Increased expression of TNFRSF14 indicates good prognosis and inhibits bladder cancer proliferation by promoting apoptosis

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Abstract. Despite advances in management, bladder cancer remains a principal cause of cancer-associated complications. Tumor necrosis factor receptor superfamily member 14 (TNFRSF14) is dysregulated in certain types of cancer; however, limited data are available on the expression and function of TNFRSF14 in bladder cancer. In the present study, the aim was to evaluate the expression and biological functions of TNFRSF14 in bladder cancer. Firstly, the expression levels of TNFRSF14 in bladder cancer tissue were examined using The Cancer Genome Atlas (TCGA) database. Secondly, reverse transcription-quantitative polymerase chain reaction was utilized to investigate the expression levels of TNFRSF14 in the T24, SW780 and EJ-M3 bladder cancer cell lines. Transfection and Cell Counting kit-8 (CCK-8) assay was used to evaluate whether TNFRSF14 overexpression or silencing would have an effect on cell proliferation of T24 and EJ-M3 cells. In addition, TNFRSF14-induced apoptotic cells were identified using Annexin V-fluorescein isothiocyanate and propidium iodide staining. Western blot analysis was used to detect proteins associated with the phosphatidylinositol 3-kinase pathway. According to the TCGA dataset, the expression levels TNFRSF14 were decreased in bladder cancer tissue compared with in normal control samples. Patients with bladder cancer exhibiting low expression levels of TNFRSF14 had a worse prognosis compared to those with high expression levels of TNFRSF14. Overexpression of TNFRSF14 in T24 cells led to increased apoptosis and inhibited cell proliferation \textit{in vitro}. Western blotting demonstrated that TNFRSF14 overexpression increased the expression levels of caspase3-p17 in T24 cells, but significantly decreased the expression levels of phosphorylated (p)-protein kinase B (AKT) and P70 S6 kinase (P70). TNFRSF14 silencing in EJ-M3 cells enhanced cell growth, inhibited cell apoptosis, increased the expression levels of p-AKT and P70, and decreased the expression levels of caspase3-p17. In conclusion, TNFRSF14 may serve a tumor suppressive role in bladder cancer by inducing apoptosis and suppressing proliferation, and act as a novel prognostic biomarker for bladder cancer.

Introduction

Bladder cancer is the fourth most common cancer in men and the ninth most common cancer in women worldwide (1). The majority of bladder cancers are urothelial cell carcinoma, which originate from the epithelial lining of the bladder wall (2). More than half of patients with bladder cancer are diagnosed with advanced stage cancer and have very poor prognosis (3). Despite improvement in treatment of bladder cancer, the incidence of the disease is still increasing (4). Although environmental and genetic factors have been demonstrated to have important roles in the development of bladder cancer, the molecular mechanisms involved in the initiation and progression of the disease remain unclear. Investigating the underlying mechanisms of bladder cancer is instrumental for providing better treatment and development of novel therapeutic agents.

The tumor necrosis factor receptor superfamily member 14 (TNFRSF14) gene, also known as herpes virus entry mediator, is located on the short arm of chromosome 1p36 and encodes a type I trans membrane molecule that serves as a molecular switch by interacting with different ligands to regulate a series of immune responses (5). Activation of TNFRSF14 is involved in the development of various tumor types, and may be used for assessing prognosis as it is closely associated with cancer growth and metastasis (6,7). In a previous study, reduced TNFRSF14 expression was suggested to contribute to the pathobiology of classical Hodgkin lymphoma (8). Another previous study indicated that TNFRSF14 deficiency promotes development of follicular lymphoma \textit{in vivo} and establishes a favorable immune environment (9). Cheung \textit{et al} (10) reported that somatic TNFRSF14 mutations are associated with worse prognosis of follicular lymphoma. However, the expression levels of TNFRSF14 in other types of cancer types have been inconsistent. To the best of our knowledge, there is no published evidence on the expression of TNFRSF14 in bladder cancer tissues and its association with clinicopathological parameters remains poorly understood.
In the present study, the aim was to investigate the expression levels of TNFRSF14 in bladder cancer and evaluate its clinical significance. In addition, the molecular mechanism of TNFRSF14 in regulating progression of bladder cancer was examined. The results indicated that increased expression of TNFRSF14 inhibited bladder cancer proliferation by promoting apoptosis.

Materials and methods

Database. The Cancer Genome Atlas (TCGA) database was utilized to obtain clinical information and Level 3 RNA sequencing (RNA-Seq) data. The search terms used in TCGA contained the following keywords: ‘cases’, ‘primary site’, ‘bladder’ and ‘TCGA-BLCA’. Based on the above search terms, the clinical information and Level 3 RNA-Seq data were obtained from 404 bladder cancer samples and 28 normal bladder tissue samples (11). TNFRSF14 expression levels in normal and bladder cancer tissue were analyzed using the limma package (version 3.32.8) from Bioconductor (12).

Cell cultures. T24 and SW780 bladder cancer cell lines, and SV-HUC-1 normal human bladder epithelium cell line were purchased from the Shanghai Cell Bank at the Chinese Academy of Sciences (Shanghai, China). SV-HUC-1 cells were cultured in Corning™ cellgro™ F12K (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). T24 and SW780 bladder cancer cell lines, and SV-HUC-1 cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C. The EJ-M3 cell line was established from the EJ cell line, which has been demonstrated to be a T24 derivative/contaminants. EJ-M3 cells are essentially highly invasive T24 derivative cells (13) and were obtained from EJ cells (14). EJ-M3 cells were generated, according to the methods described by Girnita et al. (15). Briefly, 24-well plates were filled with media containing chemokine, and Transwell inserts coated with 100 µg Matrigel were placed into the wells and 3x10⁵ EJ cells were added onto the membrane. After 7 h in culture, the insert was removed and cells invading the lower chamber were further cultured. The medium was changed after 4-6 days. After reaching lamellar fusion, the cells were digested and subcultured. This process was repeated three times and each time the amount of Matrigel coating on the insert membrane was increased by 50 µg. Cells that were retained following this selection process were identified as highly invasive EJ-M3 cells. These cancer cells were cultured in RPMI-1640 (HyClone; GE Healthcare, Chicago, IL, USA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ until 70-80% confluence was reached. Non-adherent cells were washed away after 3 days of culture and adherent cells were fed with fresh complete medium. The cells were subcultured at a 1:2 split ratio after reaching confluence.

Transient transfection and small interfering RNA (siRNA) transfection. Cells were cultured in 6-well plates in medium without antibiotics for 24 h prior to transfection, resulting in 70-80% confluence. For transient transfection, the TNFRSF14 gene was subcloned into a pSG5-HA vector, generating pSG5-HA-TNFRSF14. The empty vector was used as a negative control for transfection. Cells were transfected by Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Specifically, cells were cultured in fresh medium without antibiotics, and when the T24 cells reached 70-80% confluence, 4 µg DNA (pSG5-HA-TNFRSF14) was combined with 250 µl Opti-MEM® medium (Invitrogen; Thermo Fisher Scientific, Inc.) and 10 µl Lipofectamine® 2000 was diluted in 240 µl Opti-MEM®, which were incubated at room temperature for 5 min. DNA/Opti-MEM® and Lipofectamine® 2000/Opti-MEM® were combined and incubated at room temperature for 20 min. A total of 500 µl plasmid/Lipofectamine® complex was added to the cells, which were cultured at 37°C. Following 24 h, the transfection medium was replaced with fresh complete medium and cells were harvested for analysis of proliferation and other experiments.

siRNA against TNFRSF14 (si-TNFRSF14; cat. no. stQ0003897-1) and negative control (si-NC; cat. no. stN5851522147) were produced and purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The negative control siRNA does not match any known mammalian GenBank sequences. Cells were seeded at a density of 3x10⁵/well in 6-well plates overnight and transfected with siRNA or si-NC at a final concentration of 100 nM using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following 24 h of transfection, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to determine transfection efficiency.

RNA extraction and RT-qPCR. RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was generated using PrimeScript RT reagent kit (cat. no. RR037A; Takara Biotechnology Co., Ltd., Dalian, China) at 37°C for 15 min. Synthesized first-strand cDNA was used as template, and GAPDH was applied for normalization. Primer sequences were used as follows: TNFRSF14 forward, 5′-CCA AGTGCAGTCCAGGTAT-3′ and reverse, 5′-ATTTGAGTG GCAATGTAGG-3′; GAPDH forward, 5′-GGTGTTGAC CATGAGATGTGA-3′ and reverse, 5′-GATGCTCTTCCAC GATAACAAAG-3′. The RT-qPCR reaction was performed at 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 60°C for 45 sec and 72°C for 30 min using an ABI 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Select Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The relative expression levels of TNFRSF14 were analyzed and normalized to GAPDH using the 2⁻ΔΔCq method (16).

Cell proliferation analysis. The Cell Counting kit-8 (CCK-8) assay is a sensitive and accurate method for determining cell viability. In the present study, cell proliferation was evaluated using the CCK-8 assay at 24, 48 and 72 h after transfection, according to a previous study (17). Briefly, cells in the TNFRSF14 or si-TNFRSF14 transfection group and control transfection group were cultured in 100 µl RPMI-1640 supplemented with 10% FBS in 96-well plates and incubated at 37°C.
for 3 days. Subsequently, CCK-8 dye (BestBio, Shanghai, China), diluted 1:10 in cell culture medium, was added to each well and incubated for 2 h at 37°C. The absorbance was read using a microplate reader set at 450 nm. The 450 nm optical density value is proportional to the total number of live cells and was used to assess cell viability in transfected vs. control transfected cells. Triplicate wells were used and experiments were repeated at least three times.

Western blot analysis. Total protein was extracted from transfected and control transfected cells using 1 mM phenylmethylsulfonfyl fluoride in 1 ml ice-cold radioimmuno-precipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China). Subsequently, protein concentration was measured using the bicinchoninic acid assay. Denatured proteins (20 µg) were separated using 12% SDS-PAGE and transferred onto Immobilon-P polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked for 2 h with 5% skimmed milk powder in Tris-buffered saline containing 0.1% Tween-20 (TBST) and incubated overnight at 4°C with the following primary antibodies: Anti-protein kinase B (AKT; 1:1,000; cat. no. SAB4500797), anti-phosphorylated (p)-AKT (1:1,000; cat. no. SAB4301414), anti-P70 S6 kinase (P70; 1:1,000; cat. no. SAB4502683), anti-active caspase3-p17 (1:1,000; cat. no. SAB4503294) and anti-GAPDH (1:5,000; cat. no. SAB2108266; Sigma-Aldrich; Merck KGaA). Subsequently, membranes were washed three times with TBST at room temperature, followed by incubation with an horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:5,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 2 h. Membranes were washed a final time with TBST. GAPDH was used as the loading control. All bands were detected using Enhanced Chemiluminescent Western Blotting Detection kit (GE Healthcare Life Sciences, Little Chalfont, UK). Imaging and quantification of protein bands was conducted using the Bio-Rad Quantity One 1-D Analysis software version 4.6.9 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Apoptosis assay. Following 24 h of transfection, cells were harvested by trypsinization, washed with pre-cooled PBS and fixed overnight with 70% ethanol at -20°C. T24 or EJ-M3 cells were washed twice, resuspended in PBS containing 0.1 mg/ml RNase A and 0.1% Triton X-100 in the dark for 30 min at 37°C. A total of 1x10⁵ cells were double stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) using the Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer’s protocol. The stained cells were acquired on a FACScan flow cytometer (BD Biosciences) equipped with a 488 nm argon laser and the data were analyzed using Cell Quest software version 6.0 (BD Biosciences). Cells were categorized into viable (Annexin V-FITC+/PI−), dead (Annexin V-FITC+/PI+), early apoptotic (Annexin V-FITC+/PI+) or apoptotic cells (Annexin V-FITC+/PI+). The percentage of apoptotic cells in the experiment group was compared with the control transfection group. All the samples were measured in triplicate.

Statistical analysis. In the present study, SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA) was used to conduct all statistical analyses. Each assay was performed at least three times. The data are presented as the mean ± standard deviation. The means between two groups were compared using Student’s t-test. One-way analysis of variance followed by Student-Newman-Keuls post hoc test was used to compare the means of multiple groups. The Kaplan-Meier method was used to evaluate the prognostic value of TNFRSF14 in bladder cancer, and the Mantel-Cox log-rank test was used to determine the statistical significance of difference between survival curves. P<0.05 was considered to indicate a statistically significant difference.

Results

Downregulation of TNFRSF14 expression levels in human bladder cancer tissues is associated with poor prognosis. The association between TNFRSF14 expression levels and prognosis of bladder cancer was assessed using RNA-Seq data from the TCGA database. The results indicated that TNFRSF14 expression levels were downregulated in the bladder tissues of patients with bladder cancer compared with healthy controls (Fig. 1A; P<0.05). Based on the median value of TNFRSF14 expression, patients were divided into TNFRSF14 low and TNFRSF14 high expression groups. As shown in Fig. 1B, the overall survival was significantly longer in the TNFRSF14 high expression group compared with in the TNFRSF14 low expression group.

TNFRSF14 expression levels are downregulated in bladder cancer cell lines. TNFRSF14 expression levels in three human bladder cancer cell lines (T24, SW780 and EJ-M3) and a normal human bladder epithelium cell line (SV-HUC-1) were measured by RT-qPCR. The results demonstrated TNFRSF14 expression levels were lower in all bladder cancer cell lines compared with in the SV-HUC-1 cell line (Fig. 2). Notably, the expression levels of TNFRSF14 in T24 cells were lower compared with in SW780 and EJ-M3 cells. Therefore, T24 cells were used to perform overexpression experiments, whereas EJ-M3 cells were used to conduct silencing assays.

CC-8 analysis of cell viability. To investigate the role of TNFRSF14 in T24 or EJ-M3 cell proliferation, overexpression and silencing experiments were conducted using pSG5-HA-TNFRSF14 and si-TNFRSF14, respectively. RT-qPCR analysis was conducted 72 h after transfection to measure transfection efficacy. The results in Fig. 3A indicated that TNFRSF14 expression levels were significantly increased in T24 cells after transfection (P<0.05). As demonstrated in Fig. 4A, TNFRSF14 expression levels were decreased in EJ-M3 cells after gene silencing.

Subsequently, the effect of TNFRSF14 overexpression on cell proliferation was evaluated using the CCK-8 assay at 24, 48 and 72 h after transfection. As demonstrated in Fig. 3B, TNFRSF14 transfection led to a reduction in cell viability of T24 cells, which was statistically significant compared with control transfection group after 72 h of transfection (P<0.05; Fig. 3B). The results indicated TNFRSF14 overexpression decreased cell proliferation of bladder cancer cells.

In addition, CCK-8 assay was used to measure the effect of TNFRSF14 silencing on cell proliferation of EJ-M3
cells after 24, 48 and 72 h. Compared with control transfected cells, TNFRSF14 silencing resulted in increased cell viability of EJ-M3 cells, particularly at 72 h after transfection (P<0.05; Fig. 4B). The results indicated that TNFRSF14 silencing increased cell proliferation of bladder cancer cells.

Apoptosis assay. To evaluate the effect of alterations in TNFRSF14 expression levels on apoptosis of T24 or EJ-M3 cells, Annexin V-FITC and PI double staining was utilized to detect apoptotic cells, including early (Annexin V-FITC+/PI−) and late apoptotic (Annexin V-FITC+/PI+) cells. As presented in Fig. 5, TNFRSF14 overexpression promoted increased apoptosis in transfected cells compared with in control cells (5.52 vs. 1; P<0.05), indicating that TNFRSF14 overexpression may have a direct therapeutic effect against the human T24 bladder cancer cell line. As demonstrated in Fig. 6, TNFRSF14 silencing decreased apoptosis in transfected cells compared with in control cells (0.51 vs. 1; P<0.05). The results suggested that suppression of cell viability following TNFRSF14 overexpression was associated with induction of cell apoptosis.

Discussion

Human bladder cancer is one of the most lethal human cancers worldwide. Systematic chemotherapy and surgery are the primary treatment options for bladder cancer (18,19); however, bladder cancer cells frequently develop drug resistance and ~50% of patients with advanced bladder cancer do not respond to chemotherapy. Chemopreventative drugs may
Figure 3. TNFRSF14 overexpression inhibits cell viability. (A) T24 cells were transfected with pSG5-HA-TNFRSF14 and TNFRSF14 expression levels were evaluated using reverse transcription-quantitative polymerase chain reaction. (B) Cell proliferation was measured by Cell Counting kit-8 assay. Each assay was performed at least three times. The data are presented as the mean ± standard deviation. *P<0.05 vs. NC. NC, negative control; OD, optical density; TNFRSF14, tumor necrosis factor receptor superfamily member 14.

Figure 4. TNFRSF14 silencing enhances cell viability. (A) EJ-M3 cells were transfected with si-TNFRSF14 and TNFRSF14 expression levels were evaluated using reverse transcription-quantitative polymerase chain reaction. (B) Cell proliferation ability was determined using Cell Counting kit-8 assay. Each assay was performed at least three times. The data are presented as the mean ± standard deviation. *P<0.05 vs. NC. NC, negative control; si, small interfering RNA; OD, optical density; TNFRSF14, tumor necrosis factor receptor superfamily member 14.

Figure 5. TNFRSF14 overexpression in T24 cells increases apoptosis. Flow cytometry dot plots of (A) control and (B) pSG5-HA-TNFRSF14-transfected T24 cells stained with Annexin V-FITC and propidium iodide, 72 h after transfection. The proportion of the cell number is presented in each quadrant. In each plot, Q1 denotes dead cells, Q2 represents late apoptotic cells, Q3 denotes early apoptotic cells and Q4 represents viable cells. (C) Relative quantification of apoptotic cells. Quantification of apoptotic cells in NC group set as 1. All experiments were performed in triplicate with three technical replicates. The data are presented as the means ± standard deviation. *P<0.05 vs. NC. NC, negative control; TNFRSF14, tumor necrosis factor receptor superfamily member 14.

Figure 6. TNFRSF14 silencing in EJ-M3 decreases apoptosis. Flow cytometry dot plots of (A) control and (B) transfected EJ-M3 cells stained with Annexin V-FITC and propidium iodide, 72 h after transfection. The proportion of the cell number is presented in each quadrant. In each plot, Q1 denotes dead cells, Q2 represents late apoptotic cells, Q3 denotes early apoptotic cells and Q4 represents viable cells. (C) Relative quantification of apoptotic cells. Quantification of apoptotic cells in NC group set as 1. All experiments were performed in triplicate with three technical replicates. The data are presented as the means ± standard deviation. *P<0.05 vs. NC. NC, negative control; si, small interfering RNA; TNFRSF14, tumor necrosis factor receptor superfamily member 14.
inhibit and delay the initiation of cancer types via various mechanisms, including anti-proliferation or pro-apoptosis. Notably, TNFRSF14 activation has been demonstrated to inhibit proliferation of adenocarcinoma cells (20) and regulate apoptosis (21), suggesting a potential tumor suppressive role. To the best of our knowledge, the role of TNFRSF14 in bladder cancer has not yet been examined, and investigating its underlying mechanisms of action may aid in the development of novel diagnostic and therapeutic approaches to bladder cancer.

In the present study, it was demonstrated that expression levels of TNFRSF14 were decreased in bladder cancer tissue compared with negative control tissue, using the TCGA database. Patients with bladder cancer exhibiting low expression levels of TNFRSF14 had poorer prognosis compared with those exhibiting high expression levels of TNFRSF14. Similarly, TNFRSF14 expression levels were additionally reduced in bladder cancer cell lines. Overexpression of TNFRSF14 in T24 cells stimulated apoptosis and inhibited proliferation in vitro, which may indicate the potential protective effects of TNFRSF14 in bladder cancer. Conversely, TNFRSF14 knockdown in EJ-M3 cells enhanced cell proliferation, inhibited cell apoptosis, increased the expression levels of p-AKT and P70, and decreased the expression levels of caspase3-p17. Overall, the results demonstrated that TNFRSF14 may act as a tumor suppressor gene in bladder cancer, which is in line with a previous study (20).

Apoptosis is a form of programmed cell death, which serves a key role in tissue homeostasis and development in multicellular organisms (22). An imbalance between cell proliferation and apoptosis may lead to fatal diseases, including cancer (23). In various cell-based models of cancer, cell death triggered by a stimulus may be switched off by suppressing control points in the cell death pathway; for example, inhibition of caspase activation (24). Activation of caspase-3 results in cleavage of numerous proteins, including poly(ADP-ribose) polymerase (PARP). PARP is a nuclear DNA-binding zinc finger protein,
which is important for DNA repair as well as other cellular processes, including cell differentiation, proliferation and apoptosis (25). Cleaved PARP is a primary indicator of apoptosis. In the present study, the results demonstrated that TNFRSF14 overexpression in T24 cells increased the expression levels of caspase-3 and corresponded to an increased number of apoptotic cells. Therefore, TNFRSF14 may block bladder cancer progression by increasing the expression levels of caspase-3, thereby promoting apoptosis and suppressing proliferation of bladder cancer cells.

The PI3K pathway has a pivotal role in cell growth, proliferation and survival (26), and is upregulated in a number of different types of cancer (27). Notably, the PI3K pathway is activated in bladder cancer (28). AKT occupies an important regulatory node in the PI3K pathway, below which the pathway branches significantly to influence a wide range of cellular processes that promote cell-cycle progression, cell growth and resistance to apoptosis. PI3K-AKT signaling has been demonstrated to have an important role in transforming growth factor β-mediated epithelial-to-mesenchymal transition (29), which is a key process in bladder cancer development (30). A previous study suggested that downregulation of p-AKT induces caspase-3-dependent apoptosis in cancer cells (31,32), which is consistent with the results obtained in the present study. TNFRSF14 overexpression significantly increased the expression levels of caspase-3, but significantly decreased the expression levels of p-AKT and P70 in T24 bladder cancer cells. Silencing of TNFRSF14 yielded opposite results. The results suggested that TNFRSF14-mediated apoptosis is regulated by a caspase-dependent cascade that blocks PI3K/AKT signaling and activates other specific signaling pathways.

In conclusion, to the best of our knowledge, this is the first study of its kind to investigate the prognostic value of TNFRSF14 in bladder cancer. The results indicated TNFRSF14 expression levels were decreased in bladder cancer tissue compared with normal control tissues using the TCGA database. Patients with bladder cancer exhibiting low expression levels of TNFRSF14 had worse prognosis compared with those exhibiting high expression levels of TNFRSF14. Therefore, TNFRSF14 may act as a tumor suppressor in bladder cancer and serve as a novel prognostic biomarker. Nevertheless, there are limitations to the present study. The association between TNFRSF14 expression levels and prognosis of bladder cancer was determined using RNA-Seq data from the TCGA database. Further studies using patient tissues are required to validate the present findings.

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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

YZ performed the experiments, analyzed the data and drafted the manuscript; M-YL conceived the study and revised the manuscript. All authors read and approved the final manuscript.

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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