Rad54L promotes bladder cancer progression by regulating cell cycle and cell senescence

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Abstract
Bladder cancer (BCa) is the most prevalent cancer of the urinary system, but its pathogenesis is still poorly understood. Several reports have suggested that gene damage repair is highly correlated with tumor development and drug resistance, in which homologous recombination repair gene Rad54L seems to play an important role, through yet unclear mechanisms. Therefore, this study stratified cancer patients by Rad54L expression in BCa tissue, and high Rad54L expression was associated with a poor prognosis. Mechanistically, we demonstrate that high Rad54L expression promotes abnormal bladder tumor cell proliferation by changing the cell cycle and cell senescence. In addition, this study also suggests that Rad54L may be associated with p53, p21, and pRB in BCa tissue. In summary, this study exposes Rad54L as potential a prognostic biomarker and precision treatment target in BCa.

Keywords Bladder cancer · Rad54L · Cell cycle · Senescence

Introduction
Bladder cancer (BCa), the seventh most common male cancer in the world, leads in prevalence and mortality rate of urinary system pathologies [1]. In 2021, there were more than 83,000 patients newly diagnosed with BCa in the United State, of which about 75% were male. Moreover, in the same year, a total of 17,200 patients died because of BCa [2]. Recent studies have shown that some DNA methylation-based urine assays facilitate early detection and risk stratification of BCa [3, 4]; however, the 5-year overall survival rate for patients with muscle-invasive BCa is dismal 5–30%. What is more, in the muscular invasive urothelial carcinoma patients, the tumor will have a high possibility of invading muscle while accompanied by lymph node and surrounding tissue metastasis. On such occasion, the treatment usually includes complete bladder resection and the combination of chemotherapy and radiotherapy. Regardless, the prognosis is generally poor and about 10–60% of patients will have a relapse and distant organ metastases within 12 months after the treatment [5]. The exact mortality rate of BCa varies according to different countries, regions, detection, and treatment methods. In some remote areas, the mortality rate increases due to lack of early-stage detection and access to effective treatments [1].

In most cases, the active DNA repair mechanism can be a double-edged sword. While it assures the stability of gene and the DNA damage repair in life, it also promotes the occurrence and development of tumors [6]. Anti-tumor drugs and radiotherapy can both lead to different degrees of single-strand DNA (ssDNA) gaps and double-strand DNA (dsDNA) breaks to inhibit the growth of tumor [6]. However, the highly active homologous recombination repair (HRR), and expression of related proteins in tumor,
A huge number of cyclins have single-nucleotide polymorphic biomarker for BCa. Therefore, here we assessed the role of Rad54L in meiotic cell cycle, DNA repair, and other important processes. Blue nodes represent downregulated DEGs while red nodes represent upregulated DEGs. Color transparency represents logFC value. e 48 core genes were imported into Metascape web for functional enrichment analysis and KEGG pathway enrichment analysis. d The potential regulatory mechanisms of Rad54L were investigated via GeneMANIA. Edges of the network stand for interaction between gene pairs while color-filled nodes stand for participation in a pathway. e Rad54L expression in BCa or normal bladder tissue was analyzed from TCGA, Y: mRNA level.

According to previous reports, the DNA repair system is strongly correlated with the uncontrolled cell cycle in tumors. In BCa, heterogeneous expression of DNA damage repair proteins can alter the response of cell cycle checkpoints, such as G1 and G2 checkpoints, and this process can accelerate the development of bladder tumors [8]. Interestingly, many reports have also indicated that a huge number of cyclins have single-nucleotide polymorphisms and abnormal expression in tumor cells. Similarly, deficiency of cell cycle suppressor genes in tumor like P53, BRCA1, or p1, can also induce gene instability and failure of cell cycle checkpoint, which finally results in the malignant proliferation of tumor cells [9, 10]. So far, many cyclins, such as P21, Cyclin B1, or Aurora-A have been explored as target genes for cancer therapy, some already in clinical trials with promising outcomes [11–13].

Rad54 is an indispensable protein in HRR belonging to the Snf2-family and exerts the function of DNA ATPase activity in DNA repair. Rad54L, which shares similarity with Rad54, facilitates the specific dsDNA structure formation and can effectively repair damaged DNA in combination with Rad51 and translocation of damaged dsDNA [14]. Besides, Rad54L can also participate in homologous recombination repair of gene damage while regulating the process of meiosis. Therefore, Rad54L may be an important protein in tumorigenesis and Rad54L-deficient cells show significantly increased cell death due to DNA damage. Additionally, Rad54L has already been accessed as a key component of BCa prognosis [15]. Previous bioinformatic analysis performed in our lab showed that high RAD54L expression in bladder tumors can affect the development of disease by participating in cell division, meiotic cell cycle, DNA repair, and other important processes. Therefore, here we assessed the role of Rad54L in BCa, hoping to find a new therapeutic target and diagnostic biomarker for BCa.

**Result**

Identification of significant genes in BCa through bioinformatical analysis

Through Gene Expression Omnibus (GEO2R) database (https://www.ncbi.nlm.nih.gov/geo/), we acquired 828, 925, and 4775 differentially expressed genes (DEGs) from GSE37817, GSE42089, and GSE38264, respectively. Venn diagram analysis identified commonly DEGs in these three datasets, which resulted in 58 upregulated genes (logFC > 1; Fig. 1a) and 175 downregulated genes (logFC < −1; Supplementary Fig. 1a) in BCa tissues.

All 233 DEGs were analyzed by STRING online database (https://string-db.org/). A total of 233 DEGs were imported into the PPI network complex resulting in 206 nodes and 2094 edges, including 155 downregulated and 51 upregulated genes (Fig. S1b, c). We then applied Cyto-type MCODE app for further analysis and acquired 38 core genes (Table S1) and 10 functional related genes (Fig. 1b).

Metascape online tool (https://metascape.org) was subsequently employed to analyze the 38 core genes. The results indicated that the core genes were mainly enriched in cell division, nuclear division, microtubule cytoskeleton organization, cell cycle, meiotic cell cycle process, and DNA conformation change (Fig. 1c; Supplementary Table 1). Among them, Rad54L was involved in nuclear division, DNA damage repair, cell cycle, and other processes, which play an important role in the occurrence and development of BCa [16, 17]. GeneMANIA (http://genemania.org/) was then employed for probing the potential regulatory mechanisms of Rad54L. As shown in Fig. 1, Rad54L interacted with 20 proteins and was mainly involved in the following functions: DNA recombination, meiosis I cell cycle process, recombinational repair, meiotic cell cycle process, and meiotic nuclear division.

Finally, the Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/) public database was mined for mRNA expression profiles of BCa and normal bladder tissue. Rad54L was significantly overexpressed in BCa compared with normal bladder tissue ($\mu_{\text{mean}}$ 8.93 vs 5.76, $p < 0.001$; Fig. 1e). Altogether, this bioinformatics analysis suggests that Rad54L might be uniquely connected with proliferation of BCa cells via cell cycle and other pathways.

Rad54L promotes proliferation of BCa cells in vitro

The above bioinformatical prompted us to evaluate the impacts of Rad54L expression in BCa in vitro. To assess the biological function of Rad54L, Rad54L expression was measured in 3 BCa cell lines (ScaBER, 5637 and T24) by
qRT-PCR (Supplementary Fig. 2a), in which the expression of Rad54L was higher in 5637 and T24 cell lines. Subsequently, 5637 and T24 cells were transfected with 3 lentivirus plasmids (shRad54L#1, shRad54L#2, and shCtrl), for Rad54L knock, which was validated by qRT-PCR \((p < 0.05; \text{Fig. } 2a)\) and western blot (Fig. 2b). Cell proliferation of BCa cells was then assessed by colony formation assay, Celigo-based method, and MTT assay. These showed that Rad54L knockdown inhibited BCa cells proliferation \((p < 0.05; \text{Fig. } 2c-e)\) and the result of 5637 cells can be found in Supplementary Fig. 2b and c. Subsequently, flow cytometry analysis showed that Rad54L could inhibit apoptosis of BCa cells (Fig. 2f), and the original figure of flow cytometry can be seen in Supplementary Fig. 2d.

**Rad54L may regulate cell cycle and cell senescence in BCa cells**

Since bioinformatical analysis suggested that Rad54L participates in cell cycle pathway, we next evaluated whether Rad54L could promote proliferation of BCa cells by flow cytometry (FCM). In 5637 cells, the frequency of cells in S phase decreased after Rad54L knockdown with shRad54L, while increasing G1 phase and G2/M phase \((p < 0.05; \text{Fig. } 3a)\) compared with the scramble control. In T24 cells, G2/M phase was not significantly different \((p < 0.05; \text{Fig. } 3a)\). These results reinforce the hypothesis that Rad54L may promote proliferation by inducing cell cycle into S phase. However, the results of G2/M phase were inconsistent in the 2 tested cell lines. Incidentally, our group discovered Rad54L might regulate cell senescence in BCa cells through β-galactosidase activity assay. These results showed that the number of senescent cells in shRad54L group increased significantly compared with the control group \((p < 0.001; \text{Fig. } 3b)\).

Subsequently, the expression of P53, P21, RB1, and its four different phosphorylation sites were measured by western blot. The result displayed that the expression of P53 and P21 was increased after Rad54L knockdown, while RB1 at four different phosphorylation sites was decreased \((\text{Fig. } 3c)\), and the result of 5637 cells can be found in Supplementary Fig. 3a.

**Rad54L associated with poor prognosis in BCa**

Finally, we assessed the impact of Rad54L on BCa tumor growth in vivo with nude mouse tumor transplantation model. As represented in Fig. 4a, tumor volume and weight of nude mice transplanted with shRad54L cells were smaller and lighter than those injected shCtrl cells \((p < 0.05)\), suggesting that Rad54L can accelerate tumor growth in vivo.

To confirm this observation translates to the clinic, we then retrospectively analyzed 48 BCa patients ratified into 2 groups of low (stage = 1 or 2) and high risk (stage = 3 or 4), with 33 and 15 patients, respectively (Table 1).
Immunohistochemistry quantitative analysis showed that the expression of Rad54L in high-risk group was significantly higher than that in low-risk group (197.80 vs 133.64, \( p < 0.05 \); Fig. 4b). Patients were then stratified according to the median immunohistochemical score for survival analysis (Supplementary Table 2), suggesting that with high Rad54L expression had worse OS in BCa patients (Fig. 4c).

**Discussion**

The findings of this research demonstrated that Rad54L was associated with the occurrence and development of BCa through bioinformatical analysis. To confirm this idea, cell clone formation assay, Celigo-based method, and MTT assay were performed which corroborated that Rad54L might promote the cell proliferation on BCa cells. Subsequently, cell cycle was detected by flow cytometry, and the data indicated that Rad54L might increase the frequency of S phase cells. Additionally, \( \beta \)-galactosidase activity assay demonstrated that Rad54L significantly decreased cell senescence in BCa cells and western blot showed that P53 and P21 are highly expressed after Rad54L knockdown while RB1 in its four different phosphorylation states is decreased. Finally, the impact of Rad54L was verified in vivo in the nude mouse transplantation tumor model, and IHC staining validated high expression of Rad54L in BCa tissues showing patients with high Rad54L expression have poor clinical prognosis.

Rad54L is a DEAD-like helicase family protein with homologous recombination repair activity for double-stranded DNA (dsDNA) breaks, single-stranded DNA (ssDNA) breaks [18, 19], and collapsed replication forks [20–22]. Furthermore, Rad54L can stabilize Rad51 nucleoprotein filament formed on either ssDNA or dsDNA [18, 19] and promote DNA synthesis by isolating Rad51 from heterodimer DNA [23]. Previous research found that Rad54L has been associated with colon cancer, breast cancer, lymphoma, meningioma, and myelodysplastic syndrome [24, 25]. Mun JY et al. [26] demonstrated that Rad54L activity was mediated by E2F1, and these two
genes were associated with the progression of BCa and the risk of recurrence. This association is experimentally supported by our study, where bioinformatics analysis, in vitro and in vivo experiments verified that Rad54L can promote the proliferation and development of BCa, and its expression is negatively correlated with the prognosis of BCa. Altogether, these data suggest that Rad54L can promote progression of BCa and associate with the prognosis of BCa.

It is well known that recurrence and chemoresistance are important reasons for poor prognosis in BCa patients [27]. Therefore, it is imperative to explore novel approaches to enhance the sensitivity to chemotherapy. Chen et al. found that Inc-LBCS contributes to enhanced chemotherapy
sensitivity and OICR-9429, a WDR5 inhibitor, facilitates apoptosis and chemosensitivity to cisplatin [27, 28]. Given that high expression of Rad54L is associated with poor prognosis in BCa, Rad54L potentially serves a very important role in chemoresistance.

Cell cycle dysregulation is bound up with progression and aggressiveness of cancers [29]. The normal, cell cycle progresses underwent the gap 1 (G1), synthesis (S), gap 2 (G2), and mitosis (M) stages in turn; however, in tumors, the abnormal expression of cyclins leads to changes in the cell cycle. As previously reported, HRR-related genes like CHEK2, POLD1, CCNA1, and Rad54L are highly expressed during the S phase [30]. Flow cytometric analysis (Fig. 3a) showed a decrease in the number of cells in S phase and an increase in the number of cells in G1 phase after knockdown of Rad54L in both cell lines. Accordingly, we hypothesize that Rad54L might promote BC cells from G1 phase into S phase. A previous report suggested that Nek1 promotes HRR by phosphorylating Rad54L in late G2 phase to facilitate cell cycle, however, we only observed the high expression of Rad54L in G2/M in 5637 cell line (Table 1).

Cell cycle is regulated via a complex signaling network, where p53, p21, and RB proteins are all involved. Interestingly, here we show that p53, p21, and RB1 get involved in cell senescence [31]. Cell senescence is a stable cell cycle arrest which plays an important role in limiting tumor genesis and tissue damage [32]. Activation of the p16\textsuperscript{INK4A}/pRB and p53/p21\textsuperscript{WAF1/CIP1} tumor suppressor pathways plays a crucial role in regulating senescence [33–36]. Mechanistically, p16 can specifically inhibit the phosphorylation of RB, which finally contributes the cell senescence via cell cycle arrested in G1/S phase [37, 38]. Our results showed that the expression of 4 phosphorylation sites of RB1 decreased as a result of knockout of Rda54L (Fig. 3c); therefore, we speculate that Rad54L may upregulate Rb phosphorylation. Consequently, malignant cells enter into S stage which inhibits cell senescence and enables BC to develop. In contrast, p21\textsuperscript{WAF1/CIP1} is activated by p53 at the transcriptional level which activates cell senescence by inhibiting the phosphorylation of Rb [39]. Likewise, we observed that Rad54L knockdown induces P53 and P21 further suggesting that Rad54L might accelerate cell cycle progression and hindered cell senescence.

### Conclusion

In conclusion, we show that Rad54L can favor cell proliferation and inhibit the apoptosis of BCa. Meanwhile, Rad54L might promote progression from G1 into S phase thereby inhibiting the senescence of tumor cells through inhibition of p53 and p21 and promoting Rb phosphorylation. However, the mechanisms involved in the regulation of bladder cancer by RAD54L still need further study.

### Method

#### Ethical approval declarations

All animal experiments used in this study were proved by the Ethics Committee of Central South University Animal Ethics Committee, and the approval number is 202103337. Meanwhile, the pathological slides of BCa used in this experiment have obtained consent from patients and were approved by the Medical Ethics Committee of Xiangya Hospital of Central South University with the approval number as 202201018.

#### Bioinformatics analysis

Gene expression matrix files of GSE37817, GSE42089, and GSE382643 were obtained from the GEO database [40–42]. A total of 78 tumor samples and 21 normal samples adjacent to tumor were acquired. GEO2R online analysis tools [43] were used to identify DEGs between tumor sample and normal tissue sample, and the genes with llogFCl > 1 and p < 0.05 were deemed statistically

| Characteristics | Number (%) | Median (range) |
|-----------------|------------|----------------|
| Gender          |            |                |
| Male            | 37 (77.1%) |                |
| Female          | 11 (22.9%) |                |
| Age (years)     |            | 63 (56.0–68.5) |
| Follow-up time (month) | 58 (26.8–60.8) |
| IHC score       |            | 155 (95.5–191.5) |
| T stage         |            |                |
| T1              | 20 (41.7%) |                |
| T2              | 16 (33.3%) |                |
| T3              | 11 (20.8%) |                |
| T4              | 1 (4.2%)   |                |
| N stage         |            |                |
| N0              | 40 (83.3%) |                |
| N1              | 7 (14.6%)  |                |
| N2              | 1 (21%)    |                |
| M stage         |            |                |
| M0              | 45 (93.8%) |                |
| M1              | 3 (6.3%)   |                |
| Clinical stage  |            |                |
| I               | 20 (41.7%) |                |
| II              | 13 (27.1)  |                |
| III             | 5 (10.4%)  |                |
| IV              | 10 (20.8%) |                |
significant DEGs. Search Tool for the Retrieval of Interacting Genes (STRING) [44] was used to identify potential relationships between DEGs, followed by Cytoscape software [45] and MCODE app analysis to screen for core genes (degree cutoff = 2, node score cutoff = 0.2, k-score = 2, and max. Depth = 100). Metascape online analysis tool [46], GeneMANIA database [46], was used to visualize DEGs enrichment of GO and KEGG pathway analysis of patients with bladder tumors. Finally, TCGA database was used to determine the mRNA expression of RAD54L in bladder tumors compared to normal bladder tissue.

Immunohistochemistry

The paraffin was removed with turpentine, followed by rehydration with 100%, 90%, 85%, and 70% alcohol. Slides were then placed in the prepared EDTA antigen repair solution (ZSGB Biotech, ZLI-9071) and boiled for 30 min. After cooling, the slides were washed with secondary antibody at 37 °C for 30 min. DAB staining solution and hematoxylin were separately added for 10 s and 20 s for counterstaining. The endogenous peroxidase blocker, secondary antibody, and DAB staining solution were purchased from ZSGB Biotech. At last, we used Mantra1.0.3 and Inform 2.4 software for photography and immunohistochemical scores.

Quantitative real-time PCR

All BCa cell lines were purchased from Procell Biotech and cultured in RPMI1640 added with 10% FBS. The BCa cells (T24, ScaBER, and 5637) were seeded in 6-well plate and cultured at 37 °C with 5% CO₂, when the cell density of each well reached 80% lysed with Trizol kit (Shanghai Pufei Biotech, 3101-100) for RNA extraction, and cDNA libraries were prepared with M-MLV kit (Promega Biotech, M1705) following providers’ protocol. Quantitate real-time PCR (qRT-PCR) was prepared as follows: SYBR premix ex taq 10 μL, forward primer 2 μL, reverse primer 2 μL, cDNA 2 μL, and RNase-Free H2O 4 μL. Real-time SYBR integration was detected with MX300p. Agilent with the following program: 95 °C (30 s), 45 cycles of 95 °C (5 s) plus 60 °C (30 s), 95 °C (15 s), 55 °C (30 s), and 95 °C (15 s). Expression levels were calculated according to the formula $F = 2^{-\Delta\Delta C_T}$, relative to GADPH loading control. Rad54L

Construction and infection of shRNA lentivirus

The GV115 plasmid was used to construct RAD54L knockdown plasmid; AgeI and EcoRI were chosen as the enzyme restriction sites. The two shRad54L target sequences are as follows: TTGTAACATCCAGCTCTA and GGAGTCTCAGAAAGGAA, and the control insert sequence is as follows: TTCTCCGAAACGTCACGT. Complete DNA oligo was annealed to produce double-strand DNA which was then ligated into the vector through the restriction sites. The construction of lentivirus plasmid (shRad54L#1, shRad54L#2, and shCtrl) and transfection procedure were based on previous studies.

Cell proliferation ability assay

Cell proliferation was measured by colony formation assay, Celigo imaging, and MTT assay. For colony formation, cells were seeded in 6-well plates at 400–1000 cells per well and incubator for 14 days at 37 °C with 5% CO₂. Cells were then fixed with 1 mL polymethanol, stained with 500 μL GIEMSA (Shanghai Dimguo, AR-0752) for 10–20 min and visible colony-forming unites counted manually. For Celigo imaging assay, 100 cells/well were seeded in 96-well plate and incubated 1 day at 37 °C with 5% CO₂. Cells were then imaged with Celigo cell imaging system (Nexcelom) to calculate cell numbers over 5 consecutive days and final cell proliferation curve calculated. For MTT assay, cells were cultured in 96-well plate as described above and 20 μL of 5 mg/mL MTT (Genview, JT343) was added after 2 days of incubation. After 4 h of incubation with MTT, culture media was aspirated, 100 μL DMSO (Shanghai Shiyi, 130701) was added absorbance at 490/570 nm measure with microplate reader (Tecan infinite, M2009PR).

Flow detection of apoptosis by annexin V/PI

Cells were seeded in 6-well plate and infected with shRAD54L lentivirus. After 5 days, cells were enzymatically digested with 0.05% trypsin EDTA, washed, and resuspended in 200 μL 1× binding buffer with 10 μL Annexin V-APC staining solution (Invitrogen, A13201) for 10 min. Frequency of apoptotic cells was quantified by flow cytometer (Millipore, Guava easyCyte HT).

Senescence β-galactosidase staining

Cells were enzymatically detached as described above and fixed with 1 mL β-galacturonidase at room temperature for
15 min and 1 mL β-galactosidase staining solution was added after PBS washing. The cells were incubated in the 3-well plate overnight in the cell incubator. Total number of cells and stained cells were counted under an optical microscope the following day. For specific experimental details, please refer to the protocol in Senescence β-Galactosidase Staining Kit (Beyotime, C0602).

**Tumorigenesis experiment in mice**

All the BALB/c nude mice (4 weeks old females) were purchased from Shanghai Lingchan Biotech. Animals were housed in pairs per cage at constant room temperature (21 ± 2 °C) with a 12/12-h light/dark cycle. Logarithmic growth phase shRad54L #1 and ShCtrl T24 cells were enzymatically detached as described above, washed, and injected (10^7 cells) into the skin axilla on the right side with disposable sterile syringes. After the injection, the weight of the mice and the width, length, and volume of the tumor on 21, 24, 28, 31, and 35 days were measured. On the last day, the mice were euthanized by injection of 2% pentobarbital. The tumor was weighted and kept in polymethanol at room temperature.

**Cell cycle analysis**

Cells were fixed with 75% ethanol for 1 h and washed by centrifugation with D-Hanks. Cells were subsequently resuspended in 0.6–1 mL staining solution and data acquired at a flow rate of 300–800 Cells/s. Cell staining solution was prepared fresh as follows: 40 × PI 2 mg/mL: 100 × RNase 10 mg/mL: 1 × D-Hanks = 25:10:1000. PI was purchased from Sigma, P4170, Rase to Fermentas, EN0531.

**Western blotting**

Cells were lysed with 2× Lysis Buffer, transferred to 1.5 mL tubes and the nuclear contents lysed by ultrasound. After centrifugation, 1300 rmp for 5 min, the supernatant was collected, and protein concentration was estimated by BCA method. Protein concentration was adjusted to 2 µg/µL. Samples were separated by SDS-PAGE electrophoresis and transferred to PVDF membrane at 4 °C and 300 mA, and the loading quantity is 40µg. Membranes were blocked with skim milk at room temperature for 1 h, incubated for 2 h with primary antibody, and secondary antibody 1.5 h, the primary antibody was used to detected RAD54L (Abclonal, A20181), P53 (Abclonal, A19585), P21 (Abclonal, A19094), GADPH (Abclonal, A13014). After the final TBST washing, reactivity was detected with Pierce TMECL western blotting Substrate kit for development (Thermo, 32106).

**Statistical analysis**

All data were analyzed with prism8 software. The differences between two groups were calculated with two-tailed t-test, and the difference between multiple groups with one-way analysis of variance (ANOVA). p < 0.05 was considered statistically significant.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s12032-022-01751-7.

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**Author contributions** MC designed the study and helped to revise the manuscript. YW and TZ performed bioinformatics analysis, analyzed the data, and wrote and edited the manuscript. HC and PD performed the experiments in vitro and in vivo. SW conducted the IHC staining and scored the IHC results. All authors have read and approved the final version of the manuscript and agreed with the order of presentation of the authors.

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**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Conflict of interest** The authors declared no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

**Ethical approval** The study was approved by the Research Ethics Committee of Xiangya Hospital, Central South University (Approval No. 202201018). The animal study was reviewed and approved by the Ethics Committee of Central South University Animal Ethics Committee (Approval No. 202103337).

**Consent to participate** Informed consent was obtained from all individual participants included in the study.

**Consent for publication** The authors affirm that human research participants provided informed consent for publication.

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