Quantitative real time polymerase chain reaction (qRT-PCR) assay

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), reverse transcribed into cDNA using the PrimeScriptRT kit (Takara, Tokyo, Japan). Following this, qRT-PCR was conducted on Applied Biosystems 7900 Real-time PCR System with SYBR Green Mix II (Takara). The sequence of primers were as follows: circ_104640: 5'- AGCAACAGAGGAACAAGAG-3' and 5'-GAGAAGGAACAGGATGAACT-3'; centrosome and spindle pole associated protein 1 (CSPP1): 5'-AGCAACAGAGGAACAAGAG-3' and 5'-CTACCCTCATGAGGGGGTGC-3'; miR-145-5p: 5'-GTCCAGTTTTCCCAGGAATC-3' and 5'-AGAACAGTATTTCCAGGAAT-3'; CCL20, 5'-TGCTGTACCAAGAGTTTGCTC-3' and 5'- CGCACACAGACAACTTTTTCTTT-3'; GAPDH, 5'-GACAGTCAGCCGCATCTTCT-3' and 5'-GCGCCCAATACGACCAAATC-3'; U6: 5'-CTCGCTTCGGCAGCACA-3' and 5'-CTCGCTTCGGCAGCACA-3'; and 18s: 5'- GGAGTATGGTTGCAAAGCTGA-3' and 5'-TCCTGCTTTGGGGTTCGATT-3'. Gene expression levels were standardized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6, according to the $2^{-\Delta\Delta}$Ct method.

RNase R treatment assay

2 mg of total RNA and 5μ/μg RNase R (Geneseed Biotech, Guangzhou, China) were thoroughly mixed and incubated at 37 ℃ for 20 minutes, followed by purification using the RNeasy MinElute cleaning kit (Qiagen, California, USA). Subsequently, qRT-PCR was used to detect the levels of mRNA and circRNA expressions.

Subcellular localization

The Cytoplasmic and Nuclear RNA Purification Kits (BioVision, San Francisco, USA) were used to extract RNA from the nucleus and cytoplasm, respectively. Samples were then analyzed by qRT-PCR.

Cell transfection

The shRNA that targeted circ_104640 (sh-circ) and a negative control siRNA (sh-NC) were purchased from RiboBio (Guangzhou, China). miR-145-5p mimics, miR-145-5p inhibitors and negative controls (NC) were also obtained from RiboBio. The oligonucleotides were transfected into LUAC cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. The sequences used were as follows: sh-circ, GAUCCGGU GUCUCCCAGUGCUCCAGACAAUUCAAGAGAUU GUCUGGAGCACUGGGAGACACCUUUUUUC; sh-NC, UUCUCCGAACGUGUCACGUUUC; miR-145-5p mimics, GUCCAGUUUUCCCAGGAAUCCCU; miR-145-5p inhibitor, ACGGAUUCCUGGGAAAACUGGAC; NC, ACUACUGAGUGACAGUAGA.

CCK-8 assay

According to the manufacturer's protocol, the rate of cell proliferation was measured using the CCK-8 kit (Dojindo, Japan). The transfected cells were seeded into 96-well plates (1×10^3 cells/well). At 1, 2, 3, and 4 days, 10 μL CCK-8 solution was added to each well and incubated for 2 hours. The absorbance value of each well was measured at 450 nm with a microplate spectrophotometer (Bio-Rad, California, USA).

Colony formation assay

Transfected cells were seeded into 6-well plates and incubated for 10 days in a standard environment. 4% paraformaldehyde and 0.1% crystal violet solution (Beyotime, Shanghai, China) were added for fixation and staining, respectively.

Analysis of apoptosis

The Annexin V-FITC/PI apoptosis detection kit (Vazyme, Nanjing, China) and flow cytometry (Becton Dickinson, USA) were used to evaluate cell apoptosis. The transfected cells were collected, washed, and resuspended in the binding buffer. Then, 5μL Annexin V-FITC and 5μL propidium iodide (PI) were added to the cells and incubated in the dark. The percentage of apoptotic cells were detected by flow cytometry.

Bioinformatics prediction

The database Starbase (http://starbase.sysu.edu.cn/) was used to explore the possible binding sites between circRNA-

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miRNA and miRNA-mRNA (41).

Dual-luciferase reporter assay

The wild type (wt) and mutant type (mut) fragments of circ_104640 containing putative miR-145-5p binding sites were amplified and inserted into the pGL3 (Promega, USA) vector. Subsequently, 2×10^4 A549 and H1299 cells were plated into 24-well plates and cultured overnight at 37 ℃. miR-145-5p mimics and circ_104640-wt or circ_104640-mut were co-transfected into cells using Lipofectamine 3000 (Invitrogen). After 48 hours of co-transfection, the luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

Ago2 RNA immunoprecipitation assay

According to manufacturer's protocols, RNA immunoprecipitation (RIP) assays were performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, MA, USA). A549 and H1299 cells were lysed in lysis buffer and incubated with RIP immunoprecipitation buffer containing magnetic beads pre-incubated with the anti-Ago2 (ab186733, abcam, Cambridge, UK) and anti-IgG antibodies. RNA was purified from the RNA-protein complex and analyzed by qRT-PCR.

References

41. Li JH, Liu S, Zhou H, et al. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. Nucleic Acids Res 2014;42:D92-7.

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