Long non-coding RNA HCG22 inhibits the proliferation, invasion and migration of oral squamous cell carcinoma cells by downregulating miR-425-5p expression

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Abstract. Long non-coding RNA (lncRNA) HLA complex group 22 (HCG22) is known to be involved in the occurrence and development of cancer; however, its role in oral squamous cell carcinoma (OSCC) remains unclear. Therefore, the main aim of the present study was to investigate the role and mechanisms of action of lncRNA HCG22 in OSCC cells. The expression levels of lncRNA HCG22 and microRNA (miR)-425-5p in OSCC cells were assessed using reverse transcription-quantitative PCR analysis. Cell proliferation was detected using Cell Counting Kit-8 and colony formation assays. In addition, the expression levels of cell proliferation-related proteins, p27, cyclin E and cyclin-dependent kinase 2, were detected by western blot analysis. The cell invasive ability was detected by Transwell assay, while the cell migratory ability was detected via a wound healing assay. The expression levels of the invasion- and migration-related proteins, MMP2 and MMP9, were measured by western blot analysis. The targeted association between lncRNA HCG22 and miR-425-5p was verified by RNA immunoprecipitation and dual-luciferase reporter assays. The results revealed that lncRNA LCG22 was expressed at low levels, while miR-425-5p was highly expressed in OSCC cell lines, based on bioinformatics analysis. The overexpression of lncRNA HCG22 inhibited the proliferation, invasion and migration of OSCC cells. Moreover, lncRNA HCG22 and miR-425-5p were found to have a direct targeted association, and lncRNA HCG22 inhibited cell proliferation, invasion and migration by targeting miR-425-5p. Collectively, the findings of the present study demonstrated that lncRNA HCG22 may inhibit the proliferation, invasion and migration of OSCC cells by downregulating miR-425-5p expression.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignant tumors of the oral and maxillofacial region. It mostly occurs in adults aged 40-60 years, and is characterized by lymph node metastasis and highly aggressive local spread (1,2). The exact cause of OSCC remains unclear. Although smoking, alcohol consumption, thermal injury due to the consumption of hot foods, chronic inflammation, genetic susceptibility and human papillomavirus infection are all risk factors for the development of OSCC, the main known factors promoting OSCC development are smoking and alcohol consumption, both of which exert a potent synergistic effect on the development of oral cancer (3,4). OSCC is difficult to detect at an early stage and the disease is usually already at an advanced or metastatic stage at diagnosis. The effects of chemotherapy, radiotherapy and surgery are often unsatisfactory, and the 5-year survival rate is <60% (5). At present, the treatment of OSCC is based on surgery, combined with radiotherapy and chemotherapy, to provide targeted comprehensive treatment for the patients (6). In addition, in recent years, biological treatments based on cytokines, such as IFN-α and IL-2, as well as adjuvant treatments, such as traditional Chinese medicine and cryotherapy, have emerged; however, their therapeutic efficacy and associated prognosis remain unsatisfactory (7).

Long non-coding RNA (lncRNAs) are a class of non-protein-coding RNAs, >200 bp in length, that play an important role in a number of biological processes, such as gene transcription, post-transcriptional regulation, splicing/modification and the regulation of protein synthesis (8). The expression of lncRNAs is highly specific, and their expression varies greatly among different tissues. The abnormal expression of lncRNAs is closely associated with tumor occurrence, development and prognosis (9,10). It was previously found that a number of lncRNAs can also play a suppressive or promoting role in OSCC (11). The expression level of lncRNA HOX transcript antisense RNA (HOTAIR)
was found to be significantly increased in OSCC tissues, and interfering with HOTAIR expression can inhibit the proliferation, invasion and migration of OSCC cells (12). In a previous study, IncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was shown to be upregulated in OSCC tissues compared with normal tissues from healthy subjects, and it was proven that MALAT1 maintained epithelial-to-mesenchymal transition (EMT)-mediated cell migration and invasion. Following interference with MALAT1 expression, the level of EMT in OSCC cells decreased and tumor growth was inhibited in mice (13,14).

HLA complex group 22 (HCG22) is a mucin gene that is involved in the progression of a number of human diseases, such as steroid-induced intraocular hypertension and late-onset asthma (15,16). Through bioinformatics analysis, researchers have found that IncRNA HCG22 is involved in esophageal squamous cell carcinoma (17), head and neck squamous cell carcinoma (18), thyroid (19) and cervical cancer (20), as well as in several other types of cancer. IncRNA HCG22 has been shown to be downregulated in bladder cancer tissues according to The Cancer Genome Atlas database, and it has been shown to exert a significant inhibitory effect on the proliferation and metastasis of bladder cancer cells, and to exert tumor-suppressive effects by targeting poly pyrimidine tract-binding protein 1 (21). Li et al (22) used a variety of bioinformatics analysis methods to screen out IncRNA HCG22 related to the clinical characteristics of esophageal squamous cell carcinoma (ESCC); the clinicopathological data revealed that the expression level of IncRNA HCG22 was significantly associated with the degree of ESCC differentiation, and it inhibited tumor cell migration by targeting serine peptidase inhibitor Kazal type and ADAM metallopeptidase with thrombospondin domain 1 motif 12. In addition, previous studies have demonstrated that the expression level of IncRNA HCG22 in OSCC tissues was significantly downregulated and that this was associated with a lower survival rate of patients with OSCC (23,24).

However, the mechanisms through which IncRNA HCG22 affects the occurrence and development of OSCC have not yet been reported, at least to the best of our knowledge. Thus, the aim of the present study was to examine the role of IncRNA HCG22 in OSCC and to elucidate the underlying mechanisms.

Materials and methods

Cells and cell culture. OSCC cell lines, including CAL-27 (cat. no. CRL-2095), SCC-25 (cat. no. CRL-1628) and SCC-9 (cat. no. CRL-1629), were purchased from the American Type Culture Collection, and human oral epithelial cells (HOECs; cat. no. BNCC340217) were purchased from BeNa Culture Collection (Beijing, China). All cells were cultured in DMEM (Thermo Fisher Scientific, Inc.) containing 10% FBS (Thermo Fisher Scientific, Inc.) containing 1% antibiotics, and 1% glucose. All cell culture was performed at 37˚C in a humidified atmosphere with 5% CO₂.

Cell transfection. The full length of IncRNA HCG22 was synthesized by RiboBio Co., Ltd. and cloned into the pcDNA 3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.) to establish IncRNA HCG22 overexpression vector (pc-HCG22). The pcDNA 3.1 vector (pcDNA3.1) was used as a negative control for overexpression vectors. microRNA (miRNA/miR)-425-5p mimics (forward, 5′-AAUGACACGACUCUCGGUGUA-3′ and reverse, 5′-AACGGAGUGACUGUCAUUU-3′) and its corresponding negative control (miR-NC; forward, 5′-UUCUCC GAACUGUCACGUTT-3′ and reverse, 5′-UGUGACACGUG CGGAGAAT-3′) were provided by Guangzhou RiboBio Co., Ltd. CAL-27 cells were transfected with pc-HCG22 (15 nM), pcDNA3.1 (15 nM), miR-425-5p mimic (40 nM) or miR-NC (40 nM) using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.) following 48 h of transfection at 37°C, reverse transcription-quantitative PCR (RT-qPCR) analysis was performed to confirm the transfection efficiency.

RT-qPCR. TRIzol® reagent (Vazyme Biotech Co., Ltd.) was used to extract total RNA from CAL-27 cells. RNA was then quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). RNA was reverse transcribed into cDNA using a reverse transcription kit (cat. no. R222-01; Vazyme Biotech Co., Ltd.) according to the manufacturer’s instructions. RT-qPCR was performed using a PCR system (cat. no. 4364346; Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions. The following primer pairs were used for qPCR: HCG22: Forward, 5′-ATT GGG TGT TTT AGC CCC CT-3′ and reverse, 5′-AUG UGA CAC GUU GA-3′; GAPDH: Forward, 5′-AAC TTT GGC ATT GTG GAA GG-3′ and reverse, 5′-GGA TCG TGC AGT GAC A -3′; and U6: Forward, 5′-AATT TGG GTT TTG TTGA CCCCCT-3′ and reverse, 5′-AGCTGGTGTGTCAGGAGGTAG-3′; miR-425-5p: Forward, 5′-TGGCGGAAATGACACGATCACTCCG-3′ and reverse, 5′-CCAGTGACGGGGTCCAGGTT-3′; GAPDH: Forward, 5′-AAG TTT GGA GAG AAG GAA GAA G-3′ and reverse, 5′-GGA TCG AGG GAG TAT GAT G-3′; and U6: Forward, 5′-TGGGTCTCCTGCTTCGGCAGC-3′ and reverse, 5′-CCAGTGACGGGGTCCAGGTT-3′. The amplification parameters were as follows: Denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min. The 2−ΔΔCq method was used to calculate relative changes in gene expression (25). GAPDH and U6 served as the internal reference genes.

Western blot analysis. CAL-27 cells were lysed in RIPA buffer (Beyotime Institute of Biotechnology) containing protease inhibitors, phosphatase inhibitors and PMSF. Total protein was quantified via a BCA Protein Assay kit (Beyotime Institute of Biotechnology) containing protease inhibitors. Total protein was sonicated and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions. Following 48 h of transfection at 37°C, reverse transcription-quantitative PCR (RT-qPCR) analysis was performed to confirm the transfection efficiency.

Cell Counting Kit-8 (CCK-8) assay. Cell viability was detected using a CCK-8 assay. The cells were cultured in 96-well plates until reaching 80% confluency. Subsequently,
10 µl CCK-8 reagent (Beijing Solarbio Science & Technology Co., Ltd.) were added to each well. The cells were incubated with CCK-8 reagent for 1-4 h. The optical density value at 450 nm was detected using a microplate reader (Bio-Rad Laboratories, Inc.).

Colonies formation assay. The CAL-27 cells were placed in six-well plates with a density of 200 cells/well and cultured in DMEM at 37˚C for 14 days. The cells were then fixed with methanol for 15 min at room temperature and stained with 0.1% crystal violet solution for 20 min at room temperature. Finally, the colonies (>50 cells) were counted using ImageJ software (version 1.52; National Institutes of Health) and images were obtained under a light microscope at low magnification (x4).

Transwell assay. The invasive ability of the CAL-27 cells was measured using a Transwell chamber with pore size of 8.0-µm (MilliporeSigma). The cells (1x10⁵ cells/ml) were resuspended in serum-free DMEM and then were cultured (200 µl) cultured in the upper chambers of the Transwell chamber pre-coated with Matrigel for 30 min at 37°C. The lower chamber was filled with DMEM containing 10% FBS. The cells were cultured at 37°C for 24 h and were then fixed with 4% paraformaldehyde for 20 min at room temperature and stained with 0.5% crystal violet solution (Beijing Solarbio Science & Technology co., Ltd.) for 20 min at room temperature. Finally, the fixed cells were counted using an Olympus optical microscope (magnification, x100).

Wound healing assay. CAL-27 cells (1x10⁵ cells/well) were seeded into six-well plates overnight. To create a cell-free clear zone, the 100% confluent monolayer was scratched using a plastic apparatus (26). The cells were then incubated in DMEM without FBS. The wound distance was examined using a plastic apparatus (26). The colonies (>50 cells) were counted using ImageJ software (version 1.52; National Institutes of Health) and images were obtained under a light microscope at low magnification (x4).

Nuclear and cytoplasmic fractionation assays. In order to examine the location of HCG22, nuclear and cytoplasmic fractionation assays were carried out using a nuclear/cytosol fractionation kit (Cell Biolabs, Inc.) according to the manufacturer’s instructions.

RNA immunoprecipitation (RIP) assay. A Magna RIP assay kit (MilliporeSigma) was used to assess the association between lncRNA HCG22 and miR-425-5p. CAL-27 cells were washed with ice-cold PBS and lysed on ice with RIP lysis buffer. The lysates were incubated with magnetic beads conjugated to IgG or Argonaute-2 (Ago2; MilliporeSigma). The RNA was then extracted and purified using a RNeasy MinElute Cleanup Kit (Qiagen, Inc.). Finally, the level of RNA was quantified by RT-qPCR.

Dual-luciferase reporter assay. The interaction between lncRNA HCG22 and miR-425-5p was predicted by StarBase website (http://starbase.sysu.edu.cn/), and was then verified via a dual-luciferase reporter assay. lncRNA HCG22 wild-type or with site-directed mutation in the miR-425-5p binding site were subcloned into the pmirGLO vector (Promega Corporation) and co-transfected into CAL-27 cells with miR-425-5p mimic or miR-NC using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions (27). At 48 h post-transfection, the expression and activity of lncRNA HCG22 were determined by the luciferase activity. The luciferase activity was assessed using a dual-luciferase reporter assay kit (Promega Corporation), and the relative luciferase activity was normalized to Renilla luciferase activity.

Statistical analysis. Data are expressed as the mean ± SD. GraphPad Prism 8.0 software (GraphPad Software, Inc.) was used to analyze the data. Differences between two or more groups were estimated using unpaired Student's t-test or one-way ANOVA followed by Tukey's post hoc test, respectively. P<0.05 was considered to indicate a statistically significant difference. All experiments were carried out at least three times.

Results

Overexpression of lncRNA HCG22 inhibits the proliferation of OSCC cells. In OSCC cell lines, the expression level of lncRNA HCG22 was significantly decreased compared with that in HOECs (Fig. 1A). In order to examine the effect of lncRNA HCG22 on the proliferation of OSCC cells, cells overexpressing lncRNA HCG22 were constructed (Fig. 1B). The results of the CCK-8 assay revealed that cell proliferation was significantly decreased following overexpression of lncRNA HCG22 (Fig. 1C). The results of the colony formation assay also demonstrated that the proliferation of OSCC cells was significantly decreased following overexpression of lncRNA HCG22 (Fig. 1D and E). In addition, the expression levels of the cell proliferation-related proteins, CDK2, cyclin E and p27, were detected by western blot analysis (Fig. 1F and G). The results indicated that the overexpression of lncRNA HCG22 significantly inhibited the expression of cell proliferation-related proteins. The aforementioned results suggest that the overexpression of lncRNA HCG22 inhibits the proliferation of OSCC cells.

Overexpression of lncRNA HCG22 inhibits the invasion and migration of OSCC cells. The results of the Transwell assay revealed that the overexpression of lncRNA HCG22 inhibited the invasion of CAL-27 cells (Fig. 2A and B). The results of the wound healing assay demonstrated that the overexpression of lncRNA HCG22 inhibited the migration of CAL-27 cells (Fig. 2C and D). The expression levels of the invasion- and migration-related proteins, MMP2 and MMP9, were also decreased when lncRNA HCG22 was overexpressed, as determined by western blot analysis (Fig. 2E and F). These results suggested that the overexpression of lncRNA HCG22 inhibits cell invasion and migration.

lncRNA HCG22 directly interacts with miR-425-5p in OSCC cells. Nuclear and cytoplasmic fractionation assays indicated that lncRNA HCG22 was mainly located in the cytoplasm (Fig. 3A) and was enriched in the Ago2 complex (Fig. 3B). Thus, it was hypothesized that lncRNA HCG22 functioned by targeting miRNAs. Based on the StarBase website (http://starbase.sysu.edu.cn/), miR-425-5p was found to be the target gene of lncRNA HCG22 (Fig. 3C).
In addition, the expression level of miR-425-5p in CAL-27 cells was significantly increased following transfection with miR-425-5p mimics (Fig. 3D). After the binding sites between lncRNA HCG22 and miR-425-5p were mutated, wild-type and mutant-type luciferase reporter vectors (Fig. 3C) were constructed and transfected into OSCC cells to detect luciferase activity. The results revealed that the luciferase activity was significantly decreased following lncRNA HCG22 mutation (Fig. 3E). In addition, the expression of miR-425-5p in different OSCC cell lines was found to be significantly increased compared with that in HOECs (Fig. 3F). Following overexpression of lncRNA HCG22, the expression of miR-425-5p was significantly decreased (Fig. 3G). When miR-425-5p mimics were transfected into CAL-27 cells, the expression of lncRNA HCG22 was significantly decreased (Fig. 3H). The aforementioned results indicated that lncRNA HCG22 directly interacts with miR-425-5p in OSCC cells.

InceRNA HCG22 inhibits the proliferation of OSCC cells by targeting miR-425-5p. The results of the CCK-8 and colony formation assays revealed that cell proliferation was significantly increased when the miR-425-5p mimic was transfected into CAL-27 cells (Fig. 4A-C). However, the overexpression of lncRNA HCG22 inhibited cell proliferation (Fig. 4A-C). When miR-425-5p mimic and lncRNA HCG22 overexpression plasmids were transfected into CAL-27 cells, cell
proliferation was increased compared with that of lncRNA HCG22-overexpressing cells (Fig. 4A-C). The results of western blot analysis focusing on the protein expression of CDK2, cyclin E and p27 were consistent with those of the CCK-8 and colony formation assays (Fig. 4D and E). The aforementioned results indicated that lncRNA HCG22 inhibited cell proliferation by targeting miR-425-5p.

**lncRNA HCG22 inhibits the invasion and migration of OSCC cells by targeting miR-425-5p.**

Cell invasion and migration were assessed using Transwell and wound healing assays, respectively (Fig. 5A-D). The results of the Transwell assay revealed that cell invasion was significantly increased when miR-425-5p mimic was transfected into CAL-27 cells; however, the overexpression of lncRNA HCG22 inhibited cell invasion (Fig. 5A and C). When miR-425-5p mimic and lncRNA HCG22 overexpression plasmids were transfected into cells, cell invasion was increased compared with that of the lncRNA HCG22-overexpressing cells (Fig. 5A and C). The results of the wound healing assay revealed that cell migration was significantly increased when miR-425-5p mimic was transfected into CAL-27 cells; however, the overexpression of lncRNA HCG22 inhibited cell migration (Fig. 5B and D). When miR-425-5p mimic and lncRNA HCG22 overexpression plasmids were transfected into cells, cell migration was increased compared with that of lncRNA HCG22-overexpressing cells (Fig. 5B and D). The conclusions drawn from the expression analysis of the invasion- and migration-related proteins, MMP-2 and MMP-9, were consistent with the results of the Transwell and wound healing assays (Fig. 5E and F). The aforementioned results indicated that lncRNA HCG22 may inhibit cell invasion and migration by targeting miR-425-5p.

Figure 2. Effect of lncRNA HCG22 overexpression on the invasion and migration of CAL-27 cells. (A and B) Cell invasion was detected using Transwell assay following overexpression of lncRNA HCG22 in CAL-27 cells. (C and D) Cell migration was assessed using wound healing assay following overexpression of lncRNA HCG22 in CAL-27 cells. (E and F) Western blot analysis was used to measure the levels of the cell invasion- and migration-related proteins, MMP-2 and MMP-9. Magnification, x100. **P<0.01, ***P<0.001 vs. pcDNA3.1. lncRNA HCG22, long non-coding RNA HLA complex group 22.
Discussion

OSCC is a common malignant tumor that severely affects the quality of life of the patients and poses a major socioeconomic burden. In-depth investigations into the pathogenesis, early detection and diagnosis of OSCC, the implementation of scientific interventions and treatment strategies, and the reduction in the incidence and mortality of OSCC, have
import clinical and social implications. The present study investigated the effects and underlying mechanisms of lncRNA HCG22 in OSCC, and found that lncRNA HCG22 was expressed at low levels in OSCC cells, while miR-425-5p was highly expressed. The effects of the overexpression of lncRNA HCG22 on OSCC cell proliferation, invasion and migration were observed, and the targeted association between lncRNA HCG22 and miR-425-5p was verified in the present study.

miRNAs are small non-coding RNAs, the main function of which is to participate in post-transcriptional gene regulation. miRNAs regulate transcription and translation by binding to the complementary sequence of the 3′-untranslated region of target mRNAs. In addition to lncRNAs, it has been found that miRNAs are also involved in various biological stages of tumor development, including tumor cell proliferation, apoptosis, migration, adhesion and other important cellular activities (28). Notably, it has been found that the interaction between miRNAs and lncRNAs plays a crucial role in tumor regulation. lncRNAs can be used as competing endogenous RNAs that bind to miRNAs, thereby regulating the expression of target genes (29). There is evidence to support that some miRNAs are also abnormally expressed in OSCC. miR-26a/b expression was found to be significantly downregulated in OSCC, and the presence of miR-26a/b inhibits tumor cell invasion and migration (30). In addition, high expression of miR-1275 and miR-144 was found to be closely associated with the occurrence and development of OSCC (31).

Figure 4. lncRNA HCG22 inhibits the proliferation of CAL-27 cells by targeting miR-425-5p. (A) Cell proliferation was measured by Cell Counting Kit-8 assay. ***<i>P</i>&lt;0.001 vs. miR-NC; ###<i>P</i>&lt;0.001 vs. pcDNA3.1; Δ<i>P</i>&lt;0.05 vs. miR-425-5p mimics. (B and C) Cell colony formation was examined by colony formation assay. (D and E) Western blot analysis was used to measure the protein expression of CDK2, cyclin E and p27. *<i>P</i>&lt;0.05, **<i>P</i>&lt;0.01, ***<i>P</i>&lt;0.001. lncRNA, long non-coding RNA HLA complex group 22; CDK, cyclin-dependent kinase.
In recent years, preclinical studies and clinical trials have demonstrated that miR-425-5p plays an important role in several tumors. miR-425-5p expression has been found to be elevated in a variety of tumor tissues, such as colorectal cancer (32), cervical cancer (33), hepatocellular carcinoma (34) and gastric cancer (35). In addition, it has been reported that miR-425-5p is abnormally expressed in a variety of squamous cell carcinomas. Wang et al (36) compared the expression of miRNAs among lung squamous cell carcinoma tissues, adjacent tissues and normal tissues, and found that the expression...
level of miR-425-5p was significantly higher in cancer tissues compared with that in normal tissues. In addition, miR-425-5p expression in the blood plasma of patients was found to be therapy-responsive, and it was downregulated in primary head and neck squamous cell carcinoma cell cultures following radiochemotherapy (37). These aforementioned findings suggest that miR-425-5p may serve as a target for the diagnosis and treatment of squamous cell carcinoma and as a molecular marker for prognosis. However, the role of miR-425-5p in OSCC and the associated mechanisms have not yet been reported, to the best of our knowledge.

In the present study, miR-425-5p was found to be highly expressed in OSCC tissues. However, the role of miR-425-5p in the occurrence and development of OSCC must be further determined. It must also be determined whether there is an association between this miRNA and IncRNA HCG22. Based on the results obtained, it was hypothesized that IncRNA HCG22 may inhibit the proliferation, invasion and migration of OSCC cells by promoting the downregulation of miR-425-5p. In order to verify this hypothesis, research was conducted at the cellular and molecular levels to explore the effects of IncRNA HCG22 and miR-425-5p on OSCC, and to verify the targeting association between IncRNA HCG22 and miR-425-5p. The results revealed that overexpression of IncRNA HCG22 exerted an inhibitory effect on cell proliferation, cell viability and colony formation ability, downregulated the expression of CDK-2 and cyclin E, and upregulated p27. p27 is a type of CDK inhibitor that regulates cell cycle progression, thereby affecting cell proliferation (38). In addition, overexpression of IncRNA HCG22 also exerted strong inhibitory effects on cell migration and invasion. However, these inhibitory effects of IncRNA HCG22 were partly abolished by miR-425-5p, verifying our hypothesis that IncRNA HCG22 may inhibit the proliferation, migration and invasion of OSCC cells via targeting miR-425-5p.

In conclusion, the elucidation of the pathogenesis of OSCC is crucial for identifying novel therapeutic targets and prognostic molecular markers. The results of the present study demonstrated that IncRNA HCG22 may serve as a molecular marker for the diagnosis of OSCC, as well as a therapeutic target. The biological reagents developed around this gene are expected to resolve certain issues associated with OSCC prevention and treatment, and their clinical application is expected to be associated with significant socioeconomic benefits.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XC was responsible for the conception and design of the study; YF and YL were involved in data acquisition; YF, AN and QW were involved in the development of the study methodology, analysis and interpretation of the data. XC, YF and YL were involved in the writing, reviewing and revision of the article and analyzed the relevant literature. All the authors have read and approved the final article. XC and YF confirmed the authenticity of the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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