

1 Methods and materials

1.1 Clinical samples

This study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University (ethical review report number: 2018-L055). Fifty pairs of gastric cancer (GC) tissues and adjacent non-tumor tissues were collected by the pathology department from October 2017 to November 2021. After excision, the samples were transferred to -80°C refrigerators for storage. All patients with GC were diagnosed by pathologists, and informed consent was obtained according to the ethical guidelines.

1.2 RNA extraction, complementary DNA (cDNA) synthesis and qRT-PCR

Total RNA was extracted from GC tissue and cell samples using the TRIzol reagent (Invitrogen, Carlsbad, USA). The total quantified RNA was reverse transcribed into cDNA using the Revert Aid RT reverse transcription kit (Thermo Fisher Scientific, Waltham, USA). The reaction conditions were 42°C for 60 min and 70°C for 5 min. A total volume of 20 μL was prepared by adding 1 μL of forward and reverse primers, 5 μL of diluted cDNA, 3 μL of enzyme-free water, and 10 μL of ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China). The qRT-PCR procedure was performed on QuantStudio 5 (Thermo Fisher Scientific) to detect the expression of *tRF-23-Q99P9NDD* and messenger RNA (mRNA). RNU6B (*U6*) was used as the internal reference for *tRF-23-Q99P9NDD*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was utilized as the internal reference for mRNA. The primer sequences were as follows: *ACADSB* (forward-5'-CATAACAATGCTGCTAGGCTTTT-3', reverse-5'-ATTTACTCGTTGTTTGTCTGC-3'); *DGKD* (forward-5'-TCAGCGAGGATTCCGAGGTACAG-3', reverse-5'-CACCGTCTCACA GAGCACTTTGG-3'); *BCKDHB* (forward-5'-GGTGGCTCATTCTTTACTTTCCAG-3', reverse-5'-AGGATCTTTGGCCAATGAGTTA-3'); *NDST3* (forward-5'-TGGGGTCTGTGGATGAGTTCT GG-3', reverse-5'-CGTTGGAATGCCGTGCTCTAAGG-3'); *GAPDH* (forward-5'-AGAAGGCT GGGGCTCATTTG-3', reverse-5'-GCAGGAGGCATTGCTGATGAT-3'). The primer sequences of *tRF-23-Q99P9NDD* and *U6* were designed by RiboBio (Guangzhou, China). The expression levels of *tRF-23-Q99P9NDD* and mRNA were calculated by the $2^{-\Delta\Delta\text{Ct}}$ method according to the formula $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{tumor}}[\text{Ct}(\text{target}) - \text{Ct}(\text{reference})] - \Delta\text{Ct}_{\text{control}}[\text{Ct}(\text{target}) - \text{Ct}(\text{reference})]$.

1.3 Cell culture

Human GC cell lines (HGC-27, AGS, MKN-45, SGC-7901, and BGC-823) and the human gastric mucosal epithelial cell line (GES-1) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI 1640 medium (Gibco, Waltham, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (HyClone, Logan, USA). The solution was changed every 2 d. The incubator was set to 37°C and 5% CO_2 .

1.4 Cell transfection

Cells were inoculated into 6-well plates, and when they grew to 60%–70% confluency, lipofectamine 3000 Reagent (Thermo Fisher Scientific) was used to transiently transfect *tRF-23-Q99P9NDD* mimics/inhibitor and the corresponding negative controls (NC) (RiboBio), short hairpin NC (sh-NC), sh-*ACADSB* (GCTCGTGATAGAGGAATTAGC), overexpression NC (OE-NC), and OE-*ACADSB* (Gene Pharma, Shanghai, China).

1.5 Cell Counting Kit-8 (CCK-8) proliferation assay

Cells after transfection for 48 h were collected and inoculated into 96-well plates at a density of 3×10^3 cells/well. After the cells had adhered to the wall, CCK-8 reagent (Biosharp, Beijing, China) was added. Following incubation at 37 °C for 2–3 h, the absorbance at 450 nm and 630 nm was measured using a microplate reader and detected every 24 h in triplicate.

1.6 Colony formation assay

The transfected cells were inoculated into 6-well plates at a density of 1×10^3 cells/well, and the medium was changed every four days for about 14 d. The cells were fixed with 4% paraformaldehyde (Biosharp) at room temperature, then stained with crystal violet and photographed.

1.7 Transwell assay

For the cell migration experiment, 500 μ L of medium containing 20% FBS was added to the 24-well plates. Then, the transfected cells were quantified to 5×10^4 cells/well with the basic medium and added to the transwell chambers (Corning, New York, USA). The chambers were placed in the plates, cultured for 48 h, and then fixed with 4% paraformaldehyde. After crystal violet staining, photographs were taken under the microscope. The cell density was adjusted for the cell invasion assay to 8×10^4 cells/well. Matrigel (Corning) was mixed with the basal medium at a ratio of 1:7 and added to the upper chamber for 12 h. The remaining steps were the same as in the cell migration experiments.

1.8 5-Ethynyl-2'-deoxyuridine (EdU) incorporation assay

The transfected cells were inoculated into 24-well plates at a density of 1×10^5 cells/well. The proliferation of cells was detected using the EdU kit (RiboBio). First, the original medium was replaced with a medium containing 50 μ mol/L EdU to incubate the cells, and then cells were fixed with 4% paraformaldehyde after 2 h. Subsequently, Apollo567 and Hoechst 33342 fluorescent dyes were used for staining. Finally, the proliferation of cells was observed by fluorescence microscope.

1.9 Fluorescence in situ hybridization (FISH)

Cells were inoculated on 24-well plate cell slides. The FISH Kit (RiboBio) was used for subsequent operations. A hybridization solution containing *tRF-23-Q99P9NDD*, 18S, and U6 probes (RiboBio) was added to each well overnight. After washing the cells on the second day, 4',6-

diamidino-2-phenylindole (DAPI) was used to stain the nucleus, and the cells were fixed on the glass slides with a sealing agent and observed by fluorescence microscope.

1.10 Dual-luciferase reporter assay

The *ACADSB* wild-type (WT) and mutant (MUT) sequences containing the tRF-23-Q99P9P9NDD binding sites were ligated into the dual luciferase reporter gene pmirGLO vectors and co-transfected with tRF-23-Q99P9P9NDD mimics into GC cells. After 48 h, the dual luciferase reporter gene system of Promega was used for detection.

1.11 Western blot

The RIPA lysate and PMSF (SolarBio Life Science, Beijing, China) were mixed proportionally to lyse cells to extract the total protein. The protein concentration was measured by the bicinchoninic acid method. The proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, MA, USA). Primary antibodies against the target protein ACADSB (Proteintech, city, country) and the internal reference protein GAPDH (Abclonal, Wuhan, China) were incubated overnight at 4 °C, and the horseradish peroxidase (HRP)-labeled secondary antibody (Cell Signaling Technology, MA, USA) was incubated for 2 h at room temperature the next day. The ECL method was implemented to detect the protein expression (Millipore).

1.12 Data collection

Data on the expression of ACADSB from 323 GC tissues and 32 normal tissue samples were obtained from The Cancer Genome Atlas (TCGA; <http://cancergenome.nih.gov/>) database. The data were analyzed by R software (<https://www.r-project.org/>). The immunohistochemical staining results of the ACADSB antibody were collected from The Human Protein Atlas database (<https://www.proteinatlas.org/>).

1.13 Functional enrichment analysis

1.13.1 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses

GO and KEGG enrichment analyses were performed by using the R packages ‘cluster profile’ and ‘org.Hs.eg.db’, where both *P*-value and *q*-value of <0.05 were considered statistically significant.

1.13.2 Gene Set Enrichment Analysis (GSEA)

GC patients were divided into high- and low-expression groups according to the expression of *ACADSB*. The enrichment pathways of *ACADSB* in GC were identified by GSEA v4.2.3 software. The gene set ‘h.all.v2022.1.Hs.symbols.gmt’ was used as a reference for GSEA and considered statistically significant at *P*-value<0.05 and *q*-value<0.25.

1.14 Statistical analysis

SPSS 20.0 software and Graph Pad Prism v8.0 were utilized for statistical analysis, and all data

were expressed as mean \pm standard deviation (SD). The *t*-test or Mann-Whitney U test was implemented to evaluate the differences between the two groups, and the Paired *t*-test or Wilcoxon test was used to evaluate the differences between the paired samples. Differences between three or more groups were evaluated using one-way analysis of variance (ANOVA). The correlation of *ACADSB* with tRF-23-Q99P9P9NDD or other genes was assessed using Spearman correlation analysis. All experiments were repeated three times. A *P*-value of <0.05 was considered statistically significant.