Basic Research Paper

Effect of RNAi-mediated LRIG3 gene silencing on proliferation of glioma GL15 cells and expression of PCNA and Ki-67

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Background and Objective: Leucine-rich repeats and immunoglobin-like domains 3 (LRIG3), a member of LRIG gene family, is down-regulated in various human cancers, but its functions are still unclear. This study was to explore the effect of RNA interference (RNAi)-mediated LRIG3 gene silencing on the proliferation of glioma GL15 cells and the expression of proliferating cell nuclear antigen (PCNA) and Ki-67, and investigate possible mechanisms. Methods: The plasmids pGenesil2-LRIG3-shRNA1 and pGenesil2-LRIG3-shRNA2 which containing U6 promoter and LRIG3-specific short hairpin RNA (shRNA) and the plasmid pGenesil2-negative-shRNA containing unspecific shRNA were transfected into GL15 cells. Stable cell clones were selected by G418. The mRNA and protein levels of LRIG3 were measured by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot. Cell proliferation was detected by MTT assay. The expression of PCNA and Ki-67 in GL15 cells were examined by SABC immunohistochemistry. Results: Compared with those in control cells, the mRNA levels of LRIG3 transcripts were reduced by 52.4% and 63.8% in shRNA1- and shRNA2-transfected cells, respectively; its protein levels were reduced by 50.9% and 67.4%, respectively. Cell proliferation was enhanced by LRIG3 shRNA transfection. The positive rate of PCNA was significantly higher in shRNA1- and shRNA2-transfected cells than in control cells [(72.13 ± 5.64)% and (81.93 ± 5.23)% vs. (35.40 ± 5.69)%, p < 0.01]. The positive rate of Ki-67 was also significantly higher in shRNA1- and shRNA2-transfected cells than in control cells [(82.27 ± 5.50)% and (88.67 ± 3.52)% vs. (49.73 ± 5.73)%, p < 0.01]. PCNA expression was positively correlated to Ki-67 expression (r =0.932, p < 0.001). Conclusion: Down-regulating LRIG3 gene expression can improve the proliferation of glioma GL15 cells.

In recent studies on negative feedback regulation of human epithelial growth factor receptor (EGFR) signal system, leucine-rich repeats and immunoglobulin-like domain 1 (LRIG1) gene (GenBank No. AF381545) has been identified and cloned in normal human brain tissue,[1,2] which is also expressed in multiple human tissues. Early studies have showed that down-regulation of LRIG1 expression is likely to be somewhat associated with tumorogenesis of glioma. After the detection of LRIG1 (GenBank No. AB018349), LRIG2 (GenBank No. AY505340) and LRIG3 (GenBank No. AY505341) have been subsequently detected in normal human brain and rat brain tissues.[3,4] The three genes constitute LRIG gene family. LRIG3 is located at 12q13, which is associated with subtyping of cerebral glioblastoma, indicating the potential association of LRIG3 to cerebral glioma.

Both proliferating cell nuclear antigen (PCNA) and Ki-67 are markers of cellular proliferative activity to analyze and determine cellular growth, differentiation and oncobiological properties of glioma at molecular level. In order to explore the role of LRIG3 gene expression in glioma cell proliferation and relevant mechanism, and to provide theoretical basis for gene therapy of glioma, we detected the expression of Ki-67 nuclear antigen and PCNA in glioma GL15 cells using SABC immunohistochemistry after down-regulating LRIG3.

Materials and Methods

Materials. Glioma cell line GL15 was gifted by Professor Hakan (Umea University, Sweden). GL15 cells were cultured with DMEM complete culture medium (Gibico Co.), containing 10% fetal bovine serum (FBS), in an incubator constaining 5% CO_2 and with a relative humidity of 90% at 37°C. Short-hairpin RNA (shRNA) vector pGenesil2 plasmid (containing G418- and kanamycin-resistant genes) was purchased from Genesil Biotechnology Co., Ltd (Wuhan, China). Trizol was purchased from MRC Co., RT-PCR kit, Taq enzyme and reverse transcriptase M-MLV from Promega Co., rabbit anti-human LRIG3 polyclonal antibody from R&D Co., rabbit anti-human GAPDH antibody, HRP-labeled rabbit anti-goat secondary antibody and goat anti-rabbit antibody from Santa Cruz Co. Goldview was purchased from SBS Genetech Co. (Beijing, China), RIPA lysis buffer from Beyotime Co. and DAB from Sigma Co. Rabbit anti-human Ki-67 monoclonal antibody, mouse anti-human PCNA monoclonal antibody, DAB colorizing kit and ready-for-use SABC kit were purchased from Boster Co. (Wuhan, China).

Methods. <u>Construction and transfection of recombinant plasmid vector and selection of stable cell clone.</u> Two shRNA-transcripting DNA sequences were selected and designed in line with the nucleotide acid sequence of LRIG3 gene provided by GenBank, named as LRIG3-shRNA1 and LRIG3-shRNA2; one unspecific sequence was designed as negative control and named as pGenesil2-negative shRNA. These shRNAs were linked with pGenesil2 plasmid vector to transform competent *E. coli*. Positive clones were selected. Recombinant plasmids were extracted and correct clones were determined through restricted endonuclease Sal I digestion and DNA sequencing. GL15 cells were transfected with three types of recombinant vectors and cultured in complete media containing G418 at screening concentration (600 μg/ml) for ten days. Monoclone was selected with limited dilution and amplified to obtain stable cell clones while G418 in complete media was switched to maintenance concentration (300 μg/ml).

<u>Reverse transcription-polymerase chain reaction (RT-PCR).</u> Primers were designed in line with previous reports and LRIG3 gene sequence retrieved from PubMed and synthesized by Invitrogen Co. (Shanghai, China). The primer sequences for LRIG3 were as follows: 5'-CAC ATC AAT GGA ACC TGG GTA TTT TGA C-3' for sense primer and 5'-GTT TCG GTT CAA TTC GAG ATG TTG CAG TT-3' for antisense primer, with the product of 139 bp. Total RNA was extracted with Trizol using the one-step method. Its concentration and purity were tested with UV spectrometer. Then, 2 μg of total RNA were reversely transcribed with M-MLV reverse transcriptase and amplified through PCR. GAPDDH (230 bp) was amplified as internal control. PCR was performed as follows: pre-denatured at 95°C for 5 min, denatured at 94°C for 40 s, at 57.5°C for 40 s and at 72°C for 60 s in a total of 33 cycles, and finally extended at 72°C for 10 min, with the amplified fragment of 139 bp. LRIG3 target fragments and PCR products of internal control GAPDH were separated on 1.5% agarose gel electrophoresis containing 5% Goldview and examined under UV light.

<u>Western blot test</u>. Cells from the three groups were collected and cleaved with RIPA lysis buffer to harvest proteins for 8% polyacrylamide gel electrophoresis. Proteins were transferred onto NC membrane, blocked with TBST containing 5% fat-free milk powder, and incubated with primary antibodies (LRIG3 1:500, GAPDH 1:1000) at 4°C overnight; rinsed in TBST for three times (10 min for each time), and respectively incubated with rabbit anti-goat and goat anti-rabbit secondary antibodies (HRP-labeled) at room temperature for 1 h; rinsed in TBST again and colorized with DAB.

<u>Detection of cell proliferation with MTT assay.</u> Cells at logarithmic growth phase were dissociated with trypsin and seeded onto a 96-well plate at a density of 5,000 cells (200 μL) per well. For each row (12 wells), four wells were assigned for either shRNA group, three for negative control and one for blank control. At 4 h before test, 20 μL MTT (5 mg/mL) was added into each well. The supernatant was discarded on test and 100 μL DMSO was added into each well. The plate was shook in dark for 15 min. The absorbance at 492 nm (A_{492}) was determined using automatic enzyme labeling instrument. Cell proliferation rate was calculated as follows: $[(A_{exp} - A_{blank}) - (A_0 - A_{blank})] / (A_0 - A_{blank}) \times 100\%$, whereby A_0 referred to the A_{492} for shRNA group at the onset of experiment.

<u>Detection of PCNA and Ki-67 antigens with immunohistochemistry (IHC).</u> Poly-L-lysine-pretreated aseptic cover slips were
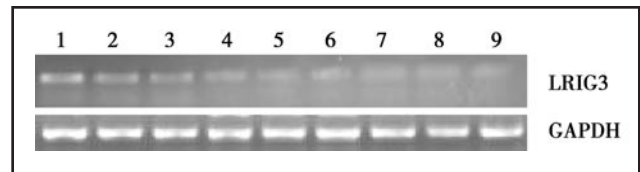

Figure 1. Expression of LRIG3 mRNA in GL15 cells after pGenesil2-LRIG3-shRNA transfection detected by RT-PCR. Lanes 1-3: GL15 cells transfected with pGenesil2-negative-shRNA; lanes 4-6: GL15 cells transfected with pGenesil2-LRIG3-shRNA1; lanes 7-9: GL15 cells transfected with pGenesil2-LRIG3-shRNA2.
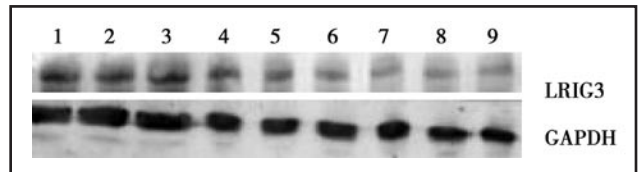

Figure 2. Expression of LRIG3 protein in GL15 cells after pGenesil2-LRIG3-shRNA transfection detected by Western blot. Lanes 1-3: GL15 cells transfected with pGenesil2-negative-shRNA; lanes 4-6: GL15 cells transfected with pGenesil2-LRIG3-shRNA1; lanes 7-9: GL15 cells transfected with pGenesil2-LRIG3-shRNA2.
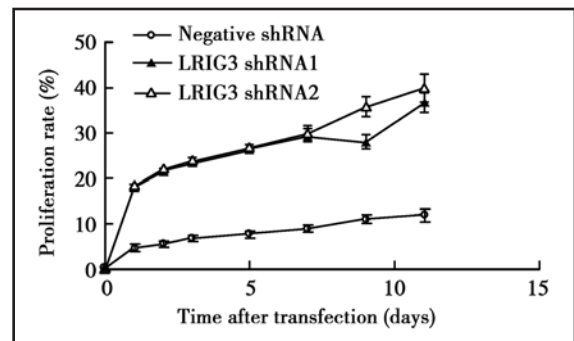

Figure 3. Effect of RNA interference on proliferation rate of GL15 cells

placed into 6-well plates and trypsin-dissociated cells at logarithmic growth phase were seeded into each well and routinely cultured for 24 h. Then, cover slips were taken out, rinsed with PBS for three times (3 min for each time), fixed in 4% paraformaldehyd for 30 min and rinsed with PBS again. IHC was performed according to SABC kit instruction. Cover slips were incubated in mixed buffer of 30% H_2O_2 and anhydrous methanol (1:50) at room temperature for 30 min to deactivate endogenous peroxidase, and rinsed with PBS for three times. Cells were blocked with 5% BSA at room temperature for 20 min and excess serum was discarded without rinse. Mouse anti-human PCNA (1:100) and rabbit anti-human Ki-67 primary antigens were added (PBS instead of primary antibody was added as blank control) for overnight incubation at 4°C. Cells were rinsed with PBS for three times (5 min for each time), incubated with biotinized rabbit anti-mouse IgG and goat anti-rabbit IgG at room temperature for 1 h, and rinsed with PBS again for three times (5 min for each time), then incubated with SABC at 20–37°C for 20 min, rinsed with PBS for four times (5 min for each time), colorized with DAB and rinsed with distilled water, mildly counterstained with
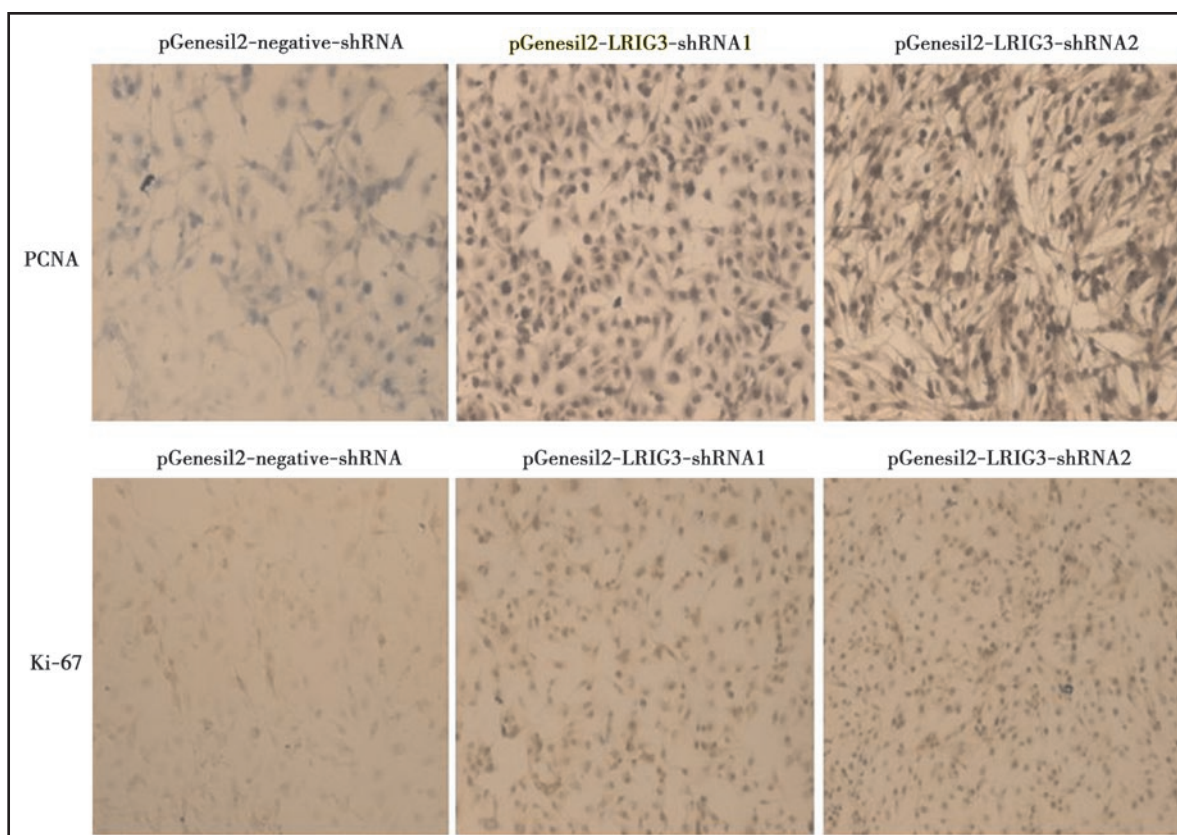
Figure 4. Expression of PCNA and Ki-67 in GL15 cells after RNA interference (Harris hematoxylin ×200). Both proliferating cell nuclear antigen (PCNA) and Ki-67 are localized in nuclei. They are intensely expressed in GL15 cells transfected with pGenesil2-LRIG3-shRNA, but weakly expressed in GL15 cells transfected with pGenesil2-negative-shRNA.

hematoxylin, and subsequently differentiated, dehydrated, hyalinized and mounted. The slips were examined under microscope at high magnification and five independent visual fields were randomly selected. Cells with intranuclear brown particles were taken as positive cells. The total number of cells, and PCNA- and Ki-67-positive cells in each visual field were counted by multi-media imaging analyzer to calculate positive rate. Mean gray value (A) for cells of 5 random visual fields on each slip was analyzed using HMZAS-2000 high-resolution color medical imaging analysis system.

Imaging analysis. Microphotographs were captured using Kodak gel imaging system and analyzed with QuantityOne analysis software Ver. 4.6.

Statistical analysis. All data were expressed as mean ± SD and analyzed with SPSS 10.0 software package. Intergroup comparisons were performed with ANOVA and Student-t test. Correlation of PCNA expression to Ki-67 expression was analyzed by linear correlation analysis.

Results

RT-PCR and Western blot detection of LRIG3 mRNA and protein expression. Compared with negative control group, the mRNA level of LRIG3 was reduced by 52.4% in shRNA1 group and by 63.8% in shRNA2 group, which were significant ($p < 0.01$) (Fig. 1); the protein level of LRIG3 was reduced by 50.9% in shRNA1 group and by 67.4% in shRNA2 group, which were also

significant ($p < 0.01$) (Fig. 2), indicating the effective inhibitory effect of RNAi on expression of LRIG3 gene.

Effects of RNA interference on cell proliferation. From Day 2, cell proliferation rates in both shRNA groups were higher than that in negative control group. After inhibition of LRIG3 expression, cell proliferation rate was significantly increased along with time with significant differences ($p < 0.01$) (Fig. 3 and Table 1).

PCNA and Ki-67 expression in GL15 cells after LRIG3 RNA interference (RNAi). Both PCNA and Ki-67 were positive in shRNA and control groups. Positive cells were distributed unevenly, varying in staining intensity. Positive cell density depended on their malignant degree, proliferation rate, and invasiveness in an increasing manner. Positive GL15 cells, with light staining, were less in negative control group; positive GL15 cells, mostly stained in brownish, were abundant in shRNA groups. The positive rates of PCNA and Ki-67 were significantly higher in shRNA1 and shRNA2 groups than in control group [(72.13 ± 5.64)% and (81.93 ± 5.23)% vs. (35.40 ± 5.69)% for PCNA, $p < 0.01$; (82.27 ± 5.50)% and (88.67 ± 3.52)% vs. (49.73 ± 5.73)% for Ki-67, $p < 0.01$]. The positive rate of PCNA was positively correlated to that of Ki-67 ($r = 0.941$, $p < 0.001$) (Fig. 4).

Correlation of PCNA expression to Ki-67 expression. Both positive rate and protein level of Ki-67 were significantly lower than those of PCNA in each group. PCNA expression was positively correlated to Ki-67 expression ($r = 0.932$, $p < 0.001$).

Discussion

As a member of LRIG gene family,[4] LRIG3 has been previously reported to be down-regulated in multiple tumors with unknown actions.

In this study, glioma GL15 cells, which were stably transfected with plasmid vector carrying U6 promoter and LRIG3-specific shRNA sequence and control plasmid containing unspecific shRNA sequence, were successfully screened out. RT-PCR and Western blot tests showed that the expression of LRIG3 was significantly reduced at both mRNA and protein levels in shRNA groups as compared with that in control group.

The prominent characteristic of cell malignant transformation is loss of growth inhibition, therefore, calculating accurately the proportion of proliferative cells in tumor, named proliferative index, is important role for evaluating tumor cell growth. PCNA, also called cyclin, is firstly detected in the serum of a systemic lupus erythema (SLE) patient by Miyachi et al.[5] in 1978. PCNA is a nuclear protein specifically expressed by proliferating cells as a specific marker of S phase, whose synthesis and expression are substantially related with DNA synthesis, cell proliferation, and growth and invasiveness of tumor cells.[6-8] Evaluating tumor cell proliferating activity can be used to predict its biological behaviors.

Ki-67 is specifically expressed by proliferating cells as a non-histone nuclear protein, which was found in Hodgkin's lymphoma L428 cell line by Gerdes et al.[9] in 1983. Ki-67 is located on the long arm of Chromosome 10 and consists of two peptide chains of 345 ku and 395 ku. Ki-67 monoclonal antibody (MIB-1) can bind proliferating cell nuclei of multiple cell lines, resulting in relocation of endogenous Ki-67 protein, indicating its capacity to control highly sequenced chromosomal structure. Ki-67 expression varies with phases of cell cycle: it isn't expressed in G_0 phase, emerges at mid/late G_1 phase, progressively increases at S and G_2 phases, reaches the peak at M phase and degrades rapidly or loses its antigenic determinants. Such expression pattern of Ki-67 is associated with mitosis-specific phosphorylation. Ki-67 has a short half-life and will be rapidly degraded after proliferative cycle, and can better reflect the proliferative activity, malignancy and prognosis of tumor.[10]

In this study, cell proliferation rate was higher in shRNA groups than in control group, both the variance and proliferation rate were increasing in a time-trend. Both PCNA and Ki-67 were expressed in each group; positive cell density was lower in negative control group than in shRNA groups. Our results showed that the positive rates of PCNA and Ki-67 were increased with the enhanced down-regulation of LRIG3 gene, the positive rate of Ki-67 was higher than that of PCNA. Both positive rate and protein level of Ki-67 were positively correlated to those of PCNA, indicating their consistence in evaluating tumor cell proliferative activity, which was also consistent with previous reports.[7] Ki-67 is a marker for cells of all-proliferative phases, which is expressed at G_1, S, G_2 and M phases except for G_0 phase. PCNA is mainly expressed by cells at late G_1 and S phases. Therefore, PCNA is generally less expressed than Ki-67.[7] Our study showed that compared with negative control group, PCNA-positive cells, with heavy brownish staining, were significantly increased in shRNA groups and the expression of PCNA was also significantly increased. Since the expression of PCNA is significantly related to the biological behaviors of tumor,[7,8] negative control GL15 cells were well differentiated with less proliferative activity and low expression of PCNA; shRNA-transfected GL15 cells were poorly differentiated and more active in DNA replication with higher cell proliferation and high expression of PCNA. PCNA and Ki-67 could be used for estimating the biological behaviors of glioma. Their expression levels could objectively reflect the proliferative activity, growth rate and invasiveness of tumor cells as a currently important approach to investigate cell proliferative kinetics and evaluate the biological behaviors of tumor.[7,8,11,12]

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