CDKN2A (p16INK4A) affects the anti-tumor effect of CDK inhibitor in somatotroph adenomas

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Abstract. The altered cell cycle is associated with aberrant growth factor signaling in somatotroph adenoma, which is the primary cause of acromegaly. The aim of the present study was to investigate the pathological role of the INK4 family and evaluate the effectiveness of CDK4 inhibitor, palbociclib, in somatotroph adenoma. RNA-Seq, RT-PCR, and immunohistochemistry were applied to measure the levels and correlations of the INK4 family with angiogenesis, CDKs, EMT, and therapeutic targets. MTS, flow cytometry, and ELISA were used to investigate the bio-activity in GH3 and GT1-1 cell lines after palbociclib treatment. Compared with lactotroph adenoma, gonadotroph adenoma, and corticotroph adenoma, somatotroph samples demonstrated higher expression of CDKN2A and SSTR2 but a lower expression of EGFR, CDK4, and CDH2 (P<0.05). CDKN2A positively correlates with SSTR2, and negatively with CDK4, EGFR, and CDH2. Patients with lower CDKN2A had larger tumor size (P=0.016) and more invasive potential (P=0.023). Palbociclib inhibited cell proliferation, induced G1 phase arrest, reduced GH/IGF-1 secretion of GH3 and GT1-1 cell lines (P<0.05), and had a more prominent role in GH3 cells (P<0.05). CDKN2A inhibited the bio-activity by modulating CDK4, and high CDKN2A predicted the insensitivity to CDK4 inhibitor, palbociclib, in somatotroph adenoma patients. In summary, the present study shows CDKN2A inhibited the bio-activity by modulating CDK4, and high CDKN2A predicts the insensitivity to CDK4 inhibitor, Palbociclib, in somatotroph adenoma patients.

Introduction

Pituitary neuroendocrine tumors (PitNETs) have five main histological subtypes: somatotroph, lactotroph, thyrotroph, corticotroph, and gonadotroph (1). Somatotroph adenomas (SOMA) are the primary cause of acromegaly, leading to severe complications such as hypertension, diabetes mellitus, cardiovascular disease, osteoarthritis, and sleep apnea (2). Transsphenoidal surgery is the first choice for patients with somatotrophic adenoma. For failed surgery, poor surgical candidates, or residual tumors, drug therapy is used as adjuvant treatment (3). However, the normalization of serum growth hormone/insulin-like growth factor 1 (GH/IGF-1) in patients treated with octreotide and lanreotide for one year was 43% and 31-35%, respectively (4,5). An international, non-interventional multicenter study of 2,090 patients with pegvisomant proved that the patient’s ratio with normal medical treatment of SOMA.

Cell cycle dysregulation results in uncontrolled proliferation in cancers, including lung cancer, hepatic carcinoma, leukemia, and gynecologic oncology (7). Ciclyn-dependent kinases (CDK) are a family of protein kinases involved in
regulating the cycle of mammalian cell division, which in turn is limited by CDK inhibitors (CKIs) (8,9). The cell cycle is negatively regulated by CKIs (8,10), which includes Cip/Kip and INK4 family (11). Somatotroph cells are usually characterized by aneuploidy, DNA damage, and cell cycle disruption, including premature cell cycle arrest (12). Overexpression of cyclin E/CDK2 and loss of p21CIP1/p27KIP1 appeared to be associated with poor prognosis in SOMA (13). The INK4 family shares similar domains that competes with Cyclin D and relieves the activation of CDK4/6 (14), leading to cell cycle arrest in the G1/S phase (15). Loss of cyclin-dependent kinase inhibitor 2A (CDKN2A) predicted sensitivity to the CDK4/6 inhibitor, PD0332991, in melanoma cell lines (16). CDKN2A specifically prevented both nucleotide probe and p Albicoc binding to CDK4 in MCF7 cell line (14).

At present, the pathogenesis of SOMA has not been well elucidated. However, disruption of the cell cycle is considered to play an essential role in pituitary tumorigenesis. The aim of the present study was to describe the characteristic profile of the INK4 family in SOMA, which was different from that of other subtype adenomas and further analyze the correlation of INK4 family with angiogenesis, CDKs activity, epithelial-mesenchymal transition (EMT) and the therapeutic target of SOMA. Based on these results, we hope to provide the evidence of combined the CDK4/6 inhibitor for the medical treatment of SOMA.

Materials and methods

Patients and samples. Tumor specimens were collected at Beijing Tiantan Hospital affiliated to Capital Medical University from May 2012 through December 2017 after transsphenoidal surgery. Isolated specimens were stored in liquid nitrogen for 30-60 min. All patients had sporadic adenomas and had no family history of adenomas. The morpho-functional classification of pituitary adenomas was diagnosed according to the 2017 World Health Organization (WHO) classification of tumors of endocrine organs (17). The patients comprised 112 (56.6%) males and 86 (43.4%) females with the average age of 46.3±13.5 years (range, 21-69). The study protocol was approved by the Internal Review Board of Beijing Tiantan Hospital Affiliated to Capital Medical University, and conformed to the ethical guidelines laid down in the Declaration of Helsinki (no. KY-2013-015-02). Knosp classification was based on coronal sections of unenhanced and gadolinium diethylene-triamine-pentaacetic acid enhanced magnetic resonance imaging scans, with the readily detectable internal carotid artery serving as the radiological landmark. Surgically proven invasion of the cavernous sinus space was present in all Grade 4 and Grade 3 cases and in all but one of the Grade 2 cases; no invasion was present in Grade 0 and Grade 1 cases (18). All the samples were obtained after informed consent of patients, following institutional review board-approved protocols. Three normal pituitary samples were obtained from the Tianjin Red Cross Society by autopsy through the human body donation program.

GH3 and GT1-1 cell lines were purchased from ATCC (Manassas) and cultured in a humidified incubator at 37°C and 5% CO₂ in F-12K medium (ATCC), supplemented with 2.5% fetal bovine serum and 10% horse serum (Gibco). RNA extractions, sequencing, RNA-Seq data processing and analysis. For RNA extractions, patient samples were assessed with AllPrep DNA/RNA Mini kit (Qiagen) according to the manufacturer's instructions. The quantity and quality of RNA was evaluated by RNA Nano6000 assay kit (Agilent Technologies) (RIN >6.8). Then, 3 µg RNA/sample was used for RNA preparations, and the ribosomal RNA was removed by Epicentre Ribo-zero™ rRNA Removal kit (Epicentre). Sequencing library was generated by NEBNext® UltraTM Directional RNA Library Prep kit (NEB). The library fragments (150-200 bp) were purified by AMPure XP system (Beckman Coulter), and assessed by Agilent Bioanalyzer 2100 system. The libraries were sequenced on an Illumina Hiseq X platform, then 150 bp paired-end reads were generated. Any reads containing adapters, containing ploy-N and low-quality reads were removed. Clean reads were mapped to the human reference genome (NCBI37/hg19) using hisat2 (v2.0.5) to get reads counts/FPKM/TPM for each identified gene. RStudio was used to analyze the correlation coefficient and significative degree. R package ggplot2 (https://github.com/tidyverse/ggplot2) was used to visualize the results.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA of 32 samples was extracted and purified using the RNeasy®Mini kit (Qiagen), following the manufacturer's instructions. Reverse transcrptase kit was used (Applied biosystems, Thermo Fisher Scientific, Inc.), and Quantitative real-time PCR (RT-qPCR) was performed on QuantStudio5 applied (Thermo Fisher Scientific, Inc.) using Power SYBR®-Green PCR Master Mix (Life Technologies). The mRNA level of crucial genes of angiogenesis, CDKs, EMT, and therapeutic targets of SOMA were measured in this study. The fold-change in differential expression for each gene was calculated using the comparative CT method (2^-ΔΔCT method) in R package with ‘PCR’ functions (https://github.com/MahShaaban/pcr) (19). GAPDH was used as the reference gene (20).

Tissue microarray construction and immunohistochemistry staining. A total of 198 formalin-fixed (4%, room temperature) paraffin-embedded tissue blocks were sectioned. Three core biopsies (2.0 mm in diameter) were selected from the paraffin-embedded tissue. The cores were transferred to tissue microarrays using a semi-automated system (Aphelys MiniCore). The microarrays were cut into 4-µm sections and incubated with anti-pI6INK4A (rabbit monoclonal, 1:1,000, ab108349, Abcam), anti-CDH2 (rabbit polyclonal, 1:300, ab18203), anti-CDK4 (rabbit polyclonal, 1:300, ab137675, Abcam), anti-EGFR (rabbit monoclonal, 1:200, ab52894), and anti-SSTR2 (rabbit monoclonal, 1:400, ab134152) primary antibodies. IHC staining was evaluated in normal pituitary and PitNETs tissue concerning the pattern of expression either cytoplasmic, membranous or nucleus. H-score was also calculated for CDK4/6/EGFR/SSTR2 using the intensity and percentage of positive cells. The intensity score (0-3) was multiplied by the percentage of cells that stained with each level of intensity and the sum of these mathematical products was expressed as a score of 0-300. H score
formula was calculated as: Strong intensity (3) x percentage + moderate intensity (2) x percentage + mild intensity (1) x percentage.

**Cell viability and cell cycle.** GH3 and GT1-1 cells were adjusted to a density of 1x10^5 cells/mL. A total of 100 µl of the cell suspension was plated into each well of a 96-well plate and cultured overnight with 37°C thermostatic. After palpociclib treatment (1, 5 or 20 µM) for 24, 48 and 72 h, 20 µl of 3-(4,5-diethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) solution was added to each well, and the cultures were further incubated for 4 h, 37°C. Absorbance was measured at 490 nm using an ELISA plate reader (Thermo Fisher Scientific, Inc.). After 72 h treatment, the cell cycle was determined with PI detection kit (Roche Diagnostics) by flow cytometry. Experiments were performed in triplicate.

**Enzyme-linked immunosorbent assay.** The levels of GH and IGF-1 in cell culture supernatant were measured by ELISA (DE3058-96T, DE2096-96T, Applygen), according to the manufacturer's protocol. A total of 10 µl of supernatant was used per well. Absorbance was read at 450 nm using an ELISA plate reader (Thermo Fisher Scientific, Inc.). Experiments were performed in triplicate.

**SDS-PAGE and western blot analysis.** Samples (up to 10 mg) were lysed in lysis buffer containing 1% Nonidet P-40 (Calbiochem, Merk) and protease and phosphatase inhibitor cocktails (Roche) overnight at 4°C. Total extracts were centrifuged at 12,000 x g for 30 min at 4°C, and protein concentration was determined with the BCA method (Pierce Biotechnology). A total of 40 µg of protein per lane was loaded onto 10% Bis-Tris SDS-PAGE gels, separated electrophoretically, and blotted onto polyvinylidene fluoride membranes (Merk). The blots were incubated with antibodies against anti-p16INK4A antibody (1:1,000), anti-CDK4 antibody (1:2,000), anti-EGFR antibody (1:1,000) and anti-CDH2 antibody (1:1,000) followed by a secondary antibody (1:10,000) tagged with horseradish peroxidase (Santa Cruz Biotechnology). Blots were visualized by enhanced chemiluminescence, and densitometry was performed using a fluorescence image analyzer (Amsherm Imager 600, GE Healthcare). GAPDH was used as a loading control.

**Statistical analysis.** Chi test and Fisher's exact test were used to determine the significance of categorical variables. One-way ANOVA (Tukey post-hoc test) and t-test (unpaired test) were applied in patients or for in vitro experiments. Spearman correlation coefficient was used for the relationship of INK4 family and crucial genes of angiogenesis, CDKs, EMT, and therapeutic targets of SOMA. P-values were two-sided, and 0.05 was applied as the significance level.

**Results**

**Clinical characteristics of patients.** The 198 patients with PitNETs included 38 (19.2%) corticotroph adenomas (CORT), 80 (40.4%) gonadotroph adenomas (GONA), 40 (20.2%) lactotroph adenomas (LACT), 40 (20.2%) SOMA and the clinical characteristics are shown in Table I. The patients comprised 112 (56.6%) males and 86 (43.4%) females with the average age of 46.3±13.5 years (range, 21-69). The distribution of tumor volume classifications was 9 (4.54%) microadenomas (diameter ≤1 cm), 127 (64.14%) macroadenomas (1 cm < diameter ≤ 4 cm), and 62 (31.32%) giant adenomas (diameter >4 cm). According to Knosp staging, there were 94 (47.47%) invasive cases and 104 (52.53%) non-invasive cases. The average follow-up time was 45.5±10.2 months (range, 23-78), and 5-year recurrence rate was 56/198 (28.28%).

**Characteristic profile of the INK4 family in SOMA.** A total of 24 SOMA specimens were sequenced to obtain the transcriptionome reading data after matching, then normalized them to transcripts per million (TPM) values. With these data, the mRNA levels correlation of INK4 family members with crucial genes of angiogenesis, CDKs, EMT, and therapeutic targets of SOMA were analyzed in Fig. 1. Among them, Spearman correlation coefficient of CDRK2A and epidermal growth factor receptor (EGFR) (r=-0.534, P=0.032), Cadherin 2 (CDH2) (r=-0.631, P=0.001), CDK4 (r=-0.441, P=0.032), Snail family transcriptional repressor 1 (SNAI1) (r=0.657, P=0.001),

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* CORT, corticotroph PitNETs; GONA, gonadotroph PitNETs; LACT, lactotroph PitNETs; SOMA, somatotroph PitNETs.
Somatostatin receptor 2 (SSTR2) \((r=-0.555, P=0.006)\) as well as CDKN2B and EGFR \((r=-0.497, P=0.014)\), CDH2 \((r=-0.545, P=0.007)\), SNAI1 \((r=0.70, P=0.001)\), CDKN2D and CDK5 \((r=0.45, P=0.029)\), dopamine receptor D2 (DRD2) \((r=0.541, P=0.007)\) had reached the significance level as shown in Fig. 2A. According to the results, EGFR, CDK4, CDH2, and SSTR2 were filtered for the next step.

Subsequently, we analyzed the mRNA levels of the INK4 family and candidate molecules in SOMA, LA cT, GONA, and c ORT in Fig. 2B via reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). The levels of CDKN2A and CDKN2B in SOMA were the highest compared to other subtypes \((P<0.01)\), and the levels of EGFR and CDK4 in SOMA were the lowest \((P<0.05)\). There was no statistical difference of CDKN2C or CDKN2D among these subtypes \((P>0.05)\). Based on these results, we chose CDKN2A for further research.

Expression profile of CDKN2A in 198 patients by tissue microarray analysis. Protein levels of CDKN2A, EGFR, CDK4, CDH2, and SSTR2 were studied in 198 specimens by immunohistochemistry (IHC) (Fig. 3). Among the five groups, SOMA had the lowest H-score compared to CDK4, CDH2, and SSTR2, but had a higher H-score compared to CDKN2A and SNAI1 \((P<0.01)\). Patients were divided into high and low groups based on the median H-score. Patients with lower CDKN2A had larger tumor size \((58/99 vs. 41/99, P=0.016)\) and...
Figure 2. The characteristic profile of the INK4 family in pituitary adenoma. (A) Correlation analysis among INK4 family with the characteristic molecule related to tumor behavior. (B) The mRNA level of INK4 family and characteristic molecule related to tumor behavior in subtypes of pituitary adenomas. *P<0.05, **P<0.01, ***P<0.001, n=8.
higher invasive behavior (55/99 vs. 39/99, P=0.023) (Table II). There was also a negative correlation between \textit{CDKN2A} and invasive behavior (r=-0.207, P=0.0004). Higher \textit{CDK4} might cause more invasive potential (54/99 vs. 40/99, P=0.046). Positive staining of \textit{SSTR2} was 25/80 (31.3\%) in GONA. The average H-scores of \textit{SSTR2} were 57.9±20.6 in \textit{SSTR2}+GONA.
compared to 143.4±9.17 in SOMA. In addition, we analyzed the correlation of DRD2 and CDKN2A in 40 SOMA patients and 4 normal pituitary samples by IHc experiment. H-scores of DRD2 in SOMA were 160.1±7.2 (range: 50 -270), and 195±25.69 (160, 210, 210 and 270) in normal pituitary. Correlation coefficient was 0.22 (P=0.151) in Fig. S1.

Inhibition induced by palbociclib in GH3 and GT1-1 cell line. The bioactivity effect of CDK4/6 inhibitor, palbociclib, was tested on the GH3 and GT1-1 cell lines. The cell viability experiment showed that inhibition by palbociclib occurred in a dose- and time-dependent manner (Fig. 4A and D). The inhibition induced by Palbociclib occurred in a time- and dose-dependent manner, whether GH3 or GTI-1 cells, **P<0.05, ***P<0.01, ****P<0.001, n=8. (B and E) Palbociclib inhibited the secretion of GH/IGF-1 in GH3 and GT1-1 cell lines. (C and F) Palbociclib induced the G1 phase arrest in GH3 and GT1-1 cell lines. (G) Statistical analysis of the change of bio-activity after 72 h treatment between GH3 and GTI-1 cell lines, compared to the control group (%). *P<0.05, **P<0.01, ***P<0.001, n=3.
of cell viability, and secretion of \(GH/IGF-1\) both showed the inhibition of palbociclib in the GH3 cell line was stronger than that in the GT1-1 cell line after 72 h treatment (Fig. 4G) (\(P<0.05\)).

Results of the RT-qPCR and western blot assays showed palbociclib undermined the level of \(CDK4\), \(CDH2\), and \(EGFR\) (Fig. 5). Western blot analysis revealed that the level of \(CDKN2A\) in GH3 cells was lower than that in GT1-1 cells. Palbociclib has more potent inhibition on the activity of \(CDK4\) in GH3 cell line than in GT1-1 cell line.

**Discussion**

SOMA accounts for 13-15% of functional PitNETs and are more common in males with a high standardized mortality ratio compared with the general population (21). Factors associated with successful treatment include tumor size, preoperative serum \(GH/IGF-1\) level, and parasellar extension (22). Disruption of cell cycle played a crucial role in pituitary tumorigenesis. Classifying the relationship of cell cycle involving SOMA biology may provide new therapeutic
molecule against SOMA. Herein, we described the profile of INK4 family in SOMA. The level of CDKN2A should be evaluated for the strategy combined with CKIs in future medical treatment.

Excess proliferation and gene dysregulation are the hallmarks of cancer. Checkpoints block cells from passing into the next phase, and CDKs control critical cell cycle checkpoints and key transcriptional events. The first checkpoint occurs at the G1-S phase, and the G1-S enzymes include CDK4, CDK6, and the D-type cyclins (23). The INK4 family regulates the G1-to-S phase transition by specifically inhibiting the activity of CDK4/6. CDKN2A/CDKN2B have unique structures and are essential tumor suppressor genes; loss of CDKN2A function was correlated with an increased risk of pancreatic cancer (24-26).

In addition, low level or loss of CDKN2A was associated with shorter disease-free survival and disease-specific survival times, independent of tumor size and WHO grade of pancreatic neuroendocrine tumors (27). In this study, patients with low CDKN2A had larger tumor volume and a higher likelihood of invasive behavior than patients with high CDKN2A. Correlation analysis of mRNA levels was measured in CDKN2A and CDKs. CDK4 was filtered as the regulatory protein of CDKN2A. The negative correlation between CDKN2A and CDH2 was identified. CDH2 knockdown markedly inhibited cell proliferation, colony formation, cell migration and invasion, and induced cell cycle arrest and apoptosis (28).

The ankyrin-repeat protein CDKN2D functions as a key regulator of G1/S transition, and phosphorylation of CDKN2D dissociates the CDK6-CDKN2D inhibitory complex, thereby, activating CDK6, which triggers entry into S-phase (29). Considering the dual role of CDKN2D in controlling differentiation and proliferation (30), the positive correlation between CDKN2D and CDK5 was not significant in SOMA. CDKN2A and CDKN2D played a converse role in the tumor proliferation and invasion of SOMA heeding the relationship with genes related to EMT, at least, albeit CDKN2D was not an inhibitor of CDK4 activity in SOMA.

Recent evidence indicates epithelial-mesenchymal transition (EMT) plays a critical role in stemness, metabolic reprogramming, immune evasion and therapeutic resistance of cancer cells. Transcriptional repressors including Snail (SNAI1), Slug (SNAI2) and the ZEB family constitute key players for EMT in cancer as well as in the developmental process (31). However, Tamura et al reported that the expression of SNAI1 was significantly associated with suprasellar expansion, which was not related to tumor invasion (32). Jia et al reported that SNAI1 had a significant correlation of PitNET proliferation, and SNAI2 had a significant correlation of sella destruction (33). In summary, the bio-activity of SNAI1 in PitNETs should be investigated to clarify its role further in future research. Therefore, we chose the most significant molecule, CDH2, which is related to EMT. RT-qPCR and IHC experiments both indicated the negative correlation between CDKN2A and CDH2.

CDK4/6 was known to promote continued cell-cycle progression and growth in cancer. The imbalance of CDK4/6 causes resistance to endocrine therapy in hormone receptor-positive breast cancer (34). Palbociclib, combined with the aromatase inhibitor, letrozole, significantly prolonged progression-free survival as compared with letrozole alone in a double-blind study of advanced breast cancer (35). An ovarian patient with loss of CDKN2A derived significant, prolonged clinical benefit from Palbociclib with letrozole (36). High CDKN2A should be one of the exclusion criteria for CDK4/6 inhibitor therapy for high CDK4 target engagement by Palbociclib in cells without functional CDKN2A and attenuated target engagement when CDKN2A is abundant (14). GH3 cells mainly secrete growth hormone and insulin-like growth factor, and GT1-1 cells mainly secrete luteinizing hormone accompanied with a little growth hormone. Theoretically, CDKN2A should be increased with the increase of Palbociclib. However, the level of CDKN2A in 20 µM group did not support our hypothesis. We speculated that the dose of Palbociclib (20 µM) was overdosed for the GH3 cell line. In summary, IC50 value of CDK4/6 inhibitor is 23 nM to 10 µM in several cell lines including MU-4, MCF7, WM2664, BV173 and H69 (37).

In this study, we identified the lower level of CDKN2A in GH3 cell line compared to that in the GT1-1 cell line based on the western blot experiment. Palbociclib showed a more potent inhibition of the level of CDK4, cell proliferation, and cell cycle in the GH3 cell line compared to the GT1-1 cell line. There was a negative correlation between CDKN2A and CDK4 in SOMA specimens. Future study aims to examine the RNAi-CDKN2A and plasmid overexpression in in vitro/vivo experiments, which can enhance the results of anti-CDKN2A antibody blockade in the current study. Palbociclib-combined SSAs should be a potent strategy in SOMA patients with low CDKN2A level. Anderson et al reported that metastatic breast cancer combined with non-functional adenaoma was treated with Palbociclib, and routine imaging demonstrated significant regression of pituitary adenaoma after one-year treatment (38).

Furthermore, we found nearly one-third of SSTR2-positive cases in GONA, which provided the evidence of 35±13% control ratio in non-functioning pituitary tumors by SSTR2 agonists (1). Combined with a low level of CDKN2A in GONA, Palbociclib-combined SSAs should be evaluated in future clinical trials. Lack of animal experiments and non-availability of biomolecular details were some of the limitations of the current study. For DNA hypomethylation and chromosomal instability in SOMA, the epigenetic signature of the INK4 family should be explored in future research.

In conclusion, correlations between CDKN2A and tumor biological behaviors were established in SOMA. Furthermore, nearly one-third of GONA had a positive SSTR2 expression. CDKN2A expression involved the inhibition of cell proliferation and GH/IGF-1 secretion induced by CDK4/6 inhibitor, and Palbociclib in in vitro experiments. Therefore, we suggest the combination of CDK4 inhibitor and SSAs, can be used as potential therapeutic candidates for targeting residual SOMA or GONA with high CDKN2A expression.

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Availability of data and materials

We agree that all datasets on which the conclusions of the manuscript rely to be either deposited in publicly available repositories (where available and appropriate) or presented in the main paper or additional supporting files, in machine-readable format (such as spreadsheets rather than PDFs) whenever possible.

Authors’ contributions

YC was involved in IHC experiments and writing the manuscript. ZL was responsible for cell functional experiments. QF conducted the PCR experiments. HW was responsible for performing cell culture and functional experiments. CL carried out RNA-seq and data analysis. HG was involved in data collection and statistical analysis thereof. YZ designed the protocol and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Informed consent was obtained from all individuals and ethical approval was obtained from the Institutional Review Board of Beijing Tiantan Hospital Affiliated to Capital Medical University (KY2013-015-02).

Patient consent for publication

All patients signed the informed consent.

Competing interests

The authors declare that they have no competing interests.

References


