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ALS2CR7 (CDK15) attenuates TRAIL induced apoptosis by inducing phosphorylation of survivin Thr34

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is considered a promising agent for medical applications because it induces apoptosis selectively in a variety of cancer cells without toxicity to normal human cells. However, its therapeutic potential has been limited by the existence of several cancer cells with TRAIL resistance. TRAIL resistance results from a variety of mechanisms, which occur at various points in the cellular signaling pathways. In this study, we demonstrate that ALS2CR7 (CDK15) can mediate resistance to TRAIL. We also demonstrate that cell viability of TRAIL sensitive HCT116 and MDA-MB-231 cells increased after TRAIL treatment in ALS2CR7 transfected cancer cells compared with vector transfected cancer cells. Furthermore, cell viability was decreased by TRAIL treatment after knockdown with ALS2CR7 siRNA in TRAIL resistant HT29 and MCF-7 cells. We also show that the activated form of apoptotic proteins such as caspase-3, -8 and -9 and PARP increased after TRAIL treatment in the control group, but decreased in the ALS2CR7 transfected group. The expression of survival proteins such as bcl2 and survivin in TRAIL sensitive cancer cells increased in the ALS2CR7 transfected group, but decreased in TRAIL resistant cancer cells treated with ALS2CR7 siRNA. Other survival proteins such as FLIP and XIAP were not affected. ALS2CR7 appears to bind with only survivin, and not bcl2. The phospho-survivin (Thr34) critical in drug resistance was increased by transfection with ALS2CR7, but the expression of death receptors such as DR4 and DR5 was not affected. ALS2CR7 did not bind with any of the death receptors in our study. In summary, our results suggest that ALS2CR7 confers TRAIL resistance to cancer cells via phosphorylation of survivin.

1. Introduction

Death receptor ligands such as FasL and TRAIL play an important role in antitumor immunity [1,2]. TRAIL is considered a promising agent for cancer therapeutics because it induces apoptosis selectively in a variety of cancer cells without toxicity to normal human cells [3]. Many types of cancer cells are resistant to TRAIL-induced apoptotic cell death, an indication that TRAIL alone may not be sufficient to treat many malignant tumors [4,5].

Serine/threonine protein kinase (STK) is well known as drug resistance kinase [6]. At least 125 of the 518 known human protein kinases are STK [7]. STK plays a role in the regulation of cell proliferation, programmed cell death (apoptosis), cell differentiation, and embryonic development [8]. Recent studies have demonstrated that several STKs such as Aurora B, Akt, CDK1, CDK2 and CDK9 are involved in the TRAIL resistant mechanism [9–12]. Besides STKs, AXL receptor tyrosine kinase also contributes to TRAIL resistance [13]. ALS2CR7 (amyotrophic lateral sclerosis 2 chromosomal region candidate gene 7 protein), also known as CDK15, is a 384 amino acid protein belonging to the STK superfamily. Although several cyclin dependent kinases are known to induce TRAIL resistant, the TRAIL resistance mechanism of ALS2CR7 has not yet been studied. The mechanism is elucidated in this study.

Several survival proteins such as survivin, bcl2, XIAP and FLIP are known to be TRAIL resistant [14]. It is well known that the Thr34 on survivin plays a prominent role in controlling survivin stability [15]. A recent study demonstrated that phosphorylation of Thr34 by p34cdc2-cyclin B1 kinase inhibits apoptosis and promotes cell division, and is therefore necessary for controlling the cell cycle [16]. Another study also demonstrated that suppression of T34 phosphorylation by inhibiting cdk1 enhances apoptosis [17]. Moreover, gene targeting using a survivin mutant incapable of Thr34 phosphorylation has been shown to inhibit tumor growth [18]. Another recent study demonstrated that phosphorylation of survivin is involved in the resistance of TRAIL-induced apoptosis

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[19]. However, the mechanism has yet to be studied. In this study, we investigate the effects and critical mechanisms of ALS2CR7 on TRAIL resistance.

2. Materials and methods

2.1. Plasmid construction

pEGFP-C3 expression vector was obtained from Clontech (Palo alto, CA, USA) and the human kinase genes obtained from Adgene (Cambridge, MA, USA). The wild type human kinase gene ALS2CR7 was amplified with PCR and cloned into pEGFP-C3 mammalian expression vectors using specific primers. The ALS2CR7 forward primer was 5′-GCTGAATTCTGATGACTTCATTTCACCCCAG-3′ and the reverse primer was 5′-GCTGGATCCTACTCATCAGGAAGCTGGTA CA-3′.

2.2. Cell culture and reagent

Human colorectal carcinoma cancer cells (HCT116 and HT29) and human breast carcinoma cancer cells (MDA-MB-231 and MCF-7) were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). Cancer cells were grown at 37 °C with 5% CO_2 humidified air in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. RPMI1640, penicillin, streptomycin and FBS were purchased from Gibco Life Technologies (Grand Island, NY, USA). Soluble Recombinant human Apo2L/TRAIL was purchased from Peprotech (Rocky Hill, NJ).

2.3. Transfection

HCT116 and MDA-MB-231 cells (5×10^4 cells/well) were plated in 24-well plates and transiently transfected with 5 μg of either the EGFP-C3 empty vector or the EGFP–ALS2CR7 vector per well, using a mixture of plasmid and the lipofectamine 2000 reagent in OPTI-MEN, according to manufacturer specification (Invitrogen, Carlsbad, CA, USA). HT29 and MCF-7 cells (5×10^4 cells/well) were plated in 24-well plates and transfected with 100 nM of either the control siRNA or the ALS2CR7 siRNA (Santa cruz biotechnology Inc., USA) using a mixture of plasmid and the lipofectamine 2000 reagent in OPTI-MEM, according to manufacturer's specification (Invitrogen, Carlsbad, CA, USA).

2.4. Cell viability

The number of viable cells was determined using a CCK-8 assay kit (Dojindo Laboratories, Tokyo, Japan). Briefly, 10 μl of the CCK-8 solution was added to each well, and the plate was incubated for 2 h. The absorbance of each well was measured at 450 nm using a microplate reader.

2.5. Western blotting

Whole cell extracts were mixed with SDS sample buffer then subjected to 12% SDS–polyacrylamide gel electrophoresis. The resolved proteins were transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The samples were immunoblotted with the following primary antibodies: mouse monoclonal antibodies directed against cleaved caspase -8 and -9 (1:1000 dilutions; Cell Signaling Technology, Beverly, MA) and β-actin (1:500 dilutions; Santa Cruz Biotechnology Inc., USA), as well as rabbit polyclonal antibodies directed against ALS2CR7 (1:1000 dilutions; abcam, UK) survivin, bcl2, phospho-survivin, XIAP, PARP, cleaved caspase-3 (1:1000 dilutions; Cell Signaling Technology, Beverly, MA) and FLIP (1:500 dilutions; Santa Cruz Biotechnology Inc., USA). The blot was then incubated with the corresponding anti-rabbit/mouse immunoglobulin G-horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., USA). Immunoreactive proteins were detected using the Enhanced Chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech, Inc., Buckinghamshire, UK).

2.6. Immunoprecipitation

Cells were transfected with the indicated constructs for 48 h, then resuspended in NP-40 lysis buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol) containing a 0.1% protease inhibitor cocktail and a 0.1% phosphatase inhibitor cocktail for 30 min at 4 °C. Cells were disrupted and centrifuged to remove insoluble debris. The antibodies indicated previously were added to the supernatants and incubated overnight at 4 °C. Protein A/G agarose beads (Santa Cruz Biotechnology Inc., USA) were added and the mixture incubated at 4 °C for 4 h. The immunocomplex bound to Protein A/G beads were washed 3 times with wash buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol) and bound proteins were eluted by boiling for 5 min in SDS sample buffer.

2.7. Statistical analysis

The data were analyzed using the GraphPad Prism 4 ver. 4.03 software (GraphPad Software, La Jolla, CA, USA). Data are presented as mean ± SD. The differences in all data were assessed by one-way analysis of variance (ANOVA). When the P value in the ANOVA test indicated statistical significance, the differences were assessed using the Dunnett's test. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. ALS2CR7 promotes survival of TRAIL sensitive cancer cells after TRAIL treatment

To investigate the effects of ALS2CR7 on TRAIL resistance in cells, we employed HCT116 and MDA-MB-231 cells with low endogenous ALS2CR7 expression to transiently overexpress ALS2CR7 (Fig. 1A). We also showed that the survival of these ALS2CR7-transfected groups after TRAIL treatment was higher than the control group (Fig. 1A). We also showed that knockdown of ALS2CR7 increased sensitivity to TRAIL-induced apoptosis in TRAIL resistant cancer cell lines such as HT29 and MCF-7 cells (Fig. 1B).

3.2. ALS2CR7 attenuates TRAIL-induced apoptosis and activation of caspases

Western blot analysis showed considerably higher protein levels of cleaved caspase-8, caspase-3 and caspase-9 in EGFP vector transfected cells compared to ALS2CR7-expressing cells in response to TRAIL treatment in TRAIL-sensitive cancer cell lines (Fig. 2A). We also showed that knockdown of endogenous ALS2CR7 substantially increased protein levels of cleaved caspase-8, caspase-3, caspase-9 and cleaved PARP relative to the control in response to TRAIL treatment (Fig. 2B). These data indicated that ALS2CR7 attenuates TRAIL-induced apoptosis and activation of caspases.

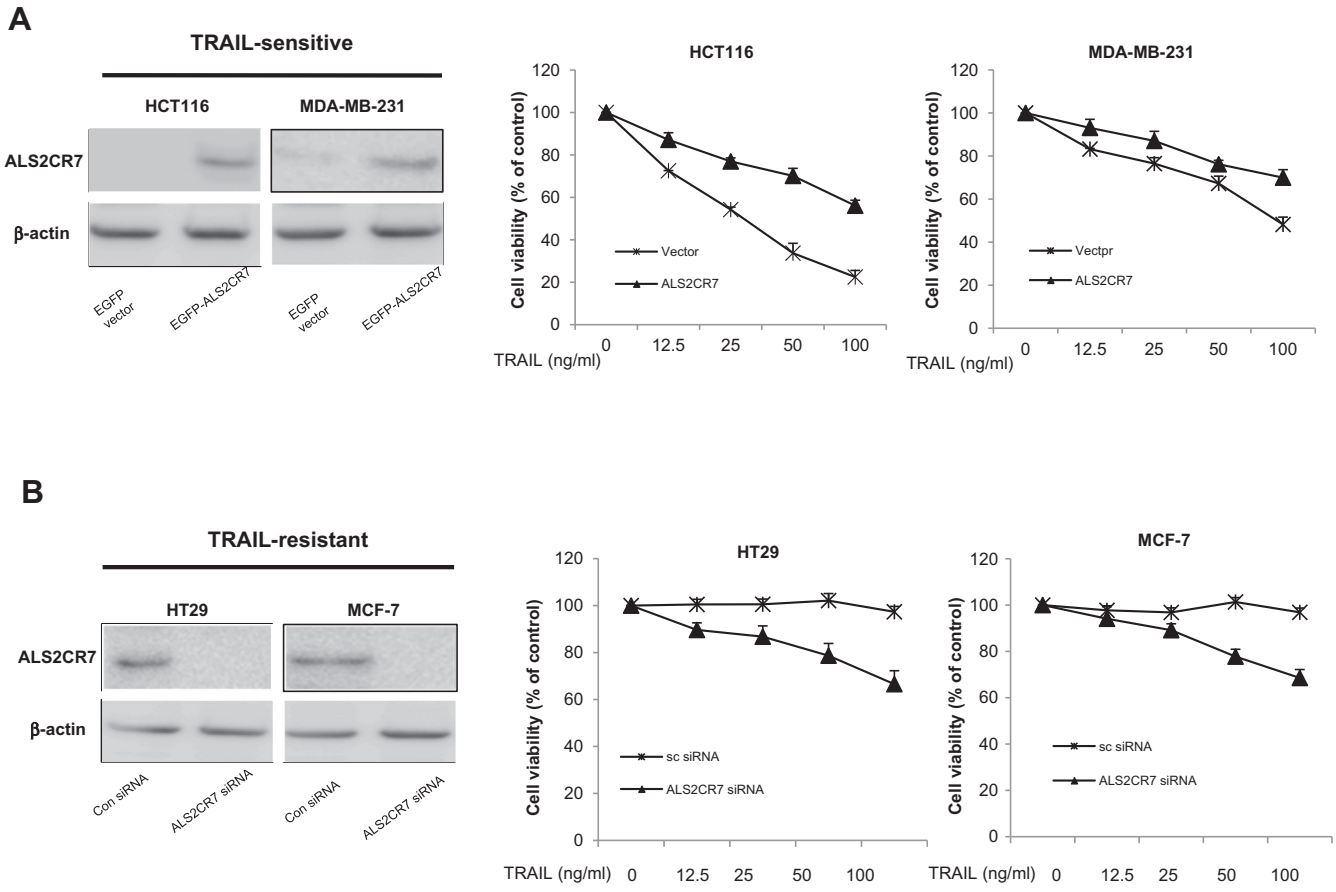

Fig. 1. Effect of ALS2CR7 on cell viability in several cancer cell lines. (A), TRAIL-sensitive HCT116 and MDA-MB-231 cells were transiently transfected with the EGFP vector or EGFP–ALS2CR7 according to the manufacturer's recommendations. Overexpression of ALS2CR7 was confirmed by Western blotting using anti-ALS2CR7 antibody. The transfected cells were treated with the indicated concentrations of TRAIL for 24 h and cell viability was measured using CCK-8. (B), TRAIL-resistant HT29 and MCF-7 cells were transfected with 100 nM of sc siRNA or ALS2CR7 siRNA. Knockdown of ALS2CR7 was confirmed by Western blotting using anti-ALS2CR7 antibody. HT29 and MCF-7 cells with or without silencing of ALS2CR7 expression were treated with the indicated concentrations of TRAIL for 24 h. At the end of treatment, cell viability was measured using CCK-8. SD. $^{*}p < 0.05$, significantly different from vector transfected control groups.

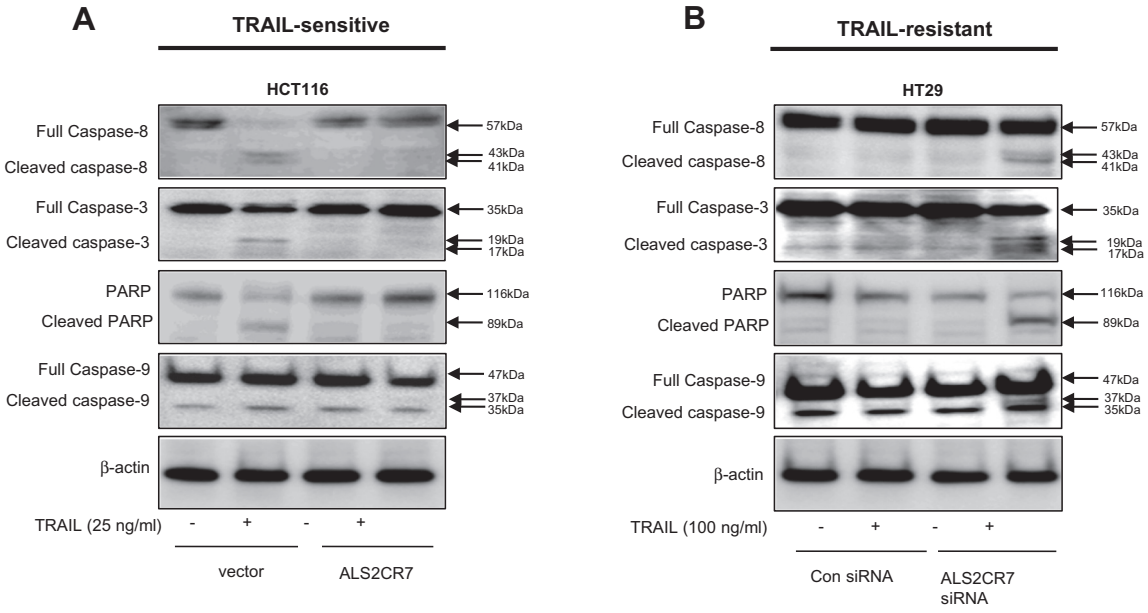

Fig. 2. Effect of ALS2CR7 on the activity of apoptotic proteins in cancer cells. (A), TRAIL-sensitive HCT116 cell was transfected with the EGFP vector or EGFP–ALS2CR7 and further treated with or without 25 ng/ml TRAIL for 24 h. The changes in the proteolytic processing of caspase-3, -8 and -9 and PARP were measured using Western blotting. β-actin was used for loading control. (B), TRAIL-resistant MCF-7 cells were transfected with either the sc siRNA or ALS2CR7 siRNA and further treated with 100 ng/ml TRAIL for 24 h. The expression of caspase-3, -8 and -9 and PARP was measured using Western blotting. β-actin was used to show equal loading of protein samples.

3.3. ALS2CR7 upregulates the expression of survivin and bcl2, but not other survival proteins

We showed that the expression of survivin and bcl2 was upregulated by ALS2CR7 (Fig. 3A) but downregulated by treatment with ALS2CR7 siRNA (Fig. 3B). However, the expression of other survival proteins was not affected by treatment with ALS2CR7 in TRAIL sensitive cancer cells nor with ALS2CR7 siRNA in TRAIL resistant cancer cells. This result suggests that ALS2CR7 is involved in TRAIL resistance via regulation of survivin and bcl2.

3.4. ALS2CR7 binds with anti-apoptotic proteins and regulates their phosphorylation

Protein phosphorylation has been implicated in the regulation of cell death pathways, influencing cell cycle transitions, subcellular localization and cytoprotection. We therefore investigated whether ALS2CR7 binds with Bcl2 or survivin to phosphorylate their critical sites. We discovered that ALS2CR7 binds with only survivin and not bcl2 (Fig. 4A). We also found that phospho-survivin (Thr34) increased after transfection with ALS2CR7 (Fig. 4B).

3.5. ALS2CR7 does not bind with death receptors

We investigated whether ALS2CR7 also binds with death receptors to further impact TRAIL resistance. Our results show that ALS2CR7 does not bind with the death receptors DR4 and DR5 (Fig. 4C). We also showed that the expression of death receptors was not affected by TRAIL treatment in both the control and ALS2CR7 transfected groups (Fig. 4D).

4. Discussion

In this study, we provide direct evidence that ALS2CR7 contributes to TRAIL resistance in cancer cells via phosphorylation of survivin. Although TRAIL induces variable cytotoxicity in several cancer cells both in vitro and in vivo, approximately one-third of cancer malignancies are resistant, and an additional one-third only have a moderate response [20]. TRAIL resistance in cancer cells appears to occur through various molecular mechanisms. Recent studies have suggested that several STKs are involved in TRAIL resistance in various cancer cell lines. However, the effect of ALS2CR7 on TRAIL resistance has not yet been studied. In this study, we demonstrated that ALS2CR7 is an important contributor to TRAIL resistance in several cancer cell lines.

To study the pathways underlying the resistance effect of ALS2CR7 on TRAIL-induced apoptosis, we first analyzed cell viability in TRAIL-sensitive cancer cells through transfection with ALS2CR7 and in TRAIL-resistance cancer cells through treatment with ALS2CR7 siRNA. We demonstrated that cancer cell growth increased after the introduction of ALS2CR7 in the TRAIL sensitive cancer cells HCT116 and MDA-MB-231 cells. However, cancer cell growth decreased following knockdown of ALS2CR7 in the TRAIL resistant cancer cells HT29 and MCF-7 cells. Next, we analyzed the expression of XIAP, survivin, Bcl2 and c-FLIP. We found that the expression of survivin and Bcl2 was increased by transfection with ALS2CR7, while the expression of XIAP and c-FLIP remained unaffected in TRAIL sensitive cancer cells such as HCT116 and MDA-MB-231. We also showed that the expression of survivin and Bcl2 was downregulated by treatment with ALS2CR7 siRNA in TRAIL resistant cancer cells such as HT29 and MCF-7 cells. We also showed that the phosphorylation of survivin was upregulated by the introduction of ASL2CR7. Based on these results, we suggest that ALS2CR7 regulates the phosphorylation of survivin (Thr34) by direct binding and subsequently regulates the expression of survivin.

We next examined the effects of ALS2CR7 on molecular signaling of apoptosis to confirm the role of ALS2CR7 in the inhibition of apoptosis in response to TRAIL. We showed that the reconstitution of ALS2CR7 expression blocked TRAIL-induced activation of

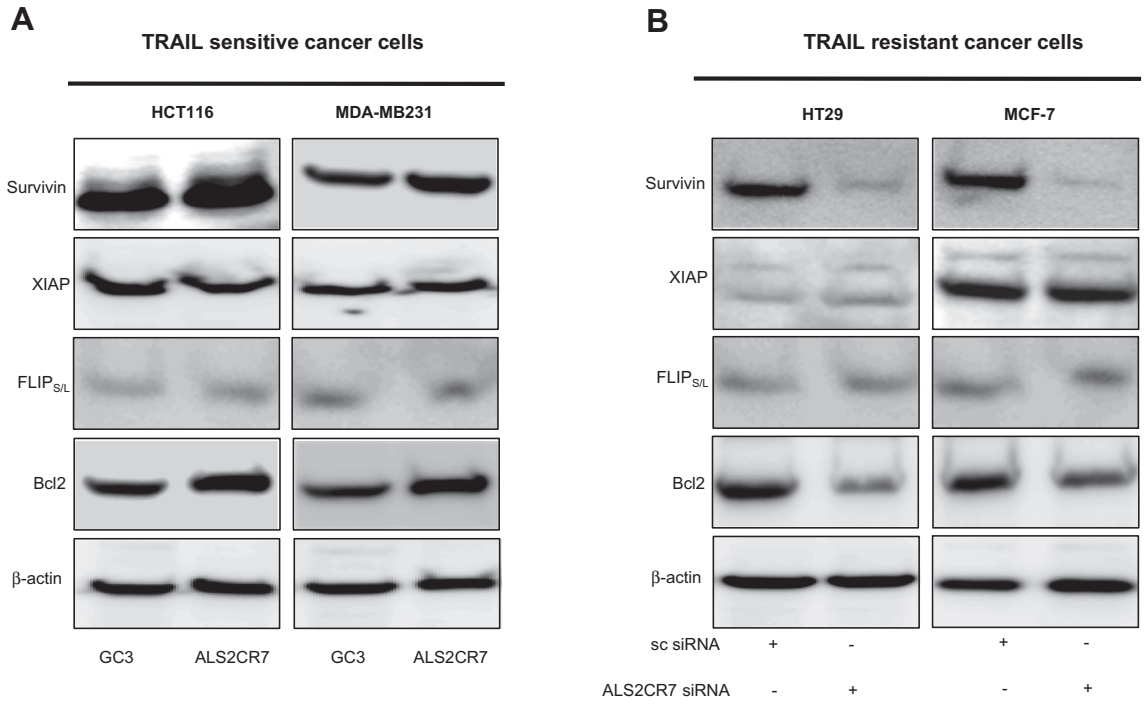

Fig. 3. Effect of ALS2CR7 on the expression of survival proteins in TRAIL-sensitive or TRAIL-resistant cancer cells. (A), TRAIL-sensitive HCT116 or MDA-MB-231 cells were transfected with either the EGFP vector or EGFP–ALS2CR7. After 24 h, the expression of survivin, XIAP, FLIP$_{S/L}$ and Bcl-2 was analyzed using Western blotting. β-actin was used as a loading control. (B), TRAIL-resistant HT29 or MCF-7 cells were transfected with 100 nM of sc siRNA or ALS2CR7 siRNA. After 48 h, the expression of survivin, XIAP, FLIPS/L and Bcl-2 was analyzed Western blotting. β-actin was used as a loading control.

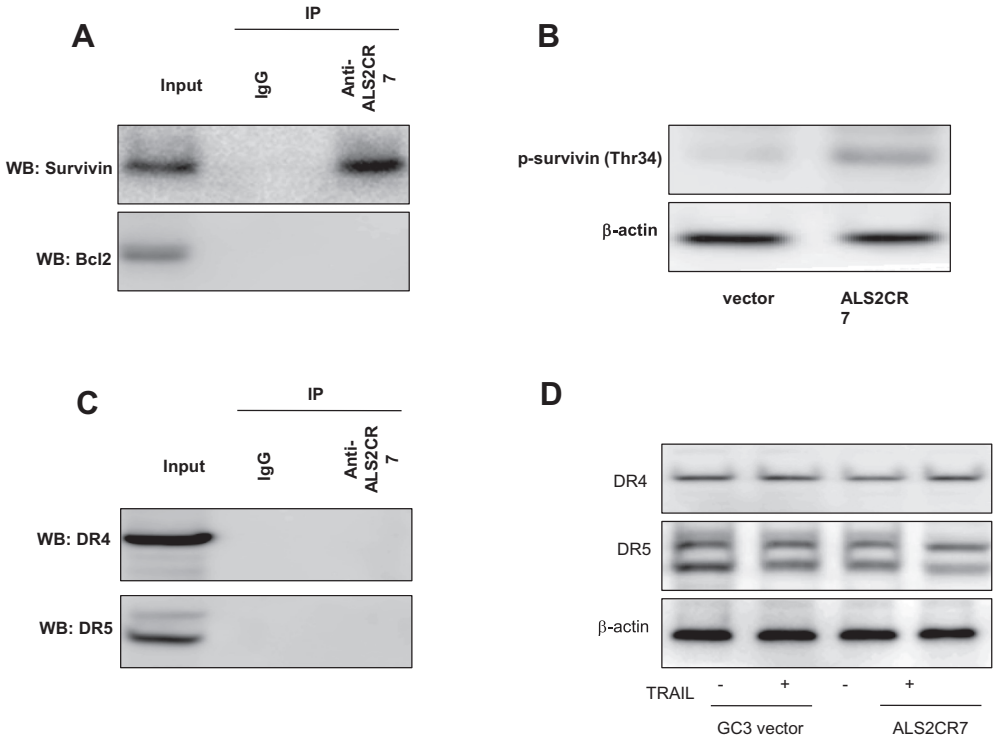

Fig. 4. ALS2CR7 binds with survivin and induces phosphorylation, but has no effect on death receptors. (A), HCT116 cell was transfected with EGFP–ALS2CR7. After 24 h, the cell extracts were immunoprecipitated with control IgG or anti-ALS2CR7 according to the Section 2. After immunoprecipitation, binding of ALS2CR7 with survivin or bcl2 was measured by Western blotting using indicated antibodies. (B), HCT116 cell was transfected with EGFP–ALS2CR7 for 24 h, and the expression of p-survivin (Thr34) detected by Western blotting. (C), HCT116 cell was transfected with EGFP–ALS2CR7 for 24 h, and the expression of DR4 and DR5 detected using the indicated antibodies. (D), HCT116 cells were transfected with EGFP vector or EGFP–ALS2CR7 for 24 h, and the extracted cells were immunoprecipitated with control IgG or anti-ALS2CR7. After that, the binding of ALS2CR7 with DR4 or DR5 was analyzed by Western blotting using indicated antibodies.

caspase-8, -3 and -9 and cleavage of PARP. Conversely, knockdown of endogenous ALS2CR7 considerably sensitized cells to TRAIL as indicated by increased activation of caspase-8, -3 and -9 and cleavage of PARP. TRAIL has been known to induce apoptosis via DR4 and DR5, and downregulation of DR4 and DR5 also involved in TRAIL resistance [21,22]. However, the expression of DR4 and DR5 was not affected by the introduction of ALS2CR7 in TRAIL sensitive cancer cells. Our findings suggest that ALS2CR7 enhances TRAIL resistance by upregulation of survival proteins and not by regulation of death receptors.

In conclusion, the current study implicated ALS2CR7 as a potential new target that can be exploited to reinforce the efficacy of TRAIL in killing tumor cells.

Conflict of interest statement

None declared.

Acknowledgments

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