, Bax and Bcl-2 measured by Western blot analysis. * $p < 0.05$ vs invasive PitNET cells transfected with si-NC or normal pituitary tissues. Statistical data were measurement data and were described as mean \pm standard deviation. The data between two groups were compared using unpaired t-test. The experiment was repeated three times.

Abbreviations: EdU, 5-Ethynyl-2'-deoxyuridine; IPA, invasive pituitary adenoma; Bcl-2, B-cell CLL/lymphoma-2; Bax, Bcl-2-associated X protein; MMP, matrix metalloproteinase; XIST, X inactive specific transcript; NC, negative control; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

was detected in cells co-transfected with miR-424-5p mimic and pISCHEK2-based bFGF-MUT vectors with recognizing sites mutated (Figure 3C), signifying that miR-424-5p could target bFGF.

Moreover, the RNA-pull down test reflected that the relative enrichment of miR-424-5p was relatively high in

the cells transfected with Bio-Wt-XIST, while no changes were found in the cells transfected with Bio-Mut-XIST (Figure 3D), proving that Bio-Wt-XIST may potentially promote the enrichment of miR-424-5p. RIP results depicted that the expression of XIST binding to AGO2 increased ($p < 0.05$), indicating that XIST could bind AGO2

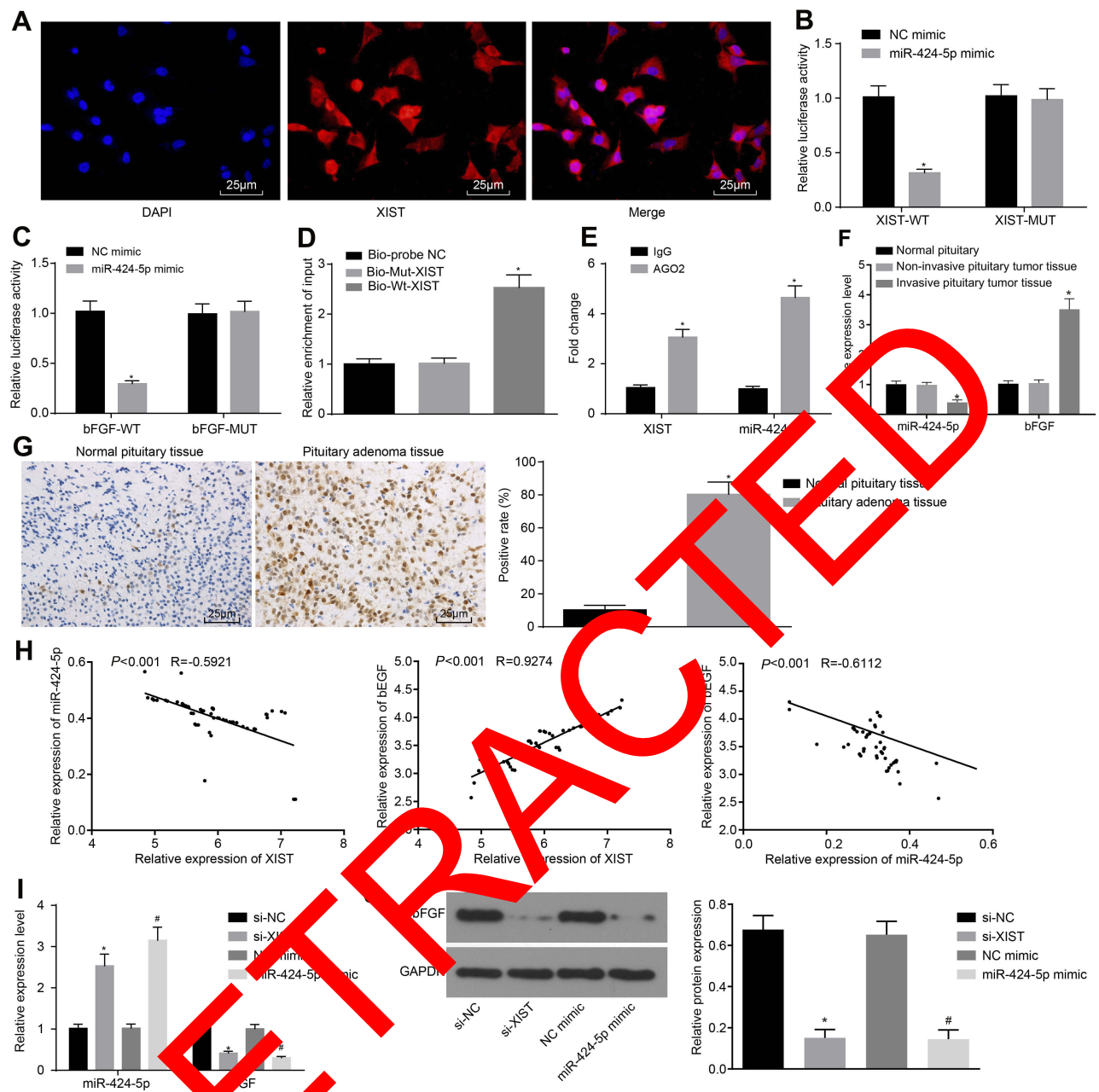


Figure 3 XIST acts as a miRNA of miR-424-5p to upregulate bFGF expression. (A) Cellular localization of XIST in invasive PitNET cells identified by FISH test ($\times 400$). (B) Relationship between XIST and miR-424-5p identified by dual-luciferase reporter gene assay. $*p < 0.05$ vs cells transfected with NC mimic. (C) Relationship between bFGF and miR-424-5p confirmed by dual-luciferase reporter gene assay. $*p < 0.05$ vs cells transfected with NC mimic. (D) the enrichment of miR-424-5p detected using RNA-pull down assay. $*p < 0.05$ vs cells transfected with Bio-probe NC. (E) the binding of XIST and miR-424-5p to AGO2 analyzed using RIP assay. $*p < 0.05$ vs IgG group. (F) Expression of miR-424-5p and bFGF in invasive PitNET tissues ($n=51$), normal pituitary tissues ($n=23$) and non-invasive PitNET tissues ($n=35$) determined by RT-qPCR. $*p < 0.05$ vs normal pituitary tissues. (G) positive expression of bFGF in Invasive PitNET tissues ($n=51$) and normal pituitary tissues ($n=23$) ($\times 200$). $*p < 0.05$ vs normal pituitary tissues. (H) Correlation analysis among XIST, miR-424-5p and bFGF in Invasive PitNET. (I) Expression of bFGF and miR-424-5p in different groups by RT-qPCR. (J) Protein expression of bFGF measured using Western blot analysis. $*p < 0.05$ vs invasive PitNET cells transfected with si-NC, $^{\#}p < 0.05$ vs invasive PitNET cells transfected with NC mimic. Statistical data were measurement data, and described as mean \pm standard deviation. Data between two groups were compared using unpaired t-test and data among multiple groups were compared using one-way ANOVA. The experiment was repeated three times.

Abbreviations: XIST, X inactive specific transcript; miR-424-5p, microRNA-424-5p; bFGF, basic fibroblast growth factor; NC, negative control; WT, wild type; MUT, mutated type; AGO2, Argonaute 2; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

protein (Figure 3E). Next, the RT-qPCR results revealed a higher expression of bFGF and lower expression of miR-424-5p in invasive PitNET tissues than in non-invasive PitNET tissues and normal pituitary tissues ($p < 0.05$) and

the expression of miR-424-5p and bFGF was not fundamentally different between normal pituitary tissues and non-invasive PitNET tissues (Figure 3F). Immunohistochemistry imaging further indicated that bFGF was mainly located in

the nucleus and the positive rate of bFGF was increased in invasive PitNET tissues ($p<0.05$; Figure 3G). In order to review whether miR-424-5p and bFGF correlate with the gender of invasive PitNET patients, the expression of miR-424-5p and bFGF in IPA tissues from 51 patients (22 males and 29 females) was determined and evaluated. To elaborate, no gender-related differences were found in relation to the expression of XIST, miR-424-5p and bFGF (Figure S1). Subsequently, the correlation among XIST, miR-424-5p and bFGF was analyzed, and the results proved a negative correlation between XIST and miR-424-5p ($R = -0.592$; $p<0.0001$) as well as a negative correlation between miR-424-5p and bFGF ($R = -0.611$; $p<0.0001$), but a positive correlation between XIST and bFGF ($R = 0.927$; $p<0.0001$) (Figure 3H). Nonetheless, it was established that depleted XIST or restored miR-424-5p led to elevated miR-424-5p expression, while reduced bFGF expression (Figure 3I,J). All in all, XIST could serve as a ceRNA of miR-424-5p to elevate the expression of bFGF.

Up-regulated miR-424-5p inhibits proliferation, migration and invasion, and promotes cell cycle arrest and apoptosis of invasive PitNET cells by decreasing bFGF expression

Hence, the effects of miR-424-5p on the progression of invasive PitNET were determined. Cell proliferation was detected by EdU assay, and results revealed that proliferation rate of cells was decreased due to transfection with miR-424-5p mimic or si-bFGF. The reduction in proliferation rate of cells induced by miR-424-5p mimic was reversed after the transfection with oe-bFGF plasmid (Figure 4A). Simultaneously, results of scratch and Transwell tests also showed that the migration and invasion abilities of invasive PitNET cells were significantly inhibited in cells transfected with miR-424-5p mimic or si-bFGF, and the decrease of the migration and invasion abilities of IPA cells induced by miR-424-5p mimic was reversed after transfection with oe-bFGF plasmid (Figure 4B,C). Flow cytometry further denoted that there was no significant change in the cell cycle distribution of cells co-transfected with miR-424-5p mimic and oe-bFGF compared with cells transfected with blank vector ($p>0.05$). Then, the transfection with miR-424-5p mimic or si-bFGF led to more cells distributed in G1 phase and fewer cells in S phase, suggesting that miR-424-5p mimic induced cell cycle arrest (Figure 4D). Flow cytometry then

displayed that the cell apoptosis rate following co-transfection with miR-424-5p mimic and oe-bFGF had no significant change compared with transfection with blank vector ($p>0.05$). Cells transfected with miR-424-5p mimic or si-bFGF exhibited significantly higher apoptosis rate than cells transfected with mimic NC or si-NC ($p<0.05$; Figure 4E), reflecting that miR-424-5p mimic could promote the apoptosis of IPA cells in vitro. Western blot analysis was carried out for the purpose of detecting the protein expression of MMP-2, MMP-9, Bax and Bcl-2. It was found that the expression of MMP-2, MMP-9, Bax and Bcl-2 in invasive PitNET cells co-transfected with both miR-424-5p mimic and oe-bFGF did not change evidently. The transfection with miR-424-5p mimic or si-bFGF contributed to down-regulated protein expression of MMP-2, MMP-9 and Bcl-2 but up-regulated protein expression of Bax ($p<0.05$; Figure 4F). With this all taken into account, the conclusion that could be arrived is that overexpressed miR-424-5p could repress invasive PitNET proliferation, invasion and migration, but also enhance cell apoptosis via decrease of bFGF.

Discussion

Resection is the preferred option for invasive PitNET treatment. However, total resection is difficult due to high risk of cerebrospinal fluid leak and damages to cranial nerves and internal carotid artery.²² Moreover, invasive PitNET is known to have a higher recurrence rate but poorer prognosis than non-invasive PitNET.²³ Thus, with the expectation to shed new light on the management of invasive PitNET, this study intended to evaluate the effects of lncRNA XIST, miR-424-5p and bFGF on IPA cell activities and further inspect the related mechanism. Collectively, this study found that lncRNA XIST could competitively bind to miR-424-5p to increase the expression of bFGF, thus promoting the progression of IPA.

Initially, the findings provided evidence that invasive PitNET tissues exhibited higher expression of XIST and bFGF, but lower expression of miR-424-5p than normal pituitary tissues and non-invasive PitNET tissues. Subsequently, it was then found that XIST could serve as a ceRNA of miR-424-5p to modulate bFGF expression. Recently, ceRNA hypothesis has been extensively proposed and numerous studies have focused on the specific interaction between lncRNA and miRNA in cancers.²⁴ For instance, high mobility group A1 non-coding pseudogene (HMGA1P), a pivotal role on the onset of PitNET,²⁵ was reported to regulate miR-483 and miR-675 through a ceRNA

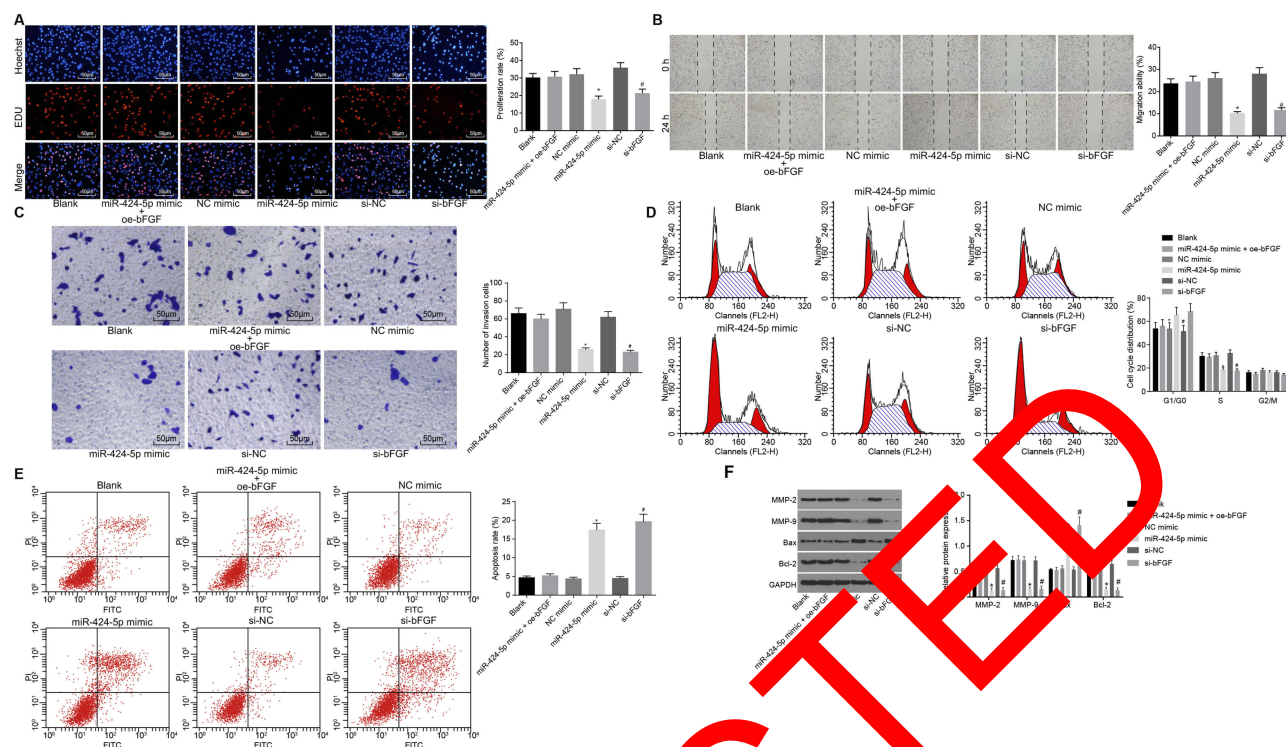


Figure 4 Restored miR-424-5p represses proliferation, migration and invasion but enhances cell cycle arrest and apoptosis of invasive PitNET cells by downregulating bFGF. Invasive PitNET cells were transfected with si-bFGF or miR-424-5p mimic or co-transfected with miR-424-5p mimic and oe-bFGF. **(A)** Cell proliferation detected by EdU assay ($\times 200$). **(B)** Cell migration detected by scratch test ($\times 40$). **(C)** Cell invasion detected by Transwell assay ($\times 200$). **(D)** Flow cytometry analysis of cell cycle distribution. **(E)** Flow cytometry analysis of cell apoptosis. **(F)** Protein expression of MMP-2, MMP-9, Bax and Bcl-2 measured using Western blot analysis. * $p < 0.05$ vs invasive PitNET cells transfected with si-NC, # $p < 0.05$ vs invasive PitNET cells transfected with miR-424-5p mimic. Statistical data were measurement data, described as mean \pm standard deviation and compared using one-way ANOVA. The experiment was repeated three times.

Abbreviations: EdU, 5-Ethynyl-2'-deoxyuridine; IPA, invasive pituitary adenoma; Bcl-2, B-cell CLL/lymphoma-2; Bax, Bcl-2-associated X protein; MMP, matrix metalloproteinase; XIST, X inactive specific transcript; NC, negative control; miR-424-5p, microRNA-424-5p; bFGF, basic fibroblast growth factor.

mechanism with Egr1.²⁶ Besides, HMC1P7 and H19 in the upregulation of H19 and Igf2 by functioning as a miRNA decoy.²⁷ XIST served as a sponge of miR-7, whereby upregulating the expression of RING1, which is implicated in the progression of non-small cell lung cancer (NSCLC).²⁸ miR-424 was also documented to be poorly expressed in various cancers such as cervical cancer, ovarian cancer, exerting tumor suppressing effects in the development of cancers.²⁹ Furthermore, miR-424-5p was confirmed to bind to the FGFR1, which is the critical receptor of bFGF.¹⁴ Similarly, elevated expression of miR-424 was found to downregulate the expression of FGFR1, thus mediating the bFGF signaling pathway.³⁰ bFGF expression was revealed to be closely correlated with tumor size, invasiveness and patient outcomes of PitNET.³¹ bFGF also acts as a significant marker of angiogenesis of PitNET via PTTG regulation.³²

Furthermore, in this study, it was intriguingly demonstrated that XIST silencing, miR-424-5p overexpression or bFGF depletion was detected to suppress the proliferation, migration, invasion, and promote the apoptosis of invasive PitNET cells,

corresponding to reduced MMP-2, MMP-9 and Bcl-2 expression, and elevated Bax expression. A previous study indicated that high expression of MMP-2 and MMP-9 can cause PitNET invasiveness.²³ Both Bax (pro-apoptosis gene) and Bcl-2 (anti-apoptosis gene) are fundamental for the regulation of the mitochondrial apoptosis process and dopamine 2 receptor short isoform infection enhance GH3 cell apoptosis in PitNET with Bcl-2 downregulation and Bax upregulation.³³ Furthermore, Jiang et al demonstrated that XIST over-expression promoted non-small cell lung cancer (NSCLC) cell viability and invasion in vitro, while XIST silencing suppressed NSCLC cell activities in vitro.⁷ Similarly, the depletion of lncRNA XIST could contribute to the suppressed proliferation and migration with an enhanced apoptosis in pancreatic cancer.³⁴ The critical role of miRNAs was implicated in tumorigenesis, progression and aggressiveness of PitNET.³⁵ miR-106b~25 cluster is asserted to be up-regulated in PitNET and share association with tumor invasion.³⁶ miR-106b exerted promotive functions on PitNET cell proliferation and invasion via targeting tumor suppressor PTEN.³⁷ By contrast, miR-15a/

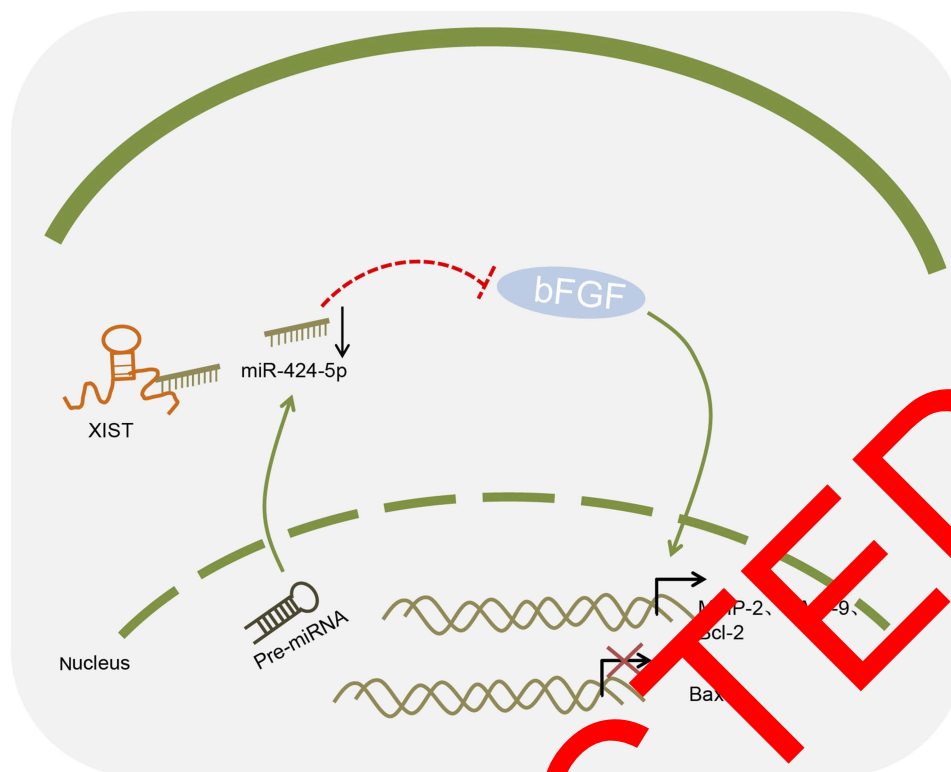


Figure 5 Mapping of the mechanism of XIST, miR-424-5p and bFGF regulating the proliferation and invasion of invasive PitNET. XIST silencing elevates miR-424-5p expression to down-regulate the expression of bFGF, thus inhibiting proliferation, migration and invasion but promoting apoptosis of IPA cells, corresponding to decreased expression of MMP-2, MMP-9 and Bcl-2 but increased that of Bax.

Abbreviations: IPA, invasive pituitary adenoma; Bcl-2, B-cell CLL/lymphoma-2; bFGF, basic fibroblast growth factor; XIST, X inactive specific transcript; miR-424-5p, microRNA-424-5p; bFGF, basic fibroblast growth factor.

16,³⁸ miR-183,³⁹ miR-148b,⁴⁰ miR-200b,⁴¹ miR-424-5p⁴² and etc. could repress PitNET cell proliferation, invasion and migration by target various oncogenic genes. miR-424 regulates biological processes in neuroblastoma, such as cell cycle, cell differentiation and proliferation.⁴³ miR-424-5p behaved as a tumor suppressor in esophageal squamous cell carcinoma by suppressing cell proliferation and invasion.⁴⁴ Zhou's research demonstrated that the upregulation of miR-424-5p contributed to repressed development of human cervical cancer by inhibiting cell proliferation and accelerating cell apoptosis.¹³ It was also widely reported that bFGF was involved in tumor angiogenesis and could enhance hepatocellular carcinoma cell growth,⁴⁵ which was partially consistent with our findings. Further, rescue experiments in this study provided evidence that miR-424-5p inhibit the development of invasive PitNET via targeting bFGF.

Conclusion

In summary, the findings in this current study suggested that lncRNA XIST silencing has the potential to upregulate miR-424-5p to downregulate bFGF expression, thus suppressing the

proliferation, migration and invasion, and promoting apoptosis of invasive PitNET cells (Figure 5). This finding exhibits the potential therapeutic targets and underlying mechanisms which may shed light on the investigation of invasive PitNET development and progression. However, more efforts and contributions are needed in order to demonstrate the role of miR-424-5p restoration in invasive PitNET or other tumors in the near future.

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Disclosure

The authors declare that they have no competing interests in this work.

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Supplementary material

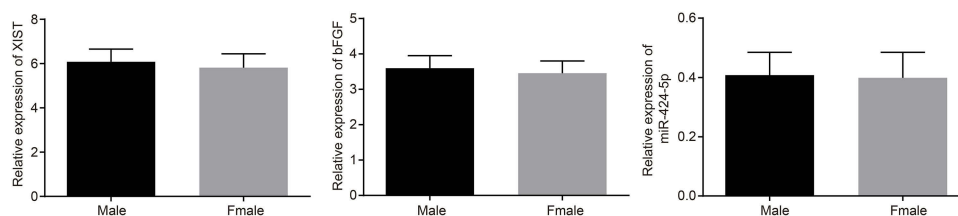


Figure S1 The expression of XIST, miR-424-5p and bFGF in male and female patients of invasive PitNET.

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