Interference with NTSR1 Expression Exerts an Anti-Invasion Effect via the Jun/miR-494/SOCS6 Axis of Glioblastoma Cells

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Supplementary Materials and methods

Plasmids, siRNAs, shRNAs, and Transfection

Control sc-siRNA and siNTSR1 were purchased from Ambion. Control sc-shRNA and shNTSR1 were purchased from GENECHEM. Control sc-siRNA, siJun, and siSOCS6 were purchased from RIBOBIO. Human Jun cDNA was purchased from OriGene and then inserted into a pCMV6-XL4 vector (OriGene). U87MG and U118MG were cultured at a density of 1×10^6 cells in 60-mm dishes in DMEM medium supplemented with 10% FBS. At 90% confluence, cells were transiently transfected with siRNA or Jun vector using transfection reagent. To obtain stable transfection shNTSR1-U87MG cell line, U87MG cells were transfected with shNTSR1 using transfection reagent, and selected with puromycin.

Wound-healing and Transwell assays

Cells were plated in 6-well plates, and wounds were made with a pipette tip after cells were attached. Images of the wound closure were photographed at 0h and 24h. For invasion assay, transwell assay was performed with the 8μm-pore chamber inserted into 24-well plates (Corning, NY, USA). Matrigel was diluted (1:7) in serum-free DMEM, and then added to the chamber and allowed to solidify completely. Transfected cells were obtained and resuspended in serum-free medium and seeded in the upper chambers, 600μL DMEM containing 30% FBS was placed at the bottom of the chamber. After 24h incubation, cells were fixed with 4% paraformaldehyde in PBS and stained with crystal violet. The images of cells that invaded to the lower surface of the membrane were taken using an inverted microscope, and the number of invaded cells was counted.

Cell proliferation assay

Cell proliferation was measured using the CCK-8 assay. Cells were seeded into 96-well plates, and 10μl CCK-8 was added to each well at 0h, 12h, and 24h. The cells were then incubated for 2h. Cell viability was assessed by OD value at 450nm using a microplate reader.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using the ChIP assay kit (Upstate Biotechnology). U87MG cells were cross-linked with formaldehyde and washed with ice-cold PBS and disrupted in SDS lysis buffer containing the protein inhibitor cocktail. Chromatin was sonicated to shear DNA to an average length between 200bp and 1000bp as verified by agarose gel. The sonicated cell supernatants were diluted in ChIP dilution buffer containing the protein inhibitor cocktail and aliquot of the solution was reserved for input control. Jun and IgG antibodies were added and the chromatin solution was gently rotated overnight on ice. The protein A agarose slurry was added to the antibody bound chromatin solution and incubated with constant rotation. The agarose beads were collected by centrifugation, washed and the antibody bound chromatin was released from the agarose beads. The DNA was purified by phenol/chloroform extraction and ethanol precipitation. The binding region was spanning the Jun binding site (18.6kb upstream of pre-miR-494), a 31kb upstream of the pre-miR-494 5' end was used as a control (Control region). ChIP primers:

Jun binding region FW 5' ATGATCGTTGTAGAGCATCAGGCCT 3'

Jun binding region RW 5' GATGAACTCTCAATTTGGATCAAACCCG 3'

Control region FW 5' GTTGGGTGGTTCATTTAAGGGTATTCCTGA 3'

Control region RW 5' TCATCAATGGGAGAATAATTTAATCAGCTC 3'

Dual-Luciferase

The pmiR-RB-Report™ luciferase miRNA expression reporter vector (RiboBio) was applied to construct the wild-type (WT) reporter vectors of the SOCS6 3' untranslated region (3' UTR). The coding sequence of miR-494 binding site was mutated to construct the mutant (MT) vector by site-directed mutagenesis. Dual-luciferase assay was performed using exponentially growing cells 24h after seeding and the luciferase assay system (Promega). Luminescence was recorded with a Fluostar Optima microplate reader (BMG Labtech).

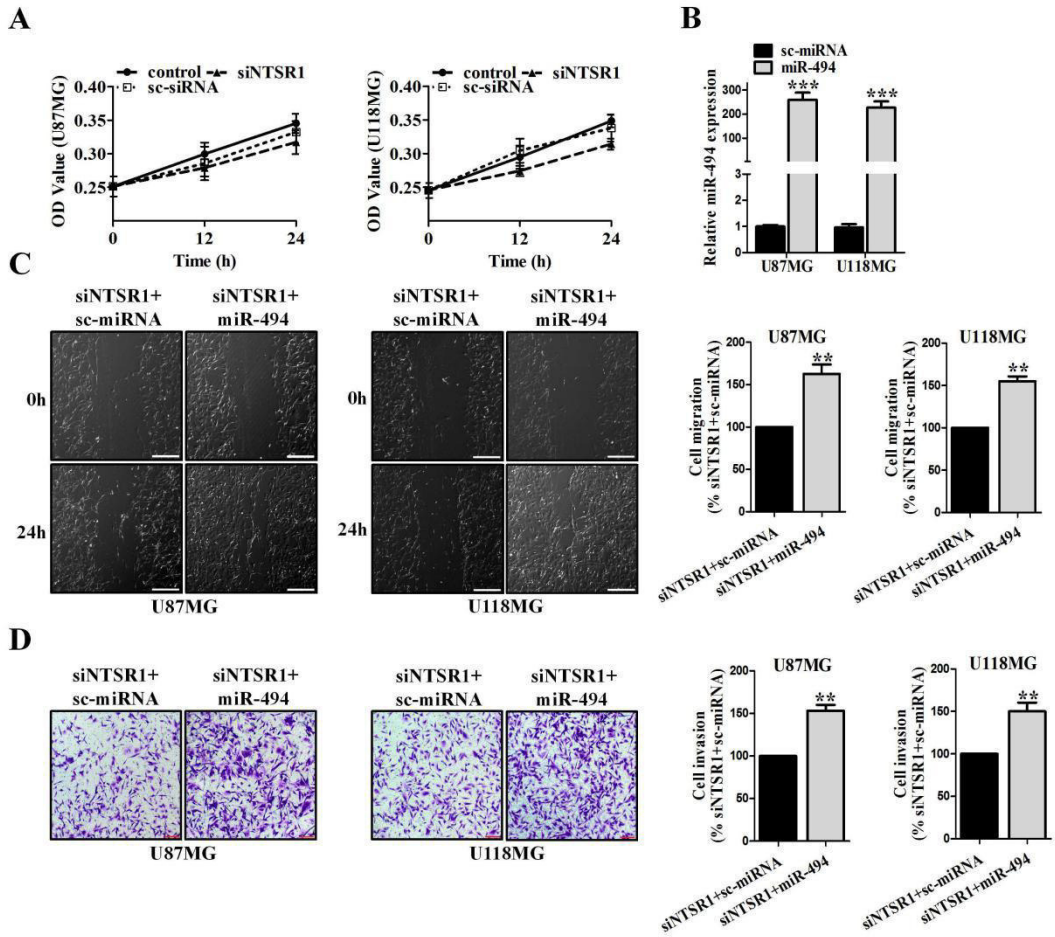
Supplementary Figure legends

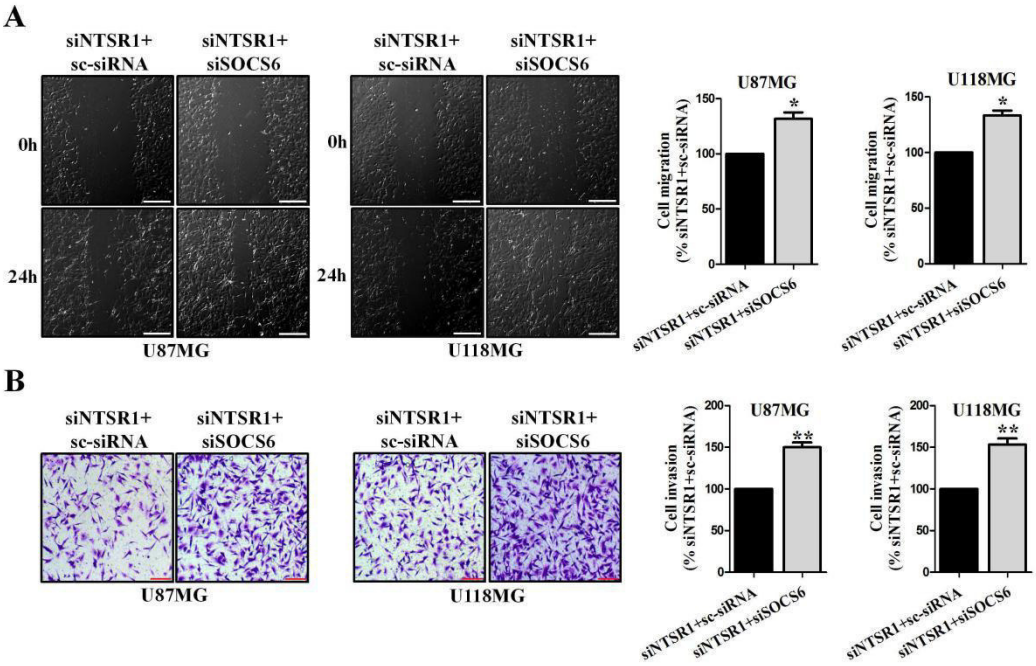
Fig. S1. A. The proliferative activity was detected by CCK-8 assay in U87MG and U118MG cells transfected with sc-siRNA or siNTSR1. B. The levels of miR-494 were measured by qRT-PCR in the indicated U87MG and U118MG cells. C. The wound-healing

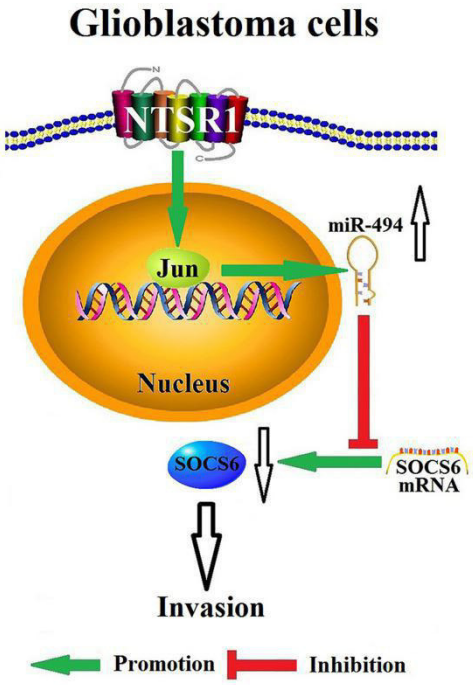
assay showed that miR-494 restored the migratory ability of U87MG and U118MG cells with siNTSR1 transfection. B. The transwell assay showed that miR-494 restored the invasive ability of U87MG and U118MG cells with siNTSR1 transfection. The histogram corresponds to the mean±S.D. of three independent experiments. **p<0.01, ***p<0.001.

Fig. S2. A. The wound-healing assay showed that SOCS6 knockdown restored the migratory ability of U87MG and U118MG cells with siNTSR1 transfection. C. The transwell assay showed that SOCS6 knockdown restored the invasive ability of U87MG and U118MG cells with siNTSR1 transfection. The histogram corresponds to the mean±S.D. of three independent experiments. *p<0.05, **p<0.01.

Fig. S3. Schematic model: NTSR1 enhances invasion via Jun/miR-494/SOCS6 axis in glioblastoma cells.




A
siNTSR1+ sc-siRNA
siNTSR1+ siSOCS6
0h
24h
U87MG
U118MG
Cell migration (% siNTSR1+sc-siRNA)
siNTSR1+sc-siRNA
siNTSR1+siSOCS6
B
Cell invasion (% siNTSR1+sc-siRNA)


Glioblastoma cells
NTSR1
miR-494
Jun
Nucleus
SOCS6
SOCS6 mRNA
Invasion
Promotion
Inhibition