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ORIGINAL RESEARCH

RETRACTED ARTICLE: Knockdown of immature colon carcinoma transcript-1 inhibits proliferation of glioblastoma multiforme cells through Gap 2/mitotic phase arrest

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Abstract: "Glioblastoma multiforme" (GBM) is the frequent form of malignant glioma. Immature colon carcinoma transcript-1 (ICT1) is essential for cell vitality and mitochondrial function and has been recognized in several human cancers. In the study reported here, we attempted to evaluate the functional role of ICT1 in GBM cells. Lentivirus-mediated RNA interference (RNAi) was applied to silence ICT1 expression in human GBM cell lines U251 and U87. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and colony formation assays. Cell-cycle progression was determined by flow cytometry with propidium iodide staining. The results revealed that lentivirus-mediated short hairpin RNA (shRNA) could specifically suppress the expression of ICT1 in U251 and U87 cells. Functional investigations showed for the first time, as far as we are aware, that ICT1 knockdown significantly inhibited the proliferation of both cell lines. Moreover, the cell cycle of U251 cells was arrested at Gap 2 (G2)/mitotic (M) phase after ICT1 knockdown, with a concomitant accumulation of cells in the sub-Gap 1 (G1) phase. This study highlights the crucial role of ICT1 in promoting GBM cell proliferation, and provides a foundation for further study into the clinical potential of lentivirus-mediated silencing of ICT1 for GBM therapy.

Keywords: glioblastoma multiforme, immature colon carcinoma transcript-1, lentivirus, proliferation, RNA interference

Introduction

"Glioblastoma multiforme" (GBM) is the frequent form of malignant glioma and the most common primary brain tumor.[1] Malignant gliomas are currently treated by surgery followed by radiotherapy and chemotherapy.[2] Multimodal treatment regimens combining radiation with the DNA alkylating agent temozolomide currently represent the standard of care for newly diagnosed GBM patients.[3–5] However, despite recent advances in therapy, GBM is invariably lethal, with afflicted patients typically dying approximately 2 years, or less, following diagnosis.[6] These data highlight the need to identify new approaches along with the current treatments that will assist in bringing about a better outcome for GBM patients.

Immature colon carcinoma transcript-1 (ICT1) was originally reported as a transcript downregulated during in vitro differentiation of colon carcinoma cell line HT29-D4.[7,8] Subsequent research identified ICT1 to be associated with mitochondrial ribosome recycling factor.[9] Richter et al further showed that ICT1 is a component of the human mitoribosome and has codon-independent peptidyl-transfer RNA hydrolysis activity via its conserved GGQ motif.[10] Depletion of ICT1 causes

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disruption of the mitoribosomal structure and subsequent de novo synthesis of mitochondrially encoded proteins. Recent studies have confirmed the position of ICT 1 at the central protuberance, close to *MRPL15*, -18, and -49.[11] Handa et al indicated that knockdown of ICT1 resulted in apoptotic cell death with a decrease in mitochondrial membrane potential and impaired cytochrome c oxidase activity, indicating that ICT1 is essential for cell vitality and mitochondrial function.[12] Recent research has identified ICT1 as a hub gene for lung cancer via network analysis of gene-expression profile.[13] Therefore, we considered that ICT1 might be worth further investigation to fully characterize its role in human cancers. In the study reported here, we aimed to investigate the biological role of ICT1 in GBM and successfully silenced ICT1 expression in the GBM cell lines U251 and U87 using RNA interference (RNAi) technology. Functional analysis showed that ICT1 knockdown significantly inhibited the cell proliferation, as well as inhibiting Gap 2 (G2)/mitotic (M) phase cell-cycle arrest. This study provides new evidence that ICT1 may play a momentous role in GBM development.

Materials and methods

Cell lines and reagents

The human GBM cell lines U251, U87, U373, and A172 and the human embryonic kidney cell line 293T (HEK293T) were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, People's Republic of China). All the cell lines were cultured in Dulbecco's Modified Eagle's Medium (HyClone™, GE Healthcare UK Ltd, Little Chalfont, UK) supplemented with 10% fetal bovine serum (BioWest, Kansas City, MO, USA) at 37°C with 5% CO_2. Short hairpin RNA (shRNA) expression vector pFH-L and helper plasmids pVSVG-I and pCMVΔR8.92 were purchased from Shanghai Hollybio (Shanghai, People's Republic of China). Lipofectamine 2000 and TRIzol® were purchased from Invitrogen (Carlsbad, CA, USA). Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase was purchased from Promega Corporation (Fitchburg, WI, USA). AgeI, EcoRI, and SYBR Green Master Mix Kits were purchased from TaKaRa Biotechnology (Dalian) Co, Ltd (Dalian, People's Republic China). All other chemicals were obtained from Sigma-Aldrich Co (St Louis, MO, USA). The antibodies used were anti-ICT1 (1:1,000 dilution; Abgent, San Diego, CA, USA), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:80,000 dilution; Proteintech Group, Inc, Chicago, IL, USA), and anti-rabbit HRP-IgG (1:5,000 dilution; Santa Cruz, Dallas, Texas, USA).

Construction of immature colon carcinoma transcript-1 short hairpin RNA containing lentivirus and transduction into glioblastoma multiforme cells

To create the ICT1 shRNA-silenced sub cell line, we used the following shRNA sequences designed against the *ICT1* gene (NM_001545): 5′-GCAGAATGTGAA CAAAGTGAACTCGAGTTCACTTTGTTCACATTC TGCTTTTTT-3′ (S1) and 5′-GCTGTTAATGCTTGTC TATAACTCGAGTTATAGACAAGCATTAACAGC TTTTTT-3′ (S2). The control shRNA sequence was 5′ -GCGGAGGGTTTGAAAGAA ATCTCG GATATT CTTTCAAACCCTCCGCTTTTT 3′. Each nucleotide sequence was inserted into a pFH-L shRNA expressing vector. Lentiviruses were generated by triple transfection of 80% confluent 293T cells with modified pFH-L plasmid and pVSVG-I and pCMVΔR8.92 helper plasmids using Lipofectamine 2000 according to the manufacturer's procedure. Then the lentiviral particles were harvested by ultracentrifugation (50,000 *g* at 4°C) for 10 minutes, filtered through a 45 μm filter, and centrifuged (4,000 *g* at 4°C) again for 5 minutes.

For cell infection, U251 cells were seeded at a volume of 2 mL at a density of 5×10^4 cells/well in six-well plates and transduced with the constructed lentiviruses containing non-silencing shRNA (Lv-shCon) and ICT1 shRNA (Lv-shICT1 [S1]/[S2]) at a multiplicity of infection of 40. The infection efficiency was observed after 96 hours through a fluorescence microscope for green fluorescence protein expression.

Real-time quantitative polymerase chain reaction

Total RNA was extracted from cells using TRIzol reagent and synthesized into complementary DNA (cDNA) by M-MLV Reverse Transcriptase according to the manufacturer's procedure. Real-time quantitative polymerase chain reaction was performed on a Bio-Rad Connect Real-Time PCR (polymerase chain reaction) platform (Bio-Rad Laboratories Inc, Hercules, CA, USA) using an SYBR Green Master Mix Kit. In brief, each PCR reaction mixture, containing 10 μL of 2× SYBR® Premix Ex Taq, 0.8 μL of sense and antisense primers (2.5 μM), 5 μL of cDNA, and 4.2 μL of double-distilled water (ddH_2O), was run for 40 cycles, with each cycle comprising initial denaturation at 95°C for 1 minute, denaturation at 95°C for 5 seconds, and extension at 60°C for 20 seconds. Beta-actin was used as an internal

control. Relative gene-expression levels were calculated using $2^{-\Delta\Delta CT}$ analysis. The primers were:

ICT1 (forward): 5′-CAGCCTGGACAAGCTCTACC-3′
ICT1 (reverse): 5′-GGAACCTGACTTCTGCCTTG-3′
β-actin (forward): 5′-GTGGACATCCGCAAAGAC-3′
β-actin (reverse): 5′-AAAGGGTGTAACGCAACTA-3′.

Western-blot analysis

Cells were lysed in 2× sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl [pH 6.8], 10 mM EDTA, 4% SDS, and 10% glycine). The protein content was measured by the Lowry method. To detect target proteins, equal amounts of protein samples (30 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were incubated with Tris-buffered saline and Tween 20® (TBST; 25 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk at room temperature for 1 hour. After washing them thrice with TBST, the membranes were probed with the primary antibody (an anti-ICT1 rabbit monoclonal antibody (mAb) or an anti-GAPDH rabbit mAb) overnight at 4°C followed by incubation with HRP-linked goat anti-rabbit immunoglobulin (Ig) G secondary antibody for 2 hours at room temperature. The blots were detected with an Electric Chemical Luminescence (ECL) detection kit according to the manufacturer's procedure. GAPDH was used as the reference control.

Cell viability assay

After lentivirus infection, U251 cells were seeded at a volume of 200 μL and density of 2×10^3 cells/well in 96-well plates and were incubated for 1, 2, 3, 4, and 5 days, respectively, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5.0 mg/mL) was added into each well and incubated with the cells for 4 hours. Then 100 μL of acidic isopropanol (10% SDS, 5% isopropanol, and 0.01 mol/L HCl) was added into each well after removing the medium and MTT from the wells. The absorbance was measured using a microplate reader (Varioskan™ LUX multimode microplate reader, Thermo Scientific, CA, USA) at 595 nm.

Colony-formation assay

After lentivirus infection, U251 cells were seeded in a volume of 2 mL at a density of 600 cells/well in six-well plates. The medium was updated every 3 days. After 9 days of culture, the cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. The fixed cells were stained with freshly prepared crystal violet stain for 20 minutes. Colony formation was observed through a light/fluorescence microscope and a count of colonies performed.

Cell-cycle analysis

The cell-cycle distribution was analyzed by flow cytometry using propidium iodide (PI) staining. After lentivirus infection, U251 cells were seeded in a volume of 5 mL at a density of 2×10^5 cells/well in 6 cm dishes. Cells were harvested after 40 hours of culture, and fixed in 70% ice-cold ethanol overnight at 4°C. After washing thrice with PBS, the cells were stained to determine DNA content using 300 μL PBS containing 50 μg/mL PI and 50 μg/mL pre-boiled ribonuclease A. The suspension was incubated in the dark at room temperature for 30 minutes and then subjected to flow cytometry using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed with the ModFit LT DNA analysis program (Version 4 for Modfit LT, Verity Software House, Topsham, ME, USA).

Statistical analysis

Data are presented as mean ± standard deviations (SD) from at least three independent experiments. Statistical analysis was performed using Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

Results

Lentivirus-mediated RNA interference suppressed immature colon carcinoma transcript-1 expression in U251 and U87 cells

To examine the function of ICT1 in GBM, we firstly detected the expression patterns of ICT1 in multiple GBM cell lines – U251, U87, U373, and A172. As shown in Figure 1A, all four cell lines expressed ICT1, but the highest level was observed in U251 cells, followed by U87 cells. Therefore, we chose U251 cells along with U87 cells for subsequent investigation. We applied lentivirus-mediated RNAi to specifically suppress ICT1 in the GBM cell line U251. As shown in Figure 1B, the ratio of cells with green fluorescence protein expression in shRNA-treated cells was more than 80%, indicating a satisfying infection. As shown in Figure 1C, the mRNA level of ICT1 was significantly ($P<0.01$) reduced in Lv-shICT1 (S1)-treated cells, compared with non-treated and Lv-shCon-treated cells. The knockdown efficiency of ICT1 was calculated to be 70% in U251 cells. Moreover, the protein level of ICT1 was obviously downregulated in

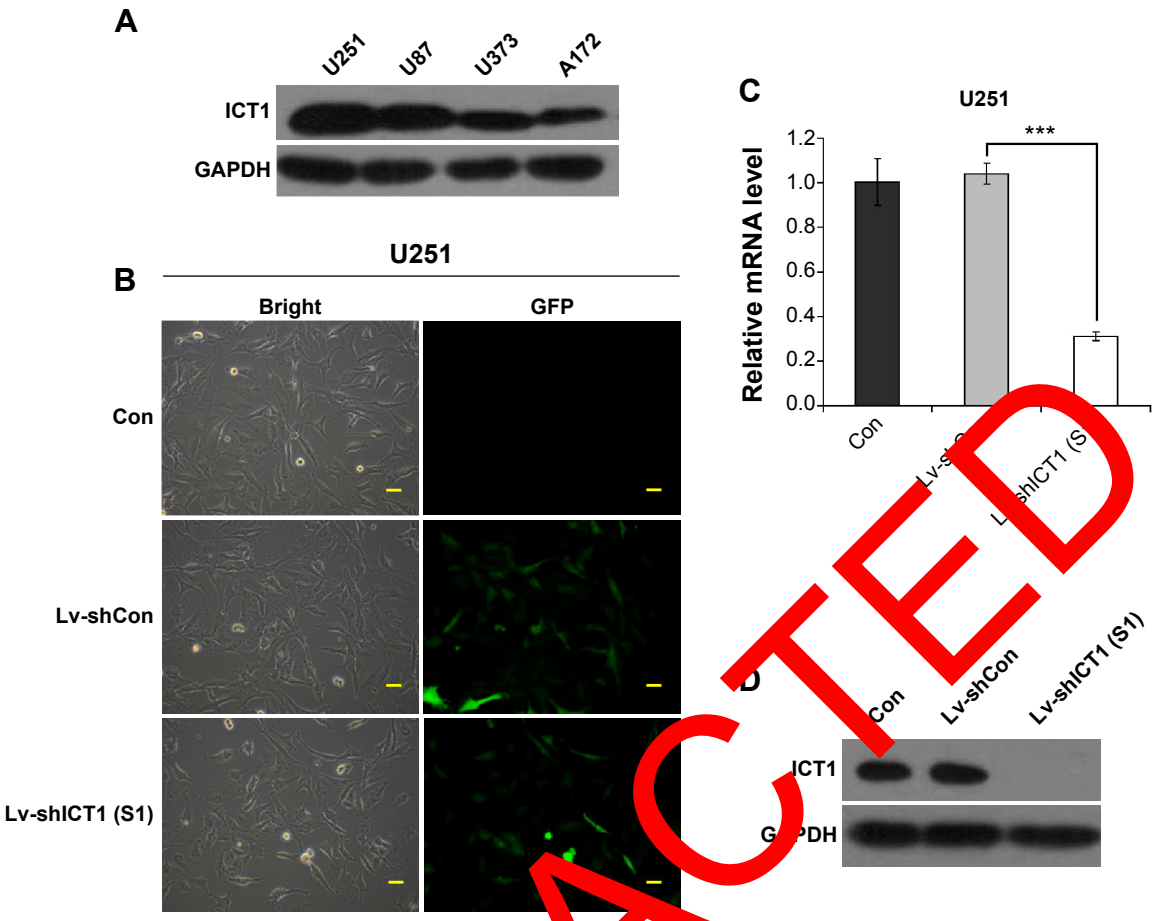

Figure 1 Effect of lentivirus-mediated small hairpin RNA (shRNA) (S1) silencing on immature colon carcinoma transcript-1 (ICT1) expression in U251 cells.
Notes: (**A**) Expression patterns of ICT1 in four glioblastoma multiforme (GBM) cell lines U251, U87, U373, and A172 determined by Western-blot analysis. (**B**) Green fluorescence protein (GFP) expression recorded under a fluorescence microscope. The representative pictures shown are from one of three independent experiments. (**C**) The messenger RNA levels of ICT1 in non-treated, control, infected lentiviruses containing non-silencing small hairpin RNA (Lv-shCon)-treated and Lv-shICT1 (S1)-treated cells determined by RT-qPCR analysis. (**D**) The protein levels of ICT1 in non-treated, Lv-shCon-treated, and Lv-shICT1 (S1)-treated cells determined by Western-blot analysis. Scale bar in (**B**): 100 μm. Data are presented as mean ± standard deviation of three independent experiments performed in triplicate. ***$P<0.01$ versus Lv-shCon.
Abbreviations: con, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Lv-shICT1 (S1)-treated cells, compared with non-treated and Lv-shCon-treated cells (Figure 1D). Similarly, more than 80% of U87 cells expressed green fluorescence protein fluorescence after treatment with Lv-shCon or Lv-shICT1 (S1) (Figure S1A). The expression of ICT1 was also obviously decreased in U87 cells after Lv-shICT1 (S1) infection (Figure S1B). These data indicate that lentivirus-mediated shRNA silencing can efficiently suppress the expression of endogenous ICT1 in U251 and U87 cells.

Silencing of immature colon carcinoma transcript-1 by Lv-shICT1 (S1) inhibited proliferation of U251 and U87 cells

The effect of ICT1 silencing on cell proliferation was firstly assessed by MTT assay. The cell viability was observed for 5 days in non-treated, Lv-shCon-treated, and Lv-shICT1 (S1)-treated cells. As shown in Figure 2A, the growth curve of Lv-shICT1 (S1)-treated cells started to drop from the second day, compared with non-treated and Lv-shCon-treated cells. The decline reached 28.1% ($P<0.001$) and 37.1% ($P<0.01$) on the fourth and fifth days, respectively, compared with Lv-shCon-treated cells, while there was no difference concerning cell viability between non-treated and Lv-shCon-treated cells. The proliferation rate of U87 cells was also markedly ($P<0.001$) decreased by ICT1 knockdown (Figure S1C). The data indicate that ICT1 knockdown significantly inhibited the proliferation of U251 and U87 cells.

The long-term effect of ICT1 silencing on cell proliferation was determined by colony-formation assay. As shown in Figure 2B, the size of independent colonies was much smaller in Lv-shICT1 (S1)-treated cells than in non-treated and Lv-shCon-treated cells. Moreover, the number of colonies that formed in U251 cells was significantly ($P<0.001$) decreased

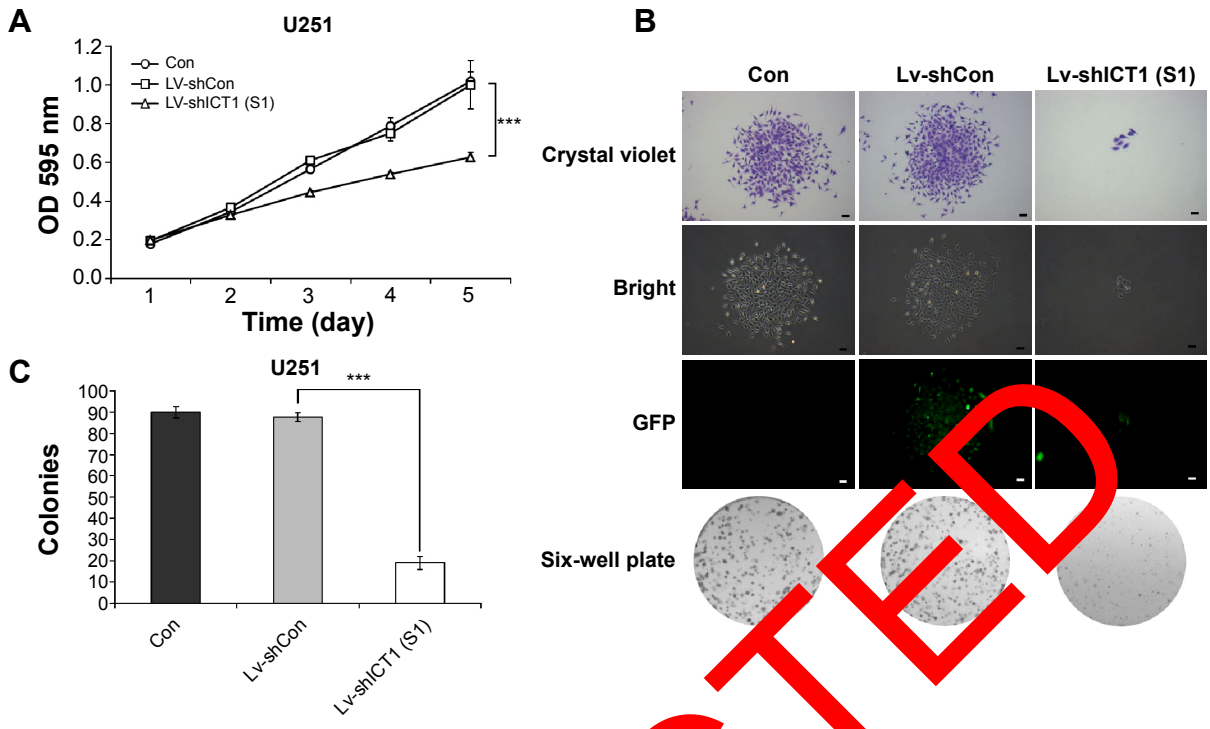

Figure 2 Effects of Lv-shICT1 (S1) on proliferation of U251 cells.
Notes: (**A**) Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. (**B**) The size and number of colonies in U251 cells recorded under a fluorescence microscope. The representative pictures shown are from one of three independent experiments. (**C**) Statistical analysis of colony numbers in non-treated, constructed lentiviruses containing non-silencing small hairpin RNA (Lv-shCon)-treated, and Lv-shICT1 (S1)-treated cells. Scale bar in (**B**): 250 μm. Data are presented as mean ± standard deviation of three independent experiments performed in triplicate. ***$P<0.001$ versus Lv-shCon.
Abbreviations: con, control; GFP, green fluorescence protein; OD, optical density.

after ICT1 knockdown (Figure 2C). The data indicate that ICT1 knockdown can also significantly inhibit the colony formation of U251 cells.

Silencing of immature colon carcinoma transcript-1 by Lv-shICT1 (S2) inhibited proliferation of U251 cells

The knockdown efficiency of ICT1 by the other recombinant lentivirus Lv-shICT1 (S2) was also detected in U251 cells by real-time quantitative polymerase chain reaction analysis. We observed a 89.3% reduction in ICT1 expression in Lv-shICT1 (S2)-treated cells (Figure 3A). Besides, the protein level of ICT1 was downregulated after Lv-shICT1 (S2) treatment (Figure 3B), which proved that the recombinant lentiviruses in our study specifically targeted ICT1 in U251 cells. MTT assay showed that cell viability significantly ($P<0.001$) declined in Lv-shICT1 (S2)-treated cells (Figure 3C). Moreover, the number of colonies was reduced by 46.1% in Lv-shICT1 (S2)-treated cells, compared with in Lv-shCon-treated cells (Figure 3D). These data indicate that ICT1 knockdown in U251 cells indeed disrupted proliferation of GBM cells.

Immature colon carcinoma transcript-1 knockdown arrested U251 cells at Gap 2/mitotic phase and Sub-Gap 1 phase

To investigate the mechanisms underlying the growth suppression effect of ICT1 knockdown, the cell-cycle distribution of U251 cells was analyzed using a flow cytometer. The results shown in Figure 4A, 4B and 4C indicate that Lv-shICT1 (S1)-treated cells presented an increased G2/M-phase population and decreased Gap 0 (G0)/Gap 1 (G1)-phase and synthesis (S)-phase populations ($P<0.001$), compared with non-treated and Lv-shCon-treated cells. The data reveal that ICT1 knockdown can arrest cell cycle at G2/M phase. Furthermore, we observed an obvious increase of cell percentage in Sub-G1 phase. Taken together, these data suggest that ICT1 knockdown can suppress GBM cell growth via blockade of cell-cycle progression.

Discussion

ICT1 has been recognized as a codon-independent but ribosome-dependent and ribosome-integrated peptidyl-transfer RNA hydrolase.[14] A recent study has reported that ICT1 played an essential role in the progression of lung cancer. In the study reported here, we identified ICT1 as a

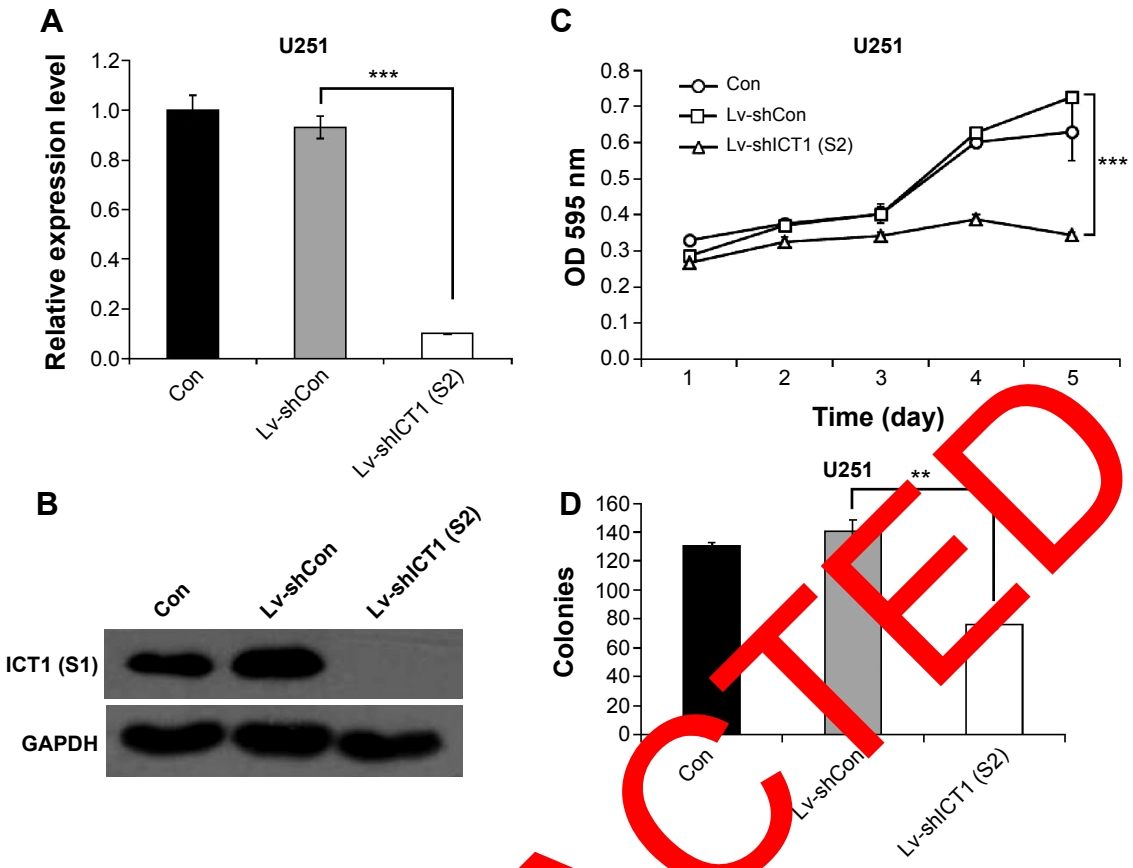

Figure 3 Effects of Lv-shICT1 (S2) on proliferation of U251 cells. The expression levels of immature colon carcinoma transcript-1 (ICT1) in non-treated, constructed lentiviruses containing non-silencing small hairpin RNA (Lv-shCon)-treated, and Lv-shICT1 (S2)-treated cells were determined by RT-qPCR analysis (**A**) and Western-blot analysis (**B**). (**C**) Statistical analysis of cell-proliferative rates in non-treated, Lv-shCon-treated, and Lv-shICT1 (S2)-treated cells. (**D**) Statistical analysis of colony numbers in non-treated, Lv-shCon-treated, and Lv-shICT1 (S2)-treated cells. Data are presented as mean ± SD of three independent experiments performed in triplicate. $^{**}P<0.01$, $^{***}P<0.001$ versus Lv-shCon.

Abbreviations: con, control; OD, optical density; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

specific molecule that can drive GBM progression. Using lentivirus-mediated shRNA silencing, we potently suppressed the expression of ICT1 both at the mRNA and protein levels in the GBM cell line U251. ICT1 knockdown significantly inhibited the proliferation of U251 and U87 cells.

Previous studies have shown that depletion of ICT1 using ICT1-specific siRNA resulted in reduction of mitochondrial protein synthesis, leading to a loss of cell viability as well as mitochondrial dysfunction.[10,12] The lack of ICT1 in HeLa cells inhibited cell proliferation, which was due to cell-cycle arrest and apoptotic cell death.[12] The lack of ICT1 also reduced translational efficiency in mitochondria.[10] Accordingly, it is thought that subunits of respiratory complexes coding for 13 genes (in mammals) of mitochondrial DNA cannot be synthesized sufficiently to make an adequate electrochemical proton gradient across the inner membranes. Accumulation of these defective mitochondria would lead to apoptosis.[15] Decreases in mitochondrial membrane potential and mitochondrial mass are often observed as an early event of apoptosis.[16,17]

Herein, PI staining combined with flow cytometry analysis was then performed to determine whether ICT1 knockdown using lentivirus-mediated RNAi blocked cell-cycle progression in U251 cells. In accordance with previous studies, ICT1 knockdown significantly arrested U251 cells at G2/M phase and Sub-G1 phase. Sub-G1 phase cells are usually considered to be the result of apoptotic DNA fragmentation: during apoptosis, the DNA is degraded by cellular endonucleases. Therefore, nuclei of apoptotic cells contain less DNA than nuclei of healthy G0/G1 cells, resulting in a sub-G1 peak in the fluorescent histogram that might be used to determine the relative amount of apoptotic cells.[18] Therefore, the growth suppression caused by ICT1 knockdown was due to cell-cycle arrest and apoptosis.

Conclusion

Our study may provide more insight into the role of ICT1 in cancer. We suggest that elevated ICT1 is a critical molecular event associated with gliomagenesis. The potential application of ICT1 targeted therapy using a lentivirus-mediated

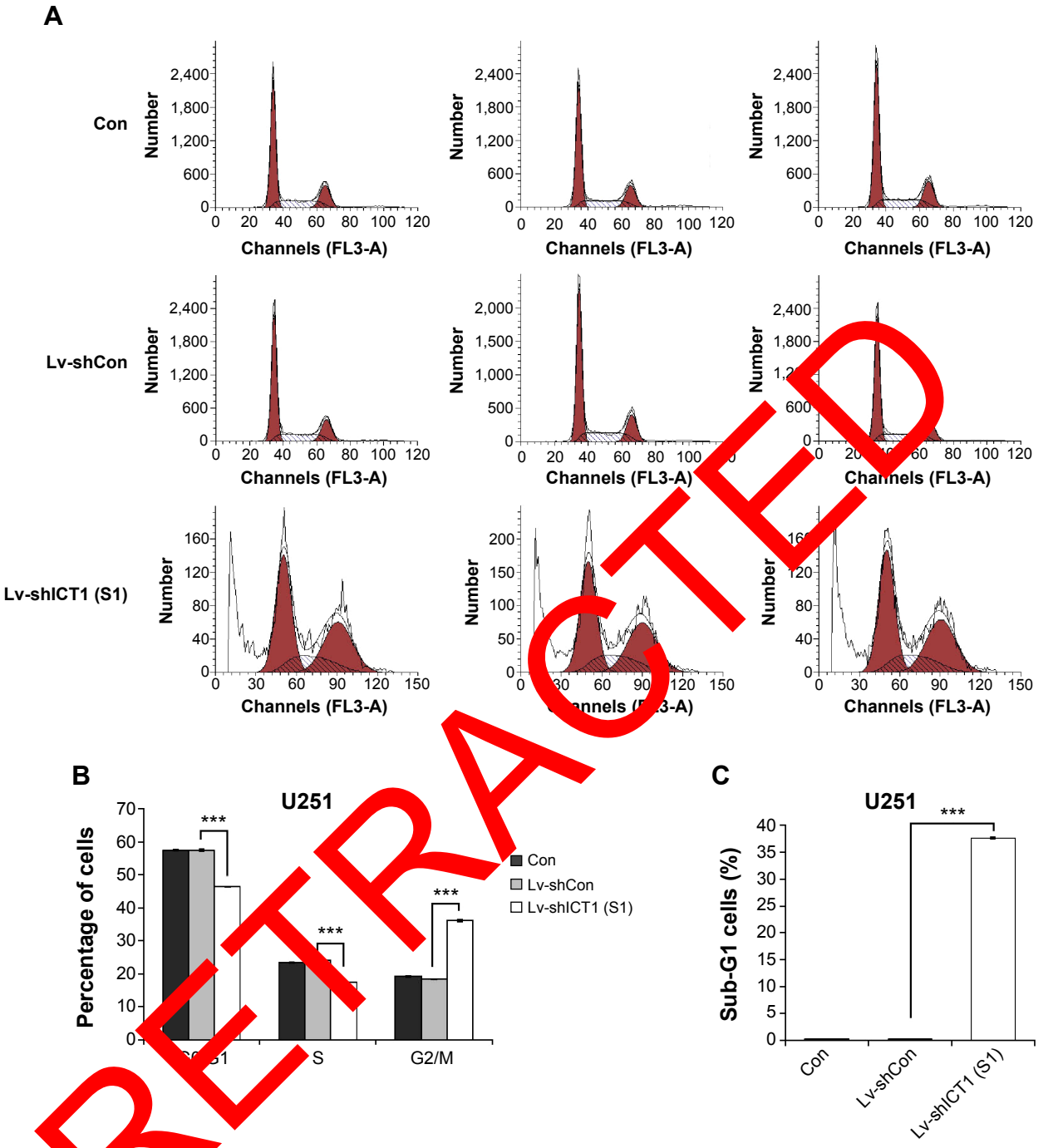

Figure 4 Effect[illegible]mmature colon carcinoma transcript-1 (ICT1) knockdown on the cell cycle of U251 cells.
Notes: (**A**) Flow c[illegible]metric analysis of the cell cycle. The representative graphs shown in Figure A are from one of three independent experiments. (**B**) Statistical analysis of G0/G1-phase, S-phase[illegible]d G2/M-phase populations in non-treated, constructed lentiviruses containing non-silencing small hairpin RNA (Lv-shCon)-treated, and Lv-shICT1 (S1)-treated cells. (**C**) Statistical analysis of Sub-G1-phase populations in non-treated, Lv-shCon-treated, and Lv-shICT1 (S1)-treated cells. Data are presented as mean ± standard deviation of three independent experiments performed in triplicate. ***$P<0.001$ versus Lv-shCon.
Abbreviation: con, control.

shRNA approach will need further investigation in preclinical and clinical studies.

Acknowledgments

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Disclosure

The authors declare no conflicts of interest in this work.

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Supplementary material

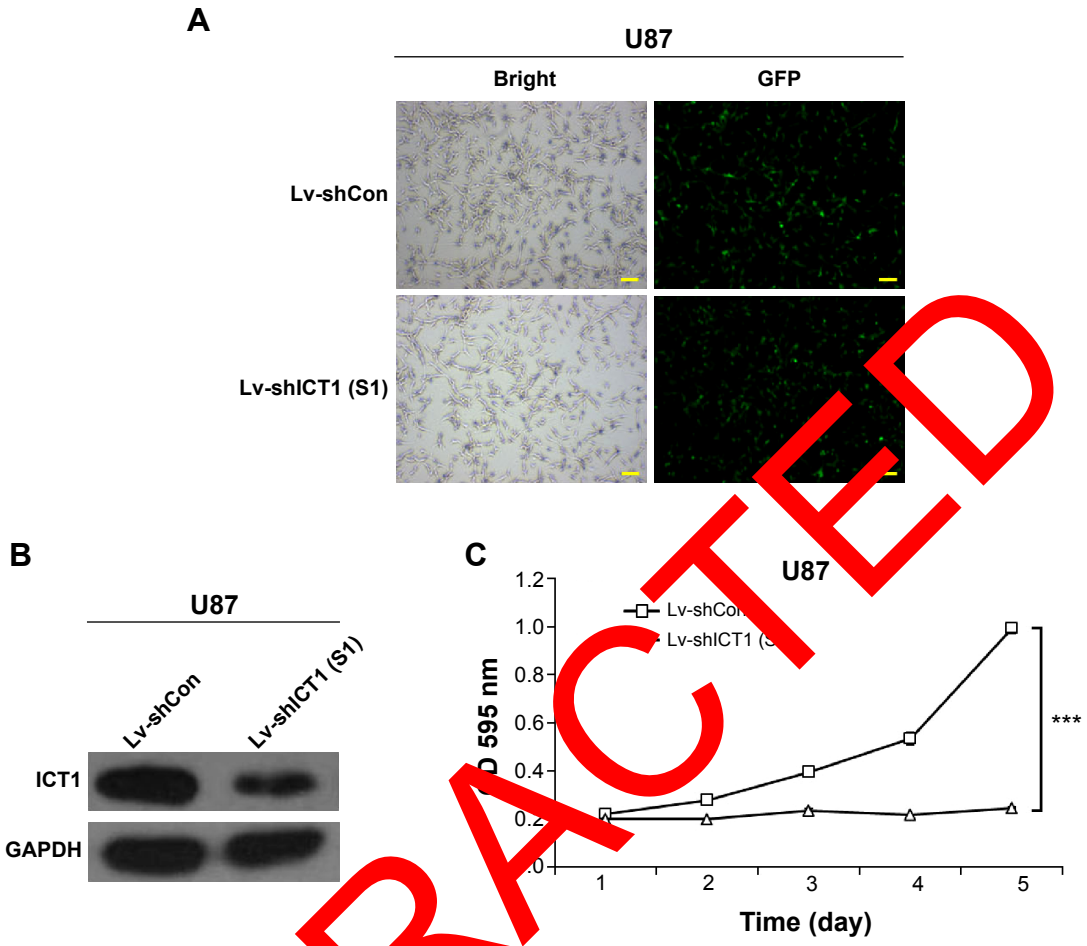

Figure S1 Effect of Lv-shICT1 (S1) on proliferat of U8
Notes: (**A**) Green fluorescence protein (GFP) expression rec ed under a fluorescence microscope. The representative pictures shown are from one of three independent experiments. (**B**) The protein levels of im colon carcino transcript-1 (ICT1) in constructed lentiviruses containing non-silencing small hairpin RNA (Lv-shCon)-treated and Lv-shICT1 (S1)-treated ce determ d by Western-b analysis. (**C**) Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Scale bar in (**A**): 100 μm. D are presented as mean ± standard deviation of three independent experiments performed in triplicate. ***$P<0.01$ versus Lv-shCon.
Abbreviations: con, control; OD, optical density; GAPD glyceraldehyde 3-phosphate dehydrogenase.