Talin1 knockdown reduces CRC tumor growth and weight in nude mice. In conclusion, Talin1 knockdown may prevent the proliferation and migration of CRC cells by downregulating various factors involved in the epithelial-to-mesenchymal transition process, such as phosphorylated STAT3 and vimentin; therefore, talin1 may provide a novel therapeutic target for CRC.

Introduction

Globally, colorectal cancer (CRC) is a leading cause of cancer-related mortality (1). In a number of low- and medium-income countries, CRC incidence and mortality rates are increasing (2). The incidence and mortality rates are 1.1 and 0.6%, respectively, in less developed areas, lower than the 2.7 and 1% incidence and mortality rate of developed countries (2). The difference is rates between developed and underdeveloped countries may be attributable to the adoption of Western diets and sedentary lifestyles (3). In the UK, an estimated 50% of patients with CRC develop metastasis, and the 5-year survival rate for all CRC patients in the UK is approximately 55% (4). As such, there is an urgent need to identify the molecular mechanisms underlying the pathophysiology of CRC and develop potential therapeutic targets.

Talin1 is a macromolecular (270 kDa) adaptor protein localized in adhesion complexes between cells and the extracellular matrix (ECM). Talin1 binds to and activates integrins, directly connecting integrins to the actin cytoskeleton and recruiting numerous proteins, such as vinculin and focal adhesion kinase (FAK), into integrin adhesion complexes (IACs) (5-7). IACs provide adhesive structures between cells and the ECM and control cell survival, proliferation, invasion and migration (8,9). Talin1 may also regulate cadherin-mediated cell adhesion in an integrin-independent manner (10).

Previous studies have demonstrated that talin1 is expressed in several human cancers, including liver (11) and prostate (12) cancer and oral squamous cell carcinoma (13), and that talin1 serves a role in tumor formation, migration and metastasis. Specifically, genes regulated by talin1 may be involved in epithelial-to-mesenchymal transition (EMT) (14). EMT is characterized by a temporary reversible shift to a mesenchymal phenotype in cancer cells, leading to cancer progression and metastasis (15).
The STAT3 signaling pathway regulates EMT, which contributes to cancer progression (16). STAT3 is activated in thymic epithelial tumors (17) and colorectal adenocarcinoma (18), whereas inhibition of the STAT3 signaling pathway represses growth and invasion and induces apoptosis in a number of different types of tumors, including pancreatic cancer, prostatic carcinomas, and head and neck tumors (19-21).

Inflammation contributes to the pathophysiology of cancer, including tumor initiation, promotion, progression and metastasis, and is involved in the immune surveillance of early cancer and response to therapy (22,23). Interleukin-6 (IL-6) exhibits pro-tumorigenic activities, affecting tumor cell proliferation, survival, differentiation, migration, invasion and metastasis, as well as serving a role in angiogenesis, inflammation and metabolism (24). IL-6 exerts pro-angiogenic activity predominantly through STAT3 signaling, which leads to the induction of hypoxia-inducible factor-1-mediated vascular endothelial growth factor A transcription, as well as endothelial cell proliferation and migration (25-27).

Currently, studies focusing on the expression levels and role of talin1 in CRC are limited. The aims of the present study were to determine the expression of talin1 by immunohistochemistry (IHC) in human CRC samples and to explore the role of talin1 in CRC using short hairpin (sh)RNA knockdown in HCT116 and mouse xenograft models.

Materials and methods

Patients and samples. CRC, adjacent normal tissues and whole blood samples were obtained from 30 patients (mean age, 53 years; range, 36-73 years; 19 males and 11 females) admitted for surgical treatment in the Department of General Surgery, The First Affiliated Hospital of Wenzhou Medical University. Written informed consent in accordance with the Declaration of Helsinki was obtained from all study subjects. The Board of the Ethical Committee of The First Affiliated Hospital of Wenzhou Medical University approved the study, and the study was conducted in strict accordance with the World Health Organization criteria (28). CRC and adjacent normal tissue samples were sectioned into two parts; One part was flash-frozen in liquid nitrogen and stored at -80˚C for 3-4 weeks. The other part was fixed in 4% paraformaldehyde for 24 h at 4˚C, subsequently embedded in paraffin and used for histopathological examination, whereas the other part was flash-frozen in liquid nitrogen and stored at -80˚C until further use. Whole blood was collected in PAXgene Blood RNA tubes (Qiagen GmbH). In July 2016, control blood samples were collected from 24 healthy donors and laboratory staff at the Department of General Surgery, The First Affiliated Hospital of Wenzhou Medical University (mean age, 51 years; range, 39-69 years; 10 males and 14 females) who had no history of malignancy, inflammatory or infectious diseases. This study was approved by the Ethical Committee of The First Affiliated Hospital of Wenzhou Medical University. Written informed consent in accordance with the Declaration of Helsinki was obtained from all study subjects.

Cell culture. HCT116 cells and 293T cells (American Type Culture Collection) were cultured in DMEM with 10% fetal bovine serum (FBS; Invitrogen, Thermo Fisher Scientific, Inc.), penicillin G (100 U/ml; Sangon Biotech Co., Ltd.) and streptomycin (100 mg/ml; Sangon Biotech Co., Ltd.) at 37˚C in a humidified atmosphere containing 5% CO2.

Preparation of viruses and cell infection. VSV-G-pseudotyped lentiviral vectors were produced by co-transfecting 6x106 293T cells with 2 pmol scrambled short hairpin (sh)RNA as a control talin1 1# TRCN0000123105, 5'-CCG GGC CTC AGA TTA TCT GGT GAA ACT CGA GTT TCA CTG CTT TTG T-3' or talin1 2# TRCN0000299020, 5'-CCG GGC TCA TAT CTT GTT GAA AACT CGA GTT TCA GA TAT CTG AGG CTT TTG T-3' shRNA (Sigma-Aldrich; Merck KGaA) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and the following plasmids (all from Invitrogen; Thermo Fisher Scientific, Inc.): 1 pmol pMDL/RRE, 0.5 pmol pMD2.G and 0.5 pmol pRSV-Rev, according to the manufacturer's protocol. The culture medium was changed after 8 h. Lentiviral supernatant was collected and filtered through a cellulose acetate membrane (0.45-µm pore) at 48 and 72 h post-transfection.

HCT116 cells were plated in a 6 well plate (1x105 cells/well) and infected with 100 µl lentivirus (multiplicity of infection, 20) for 24 h at 37˚C. Infected cells that stably expressed lentiviral shRNAs were selected with 1 mg/ml puromycin for 3-4 weeks.

Cell viability. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays were performed according to a protocol adapted from Mosmann (29). Briefly, talin1-knockdown or shRNA control-infected HCT116 cells were plated in 96-well plates at 6x103 cells/well and maintained in an incubator at 37˚C with 5% CO2 for ≤4 days. Cells in each well were treated with 20 µl MTT solution (5 mg/ml) for 3 h at 37˚C. The medium was removed, and cells were lysed with 100 µl DMSO for 2 h at 37˚C. Cell viability was measured at 570 nm using an ELISA plate reader (BioTek Instruments, Inc.). Data from three independent experiments were analyzed.

Cell migration assay. Cell migration was assessed using a wound-healing assay. Talin1-knockdown or shRNA control HCT116 cells were cultured to ~100% confluence in 6-well culture plates. A wound was generated by scratching the monolayer with the tip of a 10-µl pipette. The wound was observed using an inverted phase-contrast microscope and digital camera (magnification, x400) at 24 and 48 h. The wound healing rate was calculated as the percentage of the initial wound area that remained at each time point until total wound closure.

Invasion assay. Invasion assay was performed as previously described (30). Briefly, Matrigel-coated (BD Biosciences; 100 µl 0.35 mg/ml; 1:30 dilution in serum-free DMEM) Transwell chambers with 8-µm pore size membranes (diameter, 6 mm) were incubated at 37˚C for 6 h. Talin1-knockdown or shRNA control HCT116 cells were trypsinized, washed three times with DMEM containing 1% FBS, and resuspended in DMEM containing 1% FBS at a density of 5x105 cells/ml.
Then, 100 µl cell suspension was seeded into upper chambers; 600 µl DMEM containing 10% FBS was added to the lower chambers, and chambers were maintained in a CO₂ incubator for 12 h. Cells were fixed for 15 min at room temperature with 3.7% formaldehyde and stained using 0.1% crystal violet in 10% ethanol for 30 min at room temperature. The number of invaded cells per field in four 4 random fields per well were counted under a light microscope at x200 magnification.

**IHC.** CRC and adjacent normal tissues were embedded in paraffin. For each patient, the middle part of the CRC and normal paired tissues were punched and transferred to a recipient block to create a tissue microarray (TMA). IHC was performed on 4-µm thick sections of paraffin-embedded formalin-fixed samples of tumor and normal tissue from each patient as described previously (31). Briefly, tissue microarray (TMA) slides were baked for 1 h at 60°C, deparaffinized, deparaffinized in xylene (three washes, 10 min each), rehydrated in graded ethanol (100, 95, 85 and 75%, 1 min at each concentration), treated with citrate buffer (pH 6.0) at 100°C for 30 min, blocked using equine serum at room temperature for 20 min (Beijing Solarbio Science & Technology Co., Ltd.) and incubated with 3% hydrogen peroxide at room temperature for 1 h. TMA slides were incubated with anti-talin1 (1:25 dilution; cat no. ab71333; Abcam) primary antibody at 4°C overnight. TMA slides were incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:2000 dilution; cat no. PV-6001; OriGene Technologies, Inc.) at 37°C for 1 h, stained using hematoxylin at room temperature for 30 sec (cat no. H8070; Beijing Solarbio Science & Technology Co., Ltd.), dehydrated using a graded series of ethanol (50, 70, 80, 90, 95 and 100%, 5 min each) at room temperature, cleared in xylene and mounted on slides using Pertex mounting medium (Pioneer Research Chemicals Ltd.). Talin1 was identified in tissues using ImagePro Plus software 6.0 (Media Cybernetics, Inc.). Using an Eclipse 50i/55i optical microscope at x400 magnification. Staining intensity (I) was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. A histological grade (H-score) was determined according to a previous study as H-score=Σ (I x P) (32), and the following expression levels were assigned to each sample: ‘−’, H-score 0-1; ‘+’, H-score 2-3; ‘++’, H-score 4-5; and ‘+++’, H-score ≥6.

**Western blot analysis.** Talin1-knockdown or control HCT116 cells 2x10⁶ were seeded in 6-well plates overnight, washed with ice-cold PBS and harvested using RIPA buffer (Beyotime Institute of Biotechnology). Protein concentration was determined by BCA assay (Beyotime Institute of Biotechnology). Total protein (20 µg) was separated using 12% SDS-PAGE, transferred to a nitrocellulose membrane and blocked with 5% milk at room temperature for 1 h. Membranes were incubated with primary antibodies against talin1 (1:1,000 dilution), E-cadherin (1:1,000 dilution; cat no. ab15148; Abcam), vimentin (1:1,000 dilution; cat no. ab92547; Abcam), total STAT3 (1:1,000 dilution; cat no. ab119352; Abcam), phosphorylated STAT3 (1:1,000 dilution; cat no. ab76315; Abcam), GADPH (1:1,000 dilution; cat no. ab8245; Abcam) and tubulin (1:1,000 dilution; cat no. ab6046; Abcam) at 4°C overnight. Membranes were incubated with a goat anti-rabbit immunoglobulin G horseradish peroxidase (HRP)-conjugated antibody (1:1,000 dilution; cat. no. A0208; Beyotime Institute of Biotechnology) or goat anti-mouse immunoglobulin G HRP-conjugated antibody (1:1,000 dilution; cat. no. A0216; Beyotime Institute of Biotechnology) at room temperature for 1 h. Color was developed using SuperSignal West Pico PLUS Chemiluminescent substrate (Pierce; Thermo Fisher Scientific, Inc.). GADPH and Tubulin were used as loading controls. Protein levels were quantified using ImageJ software version 1.43u (National Institutes of Health).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from tumor, normal tissue and blood samples from each patient and talin1-knockdown or shRNA control HCT116 cells using PureLink RNA kit (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. cDNA was synthesized using 0.5-1.0 µg RNA and the SuperScript First Strand Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc) at 42°C for 50 min according to the manufacturer's instructions. qPCR was performed using SYBR® Green PCR Master mix reagents (Invitrogen; Thermo Fisher Scientific, Inc.) on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), and the thermocycling conditions were as follows: 95°C for 20 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The primers used were as follows: Talin1 forward, 5'-TGGTCCCCAGAGCACCTGCC-3' and reverse, 5'-GAAGACGCACATCAGGGGC-3'; IL-6 forward, 5'-CAATGAGAACTTGGCCTGTTG-3' and reverse, 5'-GGTTGGTCAAGGTTGTTA-3'; E-cadherin forward, 5'-TGCGCGTGAAAGTTTGCAGT-3' and reverse, 5'-ACGGTTCCGGGTGTCTCATT-3'; occludin forward, 5'-GACCCTTGGCTGGATTCTC-3' and reverse, 5'-ATCACGAGCAGCATGTATCTTC-3'; ZO-1 forward, 5'-CAACAGATGTTCAGACACGGAAGG-3' and reverse, 5'-CTGTATGGGGCCTGCTCAAGGTTC-3' and 18S rRNA forward, 5'-ACCAGCTGGTGTATCCTGCCAGT-3' and reverse, 5'-CTGACCGGGTTGTGTTGTAG-3'. PCR was performed on an ABI 7500 Realtime PCR system and analyzed using version 2.0.6 of the accompanying software (Applied Biosystems; Thermo Fisher Scientific, Inc.). Results are presented as relative quantification normalized to 18S rRNA. The 2⁻ΔΔCq method was used for the relative quantification of gene expression in each sample (33). Each experiment was repeated three times in duplicate.

**ELISA.** Serum IL-6 concentrations were detected using a Human IL-6 ELISA kit (cat. no. BMS213-2; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Concentrations of cytokines in standards and samples were measured in triplicate using an iMark microplate reader (Bio-Rad Laboratories, Inc.).

**Animal experiments.** Animal experiments were approved by the Institutional Animal Committee of Wenzhou Medical University. A total of 20 5-6-week-old BALB/c nude mice (Beijing Vital
were housed at 21-25°C with 40-70% humidity, with a 12 h dark/light cycle and free access to food and water. A total of 1x10^6 talin1 1# or shRNA control transfected HCT116 cells were subcutaneously injected. Mouse body weight was measured three times per week. Tumor size was measured every other day with calipers. Tumor volume was calculated using the following formula: Volume=(width)^2 x length/2. If the tumor burden was determined to be high, or difficulties in feeding the mice were experienced, then mice were sacrificed early. Mice were sacrificed after 4 weeks using CO_2 (20% of the chamber volume/min) until they stopped breathing, and tumors were weighed.

Statistical analysis. Statistical analyses were performed using SPSS 19.0 (IBM Corp.). Data are presented as the mean ± standard deviation. Between-group comparisons were conducted using single-factor analysis of variance (one-way ANOVA). Comparisons among multiple groups were performed using ANOVA followed by the least significant difference test. The in vitro results represent three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Talin1 expression is upregulated in CRC and regulates cancer cell proliferation. Talin1 protein expression levels were detected by IHC in tumor and adjacent normal tissues from each patient. Across all patients, the percent area stained for talin1 was 3.5-fold greater in tumor tissue compared with adjacent normal tissue (P<0.001; Fig. 1A and B), suggesting that talin1 expression was upregulated in CRC.
Stable talin1 1# or talin1 2#-knockdown HCT116 cell lines were established by infecting HCT116 cells with talin1 recombinant shRNA-expressing lentiviral vectors, screening with puromycin for 3–4 weeks and subsequently allowed to grow. Relative talin1 protein and mRNA levels were 30% lower in talin1-knockdown HCT116 cells compared with HCT116 cells transfected with shRNA control (P<0.001; Fig. 1C-E), confirming the establishment of stable talin1-knockdown HCT116 cell lines, which were used in subsequent experiments.

Cell viability was assessed using the MTT assay. At 48 h, cell proliferation was significantly inhibited in talin1 knockdown cell lines compared with shRNA control cells. The antiproliferative effects of talin1 knockdown were more pronounced after 24 h and significantly reduced after 48 h of culture (P<0.01; Fig. 1F).

**Talin1 regulates cancer cell invasion and migration.** The invasive potential of talin1 1# or talin1 2#-knockdown HCT116 cell lines and HCT116 cells transfected with the shRNA control was examined using Matrigel-coated Transwell chambers. Talin1-knockdown significantly reduced the invasiveness of talin1-knockdown HCT116 cells (Talin1 1#, 55%; Talin1 2#, 62%) compared with that of shRNA control cells (Fig. 2A and B).

The role of talin1 in the regulation of CRC cell migration was examined using a wound-healing assay. At 36 h, the rate of wound closure in the talin1-knockdown cell lines was significantly reduced compared with the control (P<0.05). At 48 h, the rate of wound closure in the talin1 knockdown cell lines was reduced by 0.579-fold (talin1 1#) and 0.584-fold (talin1 2#) compared with the control (Fig. 2C and D).
Talin1 knockdown affects the expression levels of proteins associated with the EMT signaling pathway. The levels of proteins in the EMT signaling pathway in talin1 1#- or talin1 2#-knockdown HCT116 cell lines and HCT116 cells transfected with the shRNA control were analyzed to explore the mechanism by which talin1 regulates cancer cell invasion and migration. Protein levels of phosphorylated STAT3 (pSTAT3), an oncogene that modulates cellular processes such as invasion and metastasis (34), were significantly lower in talin1-knockdown HCT116 cell lines compared with the control (Fig. 3G and H).
Protein levels of vimentin, a marker of EMT, were also significantly lower in talin1-knockdown HCT116 cell lines compared with the control, whereas mRNA and protein levels of E-cadherin were increased (Fig. 3A-D). These results suggested that talin1 knockdown inhibits CRC tumor invasion through the EMT signaling pathway.

qPCR indicated that mRNA levels of ZO-1 and occludin, which are major components of tight junctions, decreased significantly in tumor tissue compared with adjacent normal tissue (Fig. 3E-F). As a major activator of STAT3 (35), mRNA levels of IL-6 were detected in blood, tumor and adjacent normal tissue samples from each patient. IL-6 mRNA levels were significantly increased in patient blood samples compared with blood samples from healthy controls, as well as in tumor tissue compared with adjacent normal tissue (Fig. 3I and J). ELISA results demonstrated that levels of IL-6 in patient blood samples were 3.2-fold higher compared with those in healthy individuals (Fig. 3K).

Talin1 knockdown suppresses tumor growth. To evaluate the potential of talin1 as a therapeutic target, the effect of talin1 knockdown on the growth of tumor xenografts in BALB/c nude mice was examined. Mice were injected subcutaneously with talin1 1#- or talin1 2#-knockdown HCT116 cell lines or HCT116 cells transfected with the shRNA control. Talin1 knockdown inhibited tumor growth, resulting in significantly smaller tumors with reduced volumes compared to control (Fig. 4A and C). Talin1 knockdown decreased tumor mass by 63.81% compared with the control (Fig. 4B), but did not affect mouse body weight (Fig. 4D). These data suggested that talin1 knockdown may inhibit tumor growth in vivo.

Discussion

The present study determined talin1 expression levels in CRC and investigated the role of talin1 in CRC proliferation and invasion in vitro and in vivo. IHC analysis of human CRC and adjacent normal tissue demonstrated that talin1 expression was upregulated in CRC. Talin1 knockdown significantly reduced the proliferative, migratory and invasive abilities of HCT116 cells compared with the control. Talin1 knockdown affected the expression of proteins in the EMT signaling pathway in HCT116 cells. qPCR and ELISA results also showed that IL-6 levels were significantly increased in blood samples obtained from patients with tumors compared with the control. In vivo experiments revealed that talin1 knockdown reduced CRC tumor growth and weight in nude mice. These results show that Talin1 knockdown decreases proliferation and migration of CRC cells by downregulating various factors involved in EMT.

Talin1 is a ubiquitous cytoskeleton-associated protein that couples the cytoskeleton with integrins and is an essential mediator of cell-ECM adhesion. The N-terminal head domain of talin1 binds to the cytoplasmic domain of β integrin, which causes integrin activation and stimulates integrin binding to the ECM (36,37). Integrin adhesion to the ECM can be further modified, as talin1 links the ECM and actin by binding to actin filaments, and actin and/or integrins unfold talin1 domains, which results in the exposure of binding sites for additional integrin-associated proteins, such as vinculin and FAK (9,38). Talin1 can also act independently of integrins, regulating the expression of E-cadherin, which is a cell-cell adhesion molecule associated with impaired cancer progression (10). Talin1 has been identified to serve important roles in various types of disease (38-41). Specifically, Xu et al (32) demonstrated that upregulated talin-1 expression was associated with malignant behavior in prostate cancer and lymph node metastasis. Upregulation of talin1 has also been demonstrated in hepatocellular carcinoma progression and may serve as a prognostic marker (42). In the present study, Talin1 protein expression levels were determined to be upregulated in tumor tissues compared with the adjacent normal tissues.
STAT3 is an oncogene activated by phosphorylation of a conserved tyrosine residue in response to the binding of cytokines, hormones and growth factors to their receptors and activation of Janus kinase 2 or the intrinsic tyrosine kinase activity of the receptors (16). Phosphorylation of STAT3 promotes its homodimerization and translocation to the nucleus, where it regulates the transcription of target genes that modulate essential cellular processes, such as proliferation and differentiation (16). The results of the present study demonstrated that the levels of pSTAT3 were decreased in talin1-knockdown HCT116 cell lines, suggesting that talin1 knockdown inhibits CRC tumor invasion.

EMT involves dissolution of epithelial cell-cell junctions, establishment of a front-rear polarity, reorganization of the cytoskeleton, changes in cell shape and increased cell motility and invasive behavior (43). EMT is accompanied by decreased E-cadherin levels during destabilization of adherens junctions and increased vimentin levels; vimentin interacts with motor proteins and facilitates cell motility (43-46). In the present study, E-cadherin expression was increased and vimentin expression was decreased in talin1-knockdown HCT116 cell lines compared with the control. These findings suggested that talin1 knockdown inhibited cell invasive capability, possibly through the EMT signaling pathway.

Persistent infections and chronic inflammation may be a causative factor in certain types of cancer, such as colitis-associated cancer, hepatocellular carcinoma and skin cancer, and a number of tumors exhibit extensive immune cell infiltration with high levels of inflammatory mediators in the tumor microenvironment (22,47,48). In CRC, serum levels of IL-6 have been reported to be the highest in patients with advanced clinical stage or metastatic disease (49). Consistent with this, the qPCR results in the present study demonstrated that IL-6 levels were higher in human CRC tumors compared with adjacent normal tissue and the ELISA results showed that the levels of IL-6 in patient blood samples were 3.2-fold higher compared with those in healthy individuals.

In conclusion, the present study demonstrated that talin1 was upregulated in human CRC and that talin1 knockdown prevented the proliferation and migration of CRC cells via the EMT signaling pathway. EMT associated with cancer progression represents a potential treatment strategy in clinical oncology; therefore, talin1 may provide a novel therapeutic target for CRC. Future studies are to focus on the generation of a conditional knockout mouse model of talin1, selectively targeting talin1 gene expression in colorectal cells, crossed with a transgenic mouse model of colorectal cancer.

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Availability of data and materials

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

Authors’ contributions

LJ and CQ conceived and designed the experiments. LJ and XC performed the experiments. LJ, FJ, XC and CQ analyzed the data. FJ contributed the materials and analysis tools. LJ and CQ wrote the manuscript. All authors read and approved the final manuscript.

Ethical approval and consent to participate

This study was approved by the Board and Ethical Committee of The First Affiliated Hospital of Wenzhou Medical University. Written informed consent in accordance with the Declaration of Helsinki was obtained from all study subjects. Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals. All animal experimental procedures were approved by the Wenzhou Medical University Animal Policy and Welfare Committee.

Patient consent for publication

All patients provided written informed consent for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

References


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