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LRIG1 dictates the chemo-sensitivity of temozolomide (TMZ) in U251 glioblastoma cells via down-regulation of EGFR/topoisomerase-2/Bcl-2

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ABSTRACT

In the current study, we aimed to understand the potential role of leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) in TMZ-resistance of U251 glioma cells. We established TMZ-resistant U251 clones (U251/TMZ cells), which expressed low level of LRIG1, but high levels of epidermal growth factor receptor (EGFR), topoisomerase-2 (Topo-2) and Bcl-2. Depletion of LRIG1 by the targeted RNA interference (RNAi) upregulated EGFR/Topo-2/Bcl-2 in U251 cells, and the cells were resistant to TMZ. Reversely, over-expression of LRIG1 in U251 cells downregulated EGFR/Topo-2/Bcl-2 expressions, and cells were hyper-sensitive to TMZ. Our data suggested EGFR-dependent mammalian target of rapamycin (mTOR) activation was important for Topo-2 and Bcl-2 expressions in U251/TMZ cells. The EGFR inhibitor and the mTOR inhibitor downregulated Topo-2/Bcl-2 expressions, both inhibitors also restored TMZ sensitivity in U251/TMZ cells. Finally, inhibition of Topo-2 or Bcl-2 by targeted RNAi(s) knockdown or by the corresponding inhibitor re-sensitized U251/TMZ cells to TMZ, indicating that both Topo-2 and Bcl-2 were important for TMZ resistance in the resistant U251 cells. Based on these results, we concluded that LRIG1 inhibits EGFR expression and the downstream signaling activation, interferes with Bcl-2/Topo-2 expressions and eventually sensitizes glioma cells to TMZ.

1. Introduction

Glioblastoma multiforme (GBM) is the most malignant form of brain tumor with poor prognosis [1]. The current standard care for GBM is postoperative radiation and/or temozolomide (TMZ), with a median survival of approximately 14 months [2]. One key hurdle is the molecular heterogeneity of GBM, which impedes uniform application of specific molecularly targeted agents [3,4]. One pathway that is frequently dysregulated in GBM is epidermal growth factor receptor (EGFR) [5,6]. It has been reported that resistance of GBM is associated with EGFR overexpression [6]. EGFR amplification is an important poor prognostic factor in GBM patients [5].

Activation of EGFR by its ligand (i.e.EGF) promotes cancer cell progression through activation of multiple downstream signaling cascades including the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) cascade, the Ras–mitogen-activated protein kinase (MAPK) cascade and the phospholipase C γ (PLC γ) cascade [7,8]. Meanwhile, activated EGFR also undergoes self-degradation [9]. Studies have identified c-Cbl as an E3 ubiquitin ligase to tag ligand-activated EGFR with ubiquitin, thereby promoting their lysosome sorting and degradation [9].

Interestingly, recent studies have identified a group of negative regulators of EGFR, which are transcriptional up-regulated following receptor activation [10]. They form a complex with EGFR and eventually lead to EGFR degradation [10]. One of the most studied negative regulator of EGFR is leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) [11,12]. Studies first reported an inverse association between LRIG1 and EGFR in human cancer cells, which raised the possibility that LRIG1 might act as an endogenous suppressor of EGFR [13,14]. Following studies have shown that LRIG1 forms a complex with EGFR to promote receptor ubiquitination and degradation [11,12,15,16].

In the current study, we aimed to indentify TMZ resistance factors by focusing on LRIG1. We found that LRIG1 dictates the chemo-sensitivity of TMZ in cultured glioblastoma cells via down-regulation of EGFR, Topoisomerase-2 (Topo-2) and Bcl-2.

2. Material and methods

2.1. Chemical and reagents

Temozolomide (TMZ) was purchased from Sigma (St. Louis, MO, USA). Rapamycin, PD 153035 and AG 1478 were purchased from

Abbreviations: EGFR, Epidermal growth factor receptor; GBM, glioblastoma multiforme; RNAi RNA interference; LRIG1, , leucine-rich repeats and immunoglobulin-like domains 1; PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; TMZ, temozolomide; Topo-2, topoisomerase-2.

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Calbiochem (Darmstadt, Germany). ABT-737 was purchase from selleck (Shanghai, China). Anti-EGFR, LRIG1, Topo-2 and Bcl-2 antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA), Mouse monoclonal antibody against β-actin was purchased from Sigma (Sigma, Shanghai, China). Anti phospho- and total-S6 antibodies were obtained from Cell Signaling Tech (Denver, MA).

2.2. Cell Culture

U251 glioblastoma cells were maintained in RPMI 1640 (Sigma, St. Louis, MO, USA), supplemented with 10% FBS (Sigma), penicillin/streptomycin (1:100; Sigma) and 4 mM L-glutamine (Sigma), in a CO_2 incubator at 37 °C.

2.3. Generation of TMZ-resistant U251 cells

The parental U251 cells were exposed to 100 μM of TMZ for 2 weeks to generate TMZ-resistant colonies. The TMZ containing medium was switched every three days. The majority of the cells died, but a small population survived and propagated. The surviving colonies were selected and established as TMZ-resistant U251 clones (U251/TMZ cells).

2.4. Cell viability assay (MTT assay)

The cell viability was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) assay as reported[17].

2.5. Trypan blue staining "dead" cells

The trypan blue dye was used to stain the "dead" U251 cells after indicated treatment, its percentage was calculated.

2.6. TUNEL staining

The U251 cell apoptosis was detected by the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) In Situ Cell Death Detection Kit (Roche Molecular Bio-chemicals, Indianapolis, IN, USA) according to the manufacturer's protocol. Briefly, U251 cells were stained with the fluorescence dye TUNEL and Hoechst 33342. The apoptosis percentage was reflected by TUNEL percentage, which was calculated by the number of TUNEL positive cells divided by the number of Hoechst 33342 stained cells.

2.7. Western blots

Western blot were performed as previous reported [8].

2.8. RNA interference(RNAi)

The sequences of the LRIG1-targeting RNAi were 5′-GAUCAUCACCCAGCCUGAG-3′ (siLRIG1–1) [18] and 5′- GGCCUACCUUUCCUUAGAA-3′ (siLRIG1–2) [19]. The sequences of the Bcl-2-targeting RNAi were 5′-GCCCUGAUUGUGUAUAUUCA-3′ [20] and 5′-GUACGAUAACCGGGAGAUA-3′ [21]. RNAi sequences were synthesized by KeyGen Biotech (Nanjing, China). The siRNA duplexes against Topo-2 were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Fugene 6 (Roche, Mannheim, Germany) was applied to transfect RNAi (20 μM) into cultured U251 cells based on the manufacturer's protocol. Same amount of scramble non-sense siRNA (siNS, Santa Cruz Biotech, Santa Cruz, CA) was transfected into control cells. 40–80 h after transfection, the expression of target protein in transfected cells was examined by Western blots.

2.9. LRIG1. plasmid construction and transfection

The LRIG1 expression vector was constructed by inserting human LRIG1 cDNA (purchased from Shanghai Future Biotech, Shanghai, China) into the pcDNA3 (Invitrogen, Shanghai, China) expressing vector. The plasmid was amplified with JM 109 bacteria, extracted, and purified by the Plasmid Midi Kit (Qiagen, Shanghai, China). For transfection, U251 cells were cultured in antibiotic- and serum-free Opti-MEM medium (Invitrogen, Shanghai, China) with 50–60% confluence, the LRIG1 plasmid (2 μg/well) or the empty vector (pcDNA3, 2 μg/well) was transfected into U251 cells with the lipofectamine™ 2000 (Invitrogen, Shanghai, China) protocol. LRIG1 expression in transfected cells was tested by the Western blot.

2.10. Statistical analysis

The data were presented as mean ± standard deviation (SD). The statistical differences were analyzed by one-way ANOVA followed by multiple comparisons performed with post hoc Bonferroni test (SPSS version 15). Values of $p < 0.01$ were considered statistically significant. The significance of any differences between two groups was tested using paired-samples t test when appropriated.

3. Results

3.1. TMZ resistant U251 cells express low level of LRIGI, but high levels of EGFR, Topo-2 and Bcl-2

Using the method described above, we created three clones of TMZ-resistant U251 cells (U251/TMZ cells, clone-1,-2,-3). The results in Fig. 1A and B confirmed TMZ resistance in the U251/TMZ cells (clone-1), as no significant cell viability loss and apoptosis were achieved in these cells treated by TMZ (Fig. 1A and B). We tested the levels of LRIG1, EGFR, Topo-2 and Bcl-2 in U251/TMZ clones and their parental U251 cells. As shown in Fig. 1C–E, in all three resistant clones, LRIG1 was downregulated, but EGFR, Topo-2 and Bcl-2 were upregulated (F). The results in Fig. 1G (for clone-2) and Fig. 1H (for colon-3) once again confirmed the TMZ resistance in the U251/TMZ cells.

3.2. U251 cells with LRIGI overexpression are hyper-sensitive to TMZ

We next exogenously expressed LRIG1 in regular U251 cells. The Western blot results in Fig. 2A confirmed LRIG1 overexpression in the transfected cells (U251/LRIG1 cells). Meanwhile, the expressions of EGFR, Topo-2 and Bcl-2 were significantly downregulated in U251/LRIG1 cells (Fig. 1A and B). The results in Fig. 2C–E confirmed that U251/LRIG1 cells were hyper-sensitive to TMZ. And we observed more cell viability loss (Fig. 2C), trypan blue staining (Fig. 2D) and TUNEL positive cells (Fig. 2E) after TMZ treatment in these cells. These results suggested that overexpression of LRIG1 down-regulates EGFR, Topo-2 and Bcl-2, while increasing TMZ sensitivity in U251 cells.

3.3. U251 cells with LRIGI knocking-down are resistant to TMZ

Two different non-overlapping siRNAs (siLRIG1–1 and siLRIG1–2) against LRIG1 were applied to efficiently knockdown LRIG1 in U251 cells (Fig. 3A, U251/siLRIG1 cells). Correspondingly, EGFR, Topo-2 and Bcl-2 expressions were increased in the U251/siLRIG1 cells, further suggesting that LRIG1 is the negative regulator of EGFR, Topo-2 and Bcl-2. Interestingly, U251 cells with LRIG1 knocking-down (siLRIGI-1 and -2) grew faster than U251 cells transfected with non-sense siRNA (U251/siNS cells)

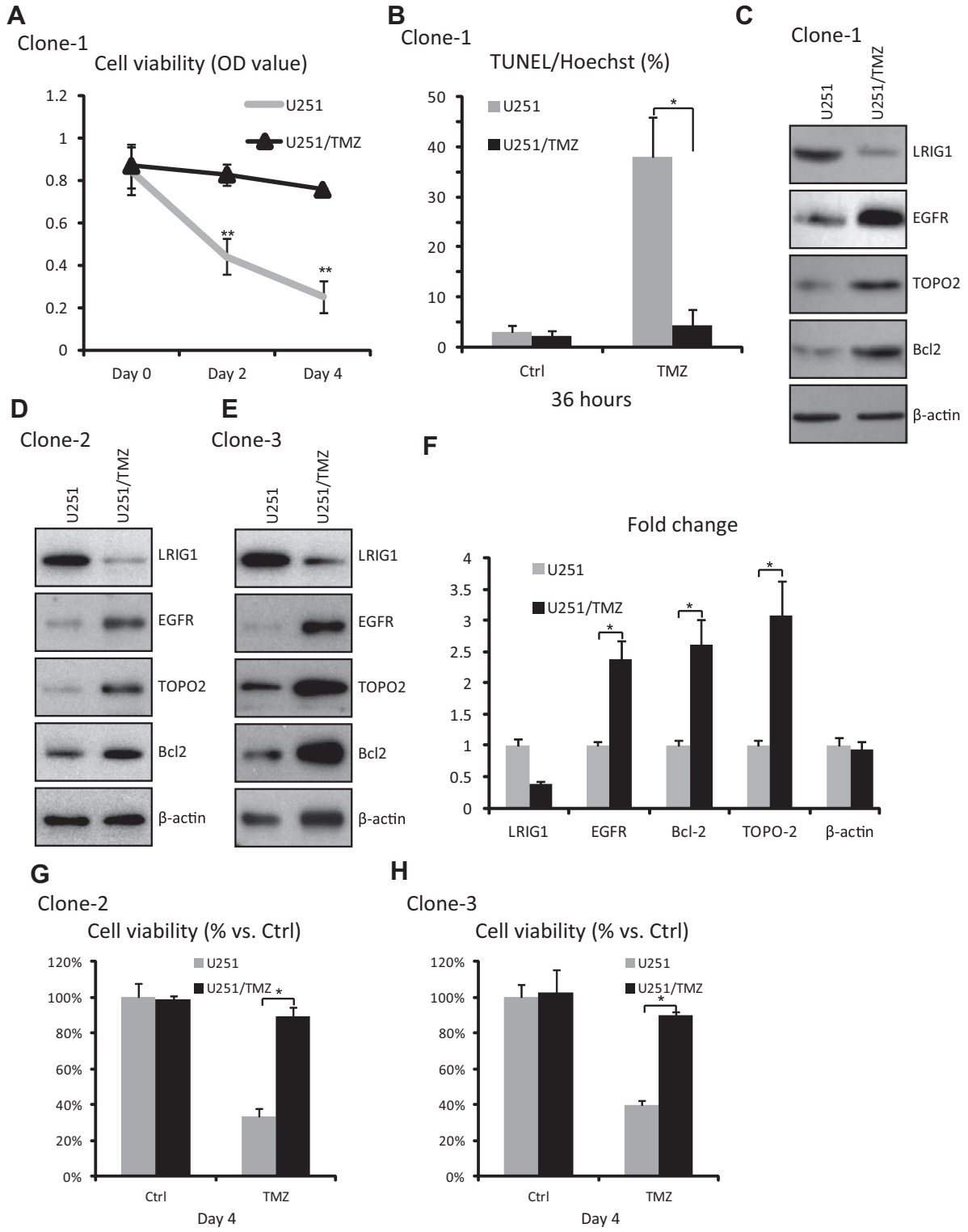

Fig. 1. TMZ resistant U251 cells have low level of LRIG1, but high levels of EGFR, Topo-2 and Bcl-2. The TMZ-resistant U251 cells (U251/TMZ, clone-1, -2 and -3) and their respective parental cells were incubated in TMZ (100 μM) containing medium for indicated time point, the cell viability was examined by MTT assay (A, G and H), and cell apoptosis was measured by TUNEL staining (B, for clone-1). Reprehensive immuno-blots showing the expressions of LRIG1, EGFR, Topo-2, Bcl-2 and β-actin in TMZ-resistant U251 cells (U251/TMZ, clone-1, -2 and -3) and theor respective parental U251 cells (C–E). The blot intensity was quantified by the densitometry using Image System (Bio-Rad) and was normalized to loading control (β-actin) (F). Experiments in this figure were repeated four times. Data were presented as mean ± SD. $^{*}p < 0.01$. $^{**}p < 0.01$ *vs.* U251/TMZ cells (A).

(Fig. 3B). More importantly, U251/siLRIG1 cells were resistant to TMZ, and we saw less cell viability loss and TUNEL staining (apoptosis) by TMZ in U251/siLRIG1 cells (Fig. 3C and D). Together, these results suggested that U251 cells with LRIG1 silencing are resistant to TMZ.

3.4. EGFR-dependent mTOR activation is important for Topo-2 and Bcl-2 expression and TMZ resistance in U251/TMZ cells

As shown in Fig. 4A and B, AG 1478 and PD 153035, two pharmacological inhibitors of EGFR, totally abolished EGFR

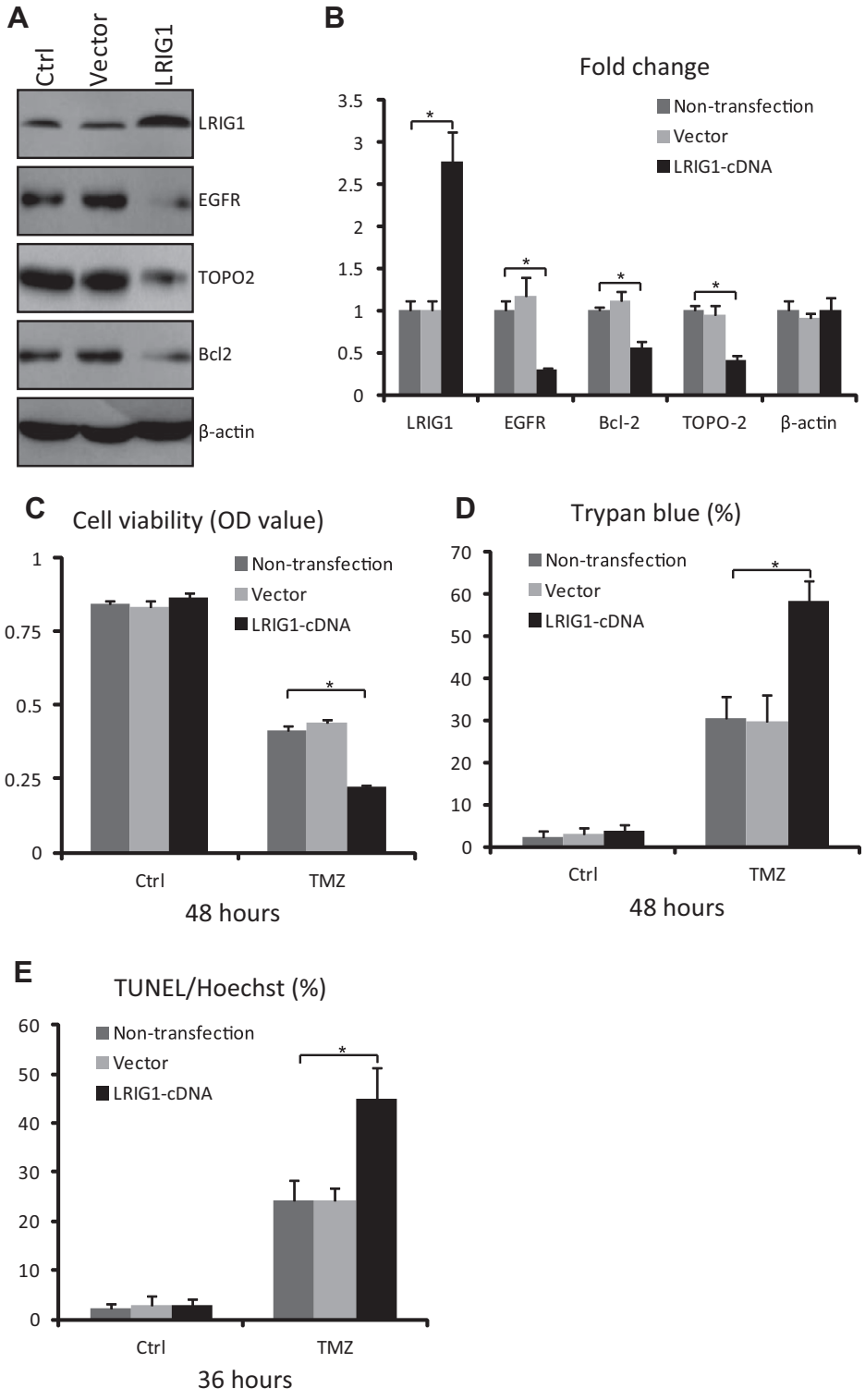

Fig. 2. U251 cells with LRIG1 overexpression are hyper-sensitive to TMZ. Reprehensive immuno-blots showing the expressions of LRIG1, EGFR, Topo-2, Bcl-2 and β-actin in non-transfected control U251 cells (Ctrl), pcDNA3 (vector, 2 μg/well) transfected U251 cells or LRIG1-cDNA (LRIG1, 2 μg/well) transfected U251 (A). The blot intensity was quantified as described (B). Ctrl, vector- or LRIG1- transfected U251 cells were treated with TMZ (100 μM), cell viability was examined by MTT assay (C), trypan blue positive cells were recorded (D), TUNEL staining was also performed (E). Experiments in this figure were repeated four times. Data were presented as mean ± SD. $^{*}p < 0.01$.

phosphorylation (activation) in U251/TMZ cells. Significantly, Topo-2 and Bcl-2 were down-regulated by EGFR inhibitors. The mTOR inhibitor rapamycin also downregulated Topo-2/Bcl-2 expression in U251/TMZ cells (Fig. 4B and C). Meanwhile, both EGFR inhibitors and rapamycin blocked S6 phosphorylation (an indicator of mTOR activation) in these cells (Fig. 4B and C). These results suggested that EGFR-dependent mTOR activation is important for Topo-2/Bcl-2 expression (Fig. 4B and C). Importantly, both AG 1478 or rapamycin re-sensitized U251/TMZ cells to TMZ (Fig. 4D and E), as significant cell viability loss and

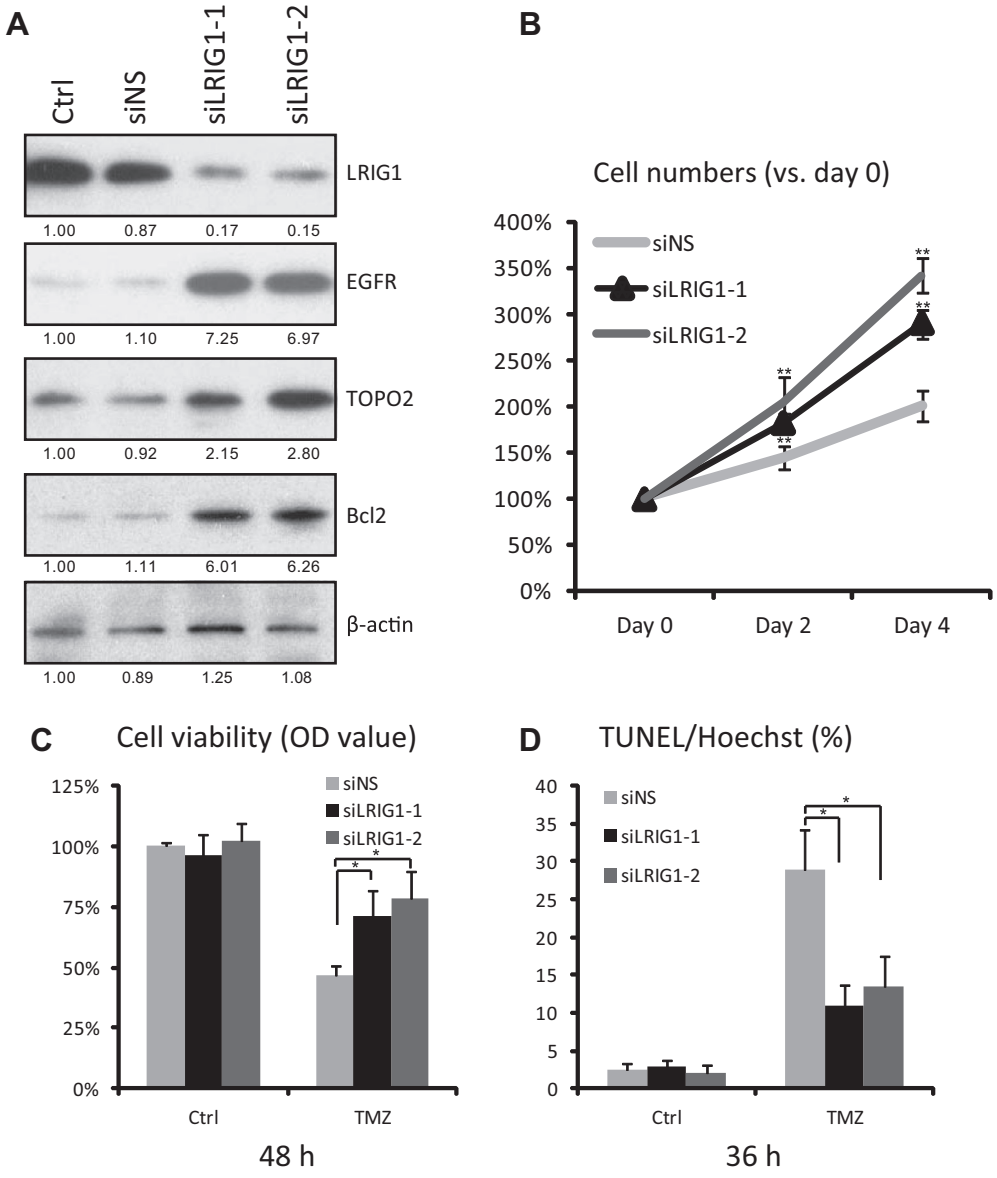

Fig. 3. U251 cells with LRIG1 knocking-down are resistant to TMZ. Reprehensive immuno-blots showing the expressions of LRIG1, EGFR, Topo-2, Bcl-2 and β-actin in control U251 cells (Ctrl), non-sense (NS) siRNA- (siNS), LRIG1 RNAi-1-(si LRIG1–1) or LRIG1 RNAi-2- (si LRIG1–2) transfected U251 cells (A). Blot intensity was quantified (A). Same number of siNS, siLRIG1–1 and siLRIG1–2 U251 cells (1×10^4) cells were cultured in 10% FBS medium for 2 and 4 days, the total cell number was counted (B). siNS, siLRIG1–1- and siLRIG1–2- transfected U251 cells were incubated in TMZ (100 μM) containing medium for indicated time point, cell viability was examined by MTT assay (C), TUNEL positive cells were also recorded (D). Experiments in this figure were repeated four times. Data were presented as mean ± SD. $^{*}p < 0.01$. $^{**}p < 0.01$ *vs.* siNS control cells (B).

apoptosis by TMZ were re-occurred when these inhibitors were presented (Fig. 4D and E).

3.5. Topo-2 and Bcl-2 inhibition restores TMZ sensitivity in resistant U251 cells

Our results showed that Topo-2 and Bcl-2 were up-regulated in U251/TMZ cells (Fig. 1). Further, Topo-2 and Bcl-2 were also up-regulated in LRIG1 knockdown cells (Fig. 3). These results suggested that Topo-2 and Bcl-2 might be the key factors contributing to TMZ-resistance in U251/TMZ cells. To test this hypothesis, we used the targeted RNAi(s) to interfere Topo-2 and Bcl-2 expressions in U251/TMZ cells. Significantly, after RNAi(s)-medicated knockdown of Topo-2 (Fig. 4F and G) or Bcl-2 (Fig. 4L), TMZ-induced cytotoxic effect was restored in U251/TMZ cells (Fig. 4H, I, M and N). Further, VP-16, the Topo-2 inhibitor, also resorted TMZ sensitivity in U251/TMZ cells (Fig. 4J and K). Also, the Bcl-2 inhibitor ABT737 exerted similar effect as VP-16 and restored TMZ cytotoxicity in U251/TMZ cells (Fig. 4M and N). Together, these results indicated that Topo-2/Bcl-2 inhibition restores sensitivity of TMZ in the resistant U251 cells, and Topo-2 and Bcl-2 might be the key downstream signals of EGFR to mediate resistance against TMZ.

4. Discussion

In the past 20 years, TMZ and many anti-cancer drugs with methylating properties have brought broad attention in the treatment of GBMs. The main target of these drugs is the DNA, TMZ is known to induce a dozen DNA methylation products. Studies with O^6-methylguanine-DNA methyltransferase (MGMT)-deficient cells [22] and MGMT-transfected isogenic cells [23] revealed that one of the lesions, namely O^6-MeG alkylation product, is the most potent

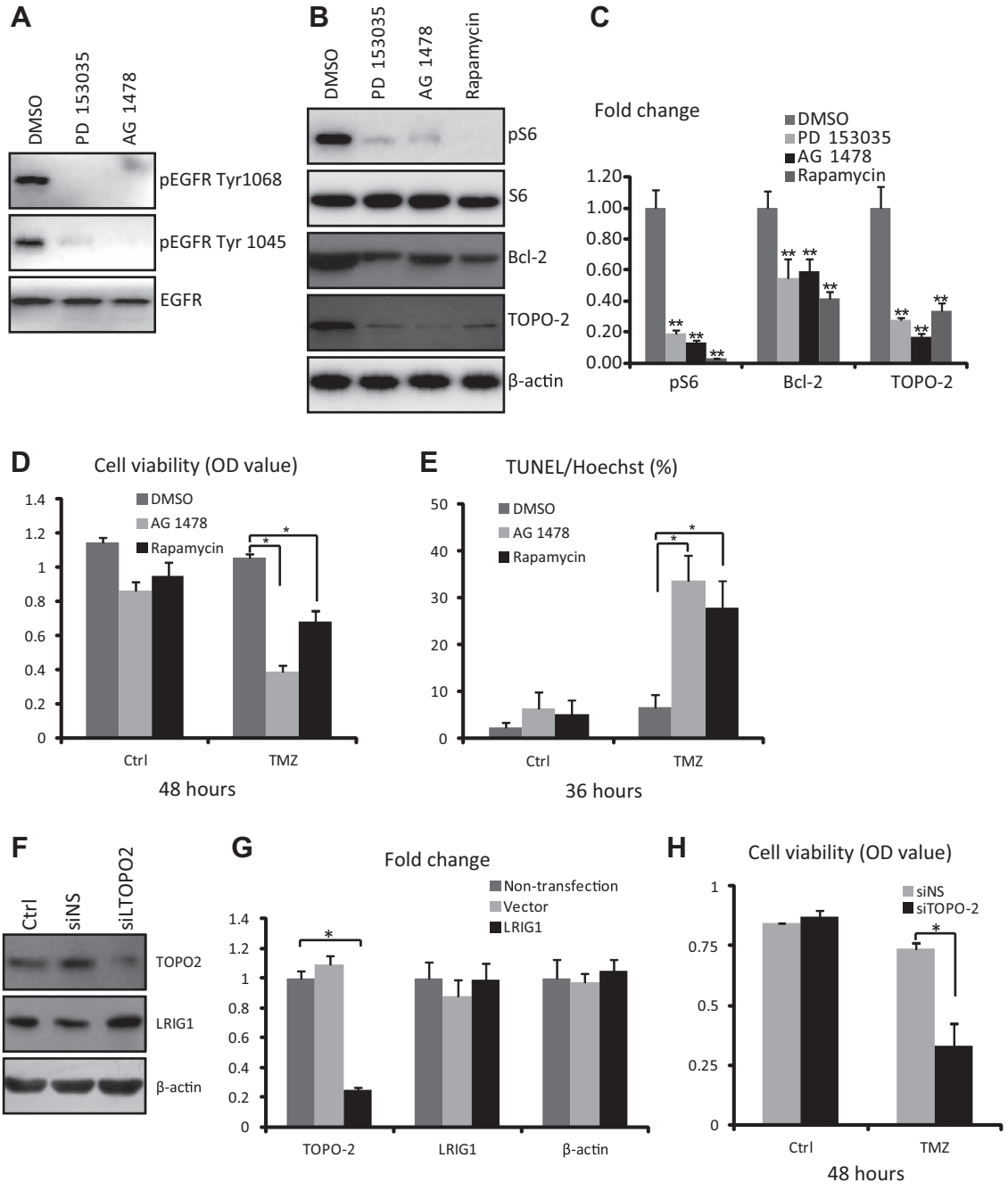

Fig. 4. EGFR-dependent mTOR activation is important for Topo-2 and Bcl-2 expressions and TMZ resistance in U251/TMZ cells. Reprehensive immuno-blots showing total and phospho- level of EGFR in U251/TMZ cells after AG 1478 and PD 153035 treatment. Note that phospho- but not total- level of EGFR were blocked by AG 1478 (1 μM) and PD 153035 (1 μM) (A). U251/TMZ cells were incubated with AG 1478 (1 μM), PD 153035 (1 μM) or rapamycin (100 nM) for 24 h, the expressions of Topo-2, Bcl-2, p-S6 (ser 235/236), S6 and β-actin were detected by Western blots (B). The blot intensity was quantified (C). U251/TMZ cells were pretreated with AG 1478 (1 μM) or rapamycin (100 nM) for 1 h, followed by TMZ (100 μM) exposure, cells were further cultured for indicated time point, cell viability and apoptosis were measured (D and E). Reprehensive immuno-blots showing the expression of LRIG1, Topo-2, and β-actin in control, non-sense siRNA-transfected (siNS) and Topo-2 siRNA (siTOPO2)-transfected U251/TMZ cells. Note that Topo-2 RNAi knockdown did not affect LRIG1 expression (F). The blot intensity was quantified (G). The siNS and siTOPO-2 U251/TMZ cells were incubated in TMZ (100 μM) containing medium for indicated time point, the cell viability and apoptosis were analyzed (H and I). U251/TMZ cells were treated with: vehicle control (DMSO 0.1%), VP-16 (25 μM), TMZ (100 μM) or VP-16 (25 μM)+TMZ (100 μM) (VP+TMZ), cells were further incubated, cell viability and TUNEL positive cells were examined (J and K). The siNS (treated with 0.1% of DMSO or 1 μM of ABT-737) and siBcl-2 U251/TMZ cells were incubated in TMZ (100 μM) containing medium for indicated time point, cell viability and apoptosis were analyzed as previously reported (M and N), expressions of Bcl-2 and β-actin were examined by Western blots (L). Data in this figure were presented as mean ± SD. $^{*}p < 0.01$. $^{**}p < 0.01$ *vs.* DMSO group (C, M and N).

killing lesion [24,25]. It acts as a powerful trigger of cell apoptosis, probably by inducing DNA double-strand breaks (DSBs) and p53 dependent cell apoptosis [26]. As a matter of fact, glioma cells mutated in DNA-dependent protein kinase catalytic subunit (DNA-PKcs) were more sensitive to TMZ-induced apoptosis [26]. While glioma cells with high DNA repair abilities may be resistant to TMZ [27,28].

Recent studies have greatly expanded the biological functions of Topo-2 in DNA replication, transcription and chromosome segregation. One of the main biological functions of Topo-2 is to insure

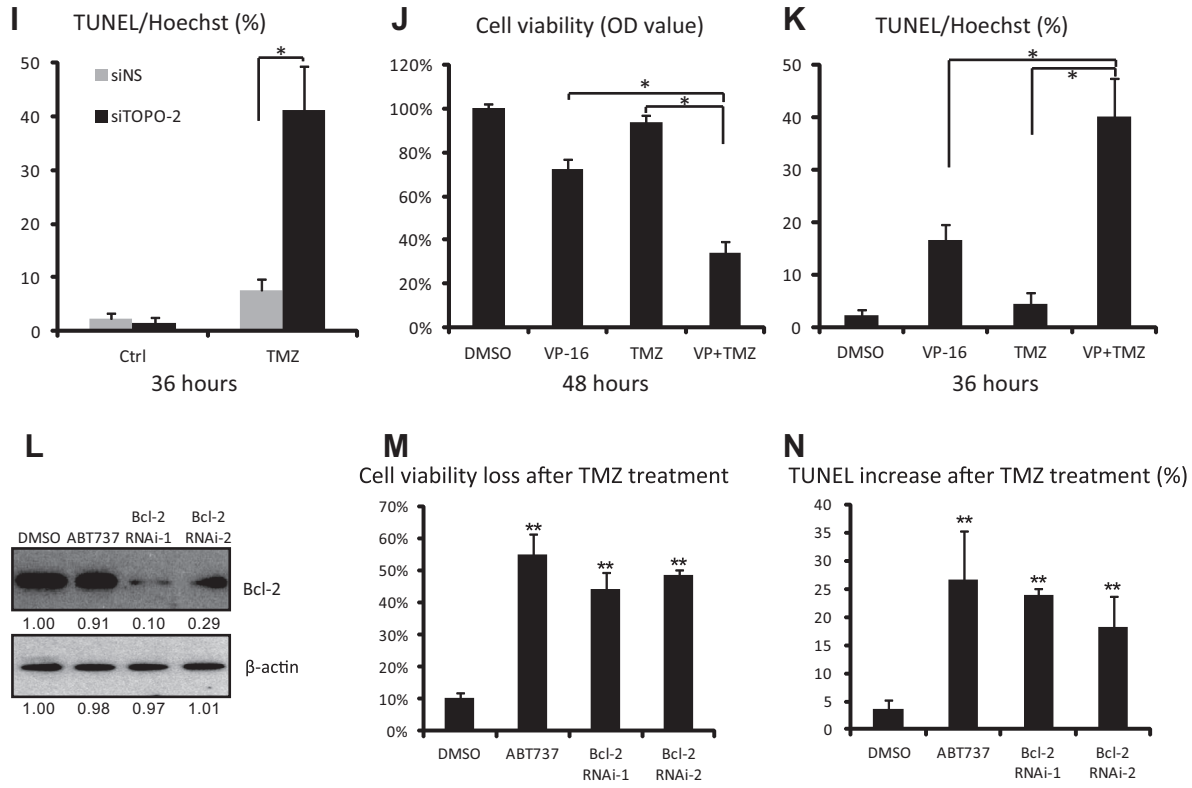

Fig. 4 (*continued*)

DNA stability and genomic integrity when the DNA facing challenges [29]. As such, the strategy to interfere with Topo-2 and generate enzyme mediated DNA damage is proven to be effective for cancer chemotherapy [29]. Recent studies have confirmed that Topo-2 is important for the DNA DSB repair [30]. Based on these information, we proposed that high level of Topo-2 might be one important factor contributing to chemo-resistance in U251/TMZ cells. The fact that TMZ sensitivity was restored in U251/TMZ cells by Topo-2 inhibitor or RNAi knockdown strongly supported our hypothesis.

It is now well established that anti-apoptotic protein Bcl-2 mediates the resistance to TMZ and other cytotoxic drugs [26,31]. Studies have shown that TMZ down-regulates Bcl-2, leading to cell apoptosis [26,31]. The fact that high Bcl-2 expression was observed in U251/TMZ cells suggested that Bcl-2 expression might also be important for the TMZ resistance in these cells. As a matter of fact, inhibition of Bcl-2 by RNAi-mediated knockdown or by the inhibitor restored TMZ sensitivity in the resistant cells supported our hypothesis. Here we found that the EGFR inhibitors and rapamycin significantly inhibited Bcl-2 expression , suggesting that EGFR-mediated mTOR activation might be responsible for Bcl-2 expression and TMZ resistance in glioma cells. In conclusion, our data suggested that LRIG1 inhibits EGFR expression and downstream signaling cascades activation, interferes with Bcl-2/Topo-2 expression and eventually sensitizes glioma cells to TMZ.

Acknowledgment

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