CCR4-NOT transcription complex subunit 2 regulates TRAIL sensitivity in non-small-cell lung cancer cells via the STAT3 pathway

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Abstract. TRAIL is an attractive candidate for anticancer therapy in a variety of tumors since it targets only tumors and not normal tissue. However, a remaining major hurdle is that the majority of tumors exhibit a resistance mechanism against the effects of TRAIL via the induction of anti-apoptotic signaling pathways. In this study, we aimed to evaluate whether the modulation of CCR4-NOT transcription complex subunit 2 (CNOT2) function can promote TRAIL sensitivity in non-small-cell lung cancer (NSCLC) cells. CNOT2 depletion partially decreased colony numbers and the proliferation of NSCLC cells. When combined with TRAIL, the suppression of CNOT2 expression markedly decreased the survival rate and increased apoptosis, as compared with TRAIL treatment alone in TRAIL-resistant NSCLC cells. Of note, CNOT2 overexpression in TRAIL-sensitive H460 cells enhanced the survival rate and decreased apoptosis when compared with TRAIL treatment alone. Gene expression analysis indicated that genes involved in the signal transducer and activator of transcription 3 (STAT3) signaling pathway were dominantly altered in the CNOT2-depleted A549 cells. Under this condition, Src homology region 2 domain containing phosphatase-1 (SHP1) was significantly upregulated and subsequently increased apoptosis. On the whole, the findings of this study demonstrate that CNOT2 participates in TRAIL sensitivity in NSCLC cells via the regulation of the STAT3 signaling pathway, and suggest that combination therapy with CNOT2 depletion and TRAIL treatment may prove to be a useful strategy for overcoming TRAIL resistance in NSCLC.

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Abbreviations: TRAIL, TNF-related apoptosis-inducing ligand; CNOT2, CCR4-NOT transcription complex subunit 2; NSCLC, non-small cell lung cancer; DR4, death receptor 4; DR5, death receptor 5; HNSCC, head and neck squamous cell carcinoma; STAT3, signal transducer and activator of transcription 3; FBS, fetal bovine serum; SHP1, Src homology region 2 domain containing phosphatase-1; Bax, BCL2 associated X protein; PARP, poly(ADP-ribose) polymerase; MAPK, mitogen-activated protein kinase; Bcl-2, B-cell lymphoma 2; CHOP, C/EBP homologous protein; GRP78, glucose-regulated protein 78; MTT, 3-(4,5-dimethylthialzol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PI, propidium iodide; RNA, ribonucleic acid; DEPC, diethyl pyrocarbonate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ECL, enhanced chemiluminescence; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; IL6, interleukin 6; IL6R, interleukin 6 receptor; CLCF1, cardiotrophin-like cytokines factor 1; LIF, leukemia inhibitory factor; ERBB4, Erb-B2 receptor tyrosine kinase 4; IL6ST, interleukin 6 signal transducer; JAK2, Janus kinase 2; ER, endoplasmic reticulum; FADD, Fas-associated protein with death domain; DISC, death-inducing signalling complex

Key words: CNOT2, TRAIL, non-small cell lung cancer, STAT3, endoplasmic reticulum stress

Introduction

Lung cancer is the most prevalent type of cancer worldwide, leading annually to the deaths of more than 1.76 million individuals (1). Non-small-cell lung cancer (NSCLC) is the major type of lung adenocarcinoma found in approximately 85% of all cases of lung cancer, with the majority of patients presenting with advanced tumor stages in the United States (2). Even though numerous types of treatment strategies including immunotherapy, chemotherapy, radiotherapy and combination therapy have been developed for NSCLC, the 5-year survival rate for patients with advanced stages of the disease (with metastasis) has only marginally improved over the past 4 decades, remaining at approximately 4% (3). Therefore, the development of novel therapeutic strategies for NSCLC is of utmost importance.

TNF-related apoptosis-inducing ligand (TRAIL) is often referred to as a 'magic bullet' as it can specifically target a broad range of tumors, including lung cancer, while avoiding detrimental effects on normal cells. TRAIL is a potent apoptotic inducer capable of binding to the death receptors, death receptor (DR)4 and DR5, which can subsequently activate an apoptotic signal pathway known as the extrinsic apoptotic pathway (4). However, a number of types of tumors evade TRAIL-mediated apoptosis, such as via an increase in the expression of anti-apoptotic molecules, by the downregulation of apoptotic proteins, or with the inhibition of apoptotic signaling pathways, leading to TRAIL resistance (5,6). To overcome this resistance, several methods have used, such as the modulation of apoptosis-related proteins or treatment with chemotherapeutic drugs, leading to an increase in TRAIL sensitivity.

CCR4-NOT transcription complex (CNOT), composed of 11 subunits, participates in ribonucleic acid (RNA) regulation, including messenger RNA (mRNA) stability and export. In particular, CNOT2, one of the CCR4-NOT subunits, plays a critical role in deadenylase activity and the structural integrity of the complex (7). In addition, this protein participates in embryonic development and transcriptional regulation via the recruitment of histone modifiers (8,9). Recent studies have suggested that CNOT2 plays important roles in tumor progression, such as in metastasis, proliferation and angiogenesis via the regulation of vascular endothelial growth factor signaling in breast cancer cells (10,11). However, to the best of our knowledge, CNOT2 has not been evaluated as a molecular target for tumor treatment to date. Hence, in the current study, the therapeutic potential through which the suppression of CNOT2 enhances TRAIL sensitivity in TRAIL-resistant NSCLC cells through the STAT3 signaling pathway was examined.

Materials and methods

Cells and cell culture. All cell culture experiments adhered to standard biosafety level 2 guidelines. All cell lines used in this study were tested for mycoplasma contamination using a mycoplasma detection kit (JCBIO). The NSCLC cell lines, A549, H1299, H596 and H460, were purchased from the Korean Cell Line Bank. The NSCLC cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640; Corning Inc.), supplemented with 10% fetal bovine serum (Corning Inc.) and 1% penicillin-streptomycin (Corning Inc.). All cells were cultured in a 37˚C humidified atmosphere containing 5% CO_2.

Reagents and antibodies. TRAIL/Apo2L (ABIN2973530) was purchased from Atgen. The antibodies for cleaved caspase-3 (sc-56053), caspase-3 (sc-7148), Src homology region 2 domain containing phosphatase-1 (SHP1) (sc-8425), DR4 (sc-8411), BCL2 associated X protein (Bax) (sc-7480) and PARP[poly(ADP-ribose) polymerase] (sc-7150) were purchased from Santa Cruz Biotechnology, while phospho-signal transducer and activator of transcription 3 (STAT3) (#9131), STAT3 (#12640), phospho-Akt(#9271), phosphor-mitogen-activated protein kinase (MAPK) (#9101), phospho-Src (#2105), Bcl-2 (#15071), DR5 (#3696), C/EBP homologous protein (CHOP) (#2895), glucose-regulated protein 78 (GRP78) (#3177) and β-actin (#4967) antibodies were purchased from Cell Signaling Technology. Finally, the antibodies for CNOT2 (ab55679) and cytochrome *c* (ab13575) were purchased from Abcam.

Cytotoxicity assay. Cell cytotoxicity was measured by way of the 3-(4,5-dimethylthialzol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. MTT was purchased from Molecular Probes. The NSCLC cells were seeded in 96-well plates at a density of $0.7x10^4$ cells per well and incubated with various concentrations (0, 3.12, 6.25, 12.5, 25, 50, 100, 200 and 400 ng/ml) of TRAIL for 24 h at 37˚C. The viability of the cells was analyzed as previously described (12,13). Optical density was measured using a microplate reader (VersaMax; Molecular Devices) at 570 nm and data were analyzed using the Softmax Pro software program (Molecular Devices). The results are expressed as the means and standard deviations after at least 3 independent experiments performed in triplicate.

Apoptosis assay. Apoptosis was measured by flow cytometry after staining with Annexin V-FITC and propidium iodide (PI). The cells ($2x10^5$) were incubated with 0, 25 and 100 ng/ml of TRAIL for 24 h at 37˚C prior to analysis. The cells were stained using an Annexin V-FITC apoptosis detection kit (Biovision) according to the manufacturer's instructions. Apoptotic cells were measured using the FACSCalibur platform (BD Biosciences). Data were analyzed using the Cell Quest program (BD Biosciences). The experiment was repeated in triplicate.

Stable cell lines. To establish a stable CNOT2-overexpressing H460 cell line, the cells were seeded in the manner of $1.5x10^5$ cells in 6-well plates at 1 day prior to transfection. Transfection was performed using pcDNA3.1-CNOT2 and vector with X-treme GENE HP DNA transfection reagent (Roche Holding AG), following the manufacturer's instructions. Following 72 h of transfection, the cells were treated with 500 μg/ml of G418 (Sigma-Aldrich) every 2 days for 10 days total to establish a stable cell line.

To ensure the stable knockdown of CNOT2 in the NSCLC cells, the A549, H1299 and H596 cells were transfected with pRS vector containing CNOT2 shRNA (cat. no. TF81001; OriGene Technologies, Inc.) or control shRNA (Cat. no. TF81001; OriGene Technologies, Inc.). After transfection, the cells were incubated for 2 days followed by selection with 1.5 to 2 μg/ml puromycin for 3-5 days. Thereafter, cells were cultured with normal media.

Transfection with siRNA. SHP1 siRNA and control siRNA were designed and purchased from Santa Cruz Biotechnology. At 1 day prior to transfection, the cells were seeded in the manner of $1x10^5$ cells in a 6-well plate and incubated at 37˚C. The cells were transfected using the INTERFERin *in vitro* siRNA transfection reagent (Polyplus-transfection SA) according to the manufacturer's instructions. The mixture was applied to the cells and incubated for 48 h at 37˚C in the presence of 5% CO_2.

Colony-forming assay. The cells were seeded in the manner of $5x10^2$ cells in 6-well plates and incubated for 7 days at 37˚C

in the presence of 5% CO_2. Subsequently, 10% buffered formaldehyde (Sigma-Aldrich) was added to the cells followed by incubation for 10 min at room temperature, while 1 ml of 0.1% crystal violet (Sigma-Aldrich) was also added to the cells and shaken for 30 min at room temperature. The cells were washed 3 times with distilled water and dried.

Cell count. The cells were seeded in the manner of $1x10^5$ cells in six6well plates and incubated for 7 days at 37˚C in the presence of 5% CO_2. The numbers of cells were counted using a light microscope (Olympus) and a hemocytometer (Hausser Scientific Co.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells using TRIzol reagent. Cells were mixed with 1 ml of TRIzol (Invitrogen; Thermo Fisher Scientific) and incubated at room temperature for 10 min following vortexing. Subsequently, 300 μl of chloroform was mixed gently by inverting. Centrifugation was performed at 12,000 x g for 15 min at 4˚C and the resultant supernatant was collected. At this point, an equal amount of isopropanol was added, mixed gently, and incubated at room temperature for 10 min. Centrifugation was performed for 20 min at 10,000 x g at 4˚C to remove the supernatant. After washing with 70% ethanol, the RNA pellet was dissolved in diethyl pyrocarbonate (DEPC) water. The RNA concentration and purity of each sample were determined using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized using the PrimeScript first-strand cDNA synthesis kit (Takara Korea Biomedical Inc.) according to the manufacturer's instructions. The amplification of each cDNA was monitored using the Sensi FAST SYBR No-ROX kit (Bioline) on a StepOnePlus instrument (Thermo Fisher Scientific). All specific forward and reverse primers used were as follows: Cardiotrophin-like cytokines factor 1 (CLCF1), forward, 5'-TATGACCTCACCCGCTACCT-3' and reverse, 5-'GGGGCCCAGGTAGTTCAG-3'; interleukin (IL)6 forward, 5'-CCACCGGGAACGAAAGAGAA-3' and reverse, 5'-GAGAAGGCAACTGGACCGAA-3'; IL6 receptor (IL6R) forward, 5'-GGGTCTCTACCATCCCCTGT-3' and reverse, 5'-AGAAATGGCAGAAGCCCTCC-3'; IL6 signal transducer (IL6ST) forward, 5'-CAGTGGTCACCTCACACTCC-3' and reverse, 5'-TGACATGCATGAAGACCCCC-3'; Janus kinase 2 (JAK2) forward, 5'-TGGGGTTTTCTGGTGCCTTT-3' and reverse, 5'-TAGAGGGTCATACCGGCACA-3'; leukemia inhibitory factor (LIF) forward, 5'-CTCGCCCATCACCTCATCTC-3' and reverse, 5'-GCAGAGCTGTTTCACGCAAA-3'; Erb-B2 receptor tyrosine kinase 4 (ERBB4) forward, 5'-CCTGGAAGAAAGACGACTCGTTC-3' and reverse, 5'-CGTCACTCTGATGGGTGAATTTCC-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-CTGCACCACCAACTGCTTAG-3' and reverse, 5'-AGGTCCACCACTGACACGTT-3'. GAPDH was used as an internal control. The relative mRNA expression change was expressed as a fold change in comparison with the GAPDH control. Data are presented as the means ± standard deviations from at least 3 independent experiments in triplicate.

RNA isolation and gene expression profiling. In the present study, we performed global gene expression analyses using Affymetrix GeneChip® Human Gene 2.0 ST oligonucleotide arrays. Total RNA was isolated from the CNOT2-depleted or control sh-RNA transfected-A549 cells using TRIzol reagent (Qiagen). RNA quality was assessed with the Agilent 2100 Bioanalyser (Agilent Technologies) and quantity was determined with the ND-2000 spectrophotometer (Thermo Fisher Scientific). For each RNA, the synthesis of target cRNA probes and hybridization were performed using Agilent's Low-input QuickAmp Labeling kit (Agilent Technologies) according to the manufacturer's instructions. The gene expression data were analyzed using GeneSpringGX 7.3.1 software (Agilent Technologies). A hierarchically clustered heatmap was generated using MeV version 4.9.0 software. The genes related to cell proliferation [Gene Ontology (GO):0008283) and STAT3 GO:0042509] were classified and visualized in a Venn diagram. Gene array hybridization raw data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database under accession no. GSE131518.

Western blot analysis. The cells were rinsed with ice-cold PBS and harvested with a cell scraper followed by centrifugation at 300 x g for 5 min at 4˚C. The cell pellets were lysed as previously described (13,14). Protein samples were quantified by using a Bio-Rad DC protein assay kit II (Bio-Rad) and 10-20 μg of protein lysates were separated on 8 to 15% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked in 5% nonfat skim milk (in TBST buffer) for 1 h at RT and then probed with primary antibodies at 1/1,000-2,000 diluted in 3-5% non-fat skim milk for 1 h at room temperature. After washing 3 times for 5 min with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (#7076 for anti-mouse, #7074 for anti-rabbit from Cell Signaling Technology), 1/5,000 diluted in 5% non-fat skim milk, for 1 h at room temperature. The protein expression levels were detected using an enhanced chemiluminescence (ECL) system (Amersham Pharmacia), according to the manufacturer's instructions.

Statistical analysis. All experiments were repeated 3 times. Data are presented as the means ± standard deviation (SD). Differences between the means of each group were analyzed using a t-test, while P-values <0.05 were considered significant to indicate statistically significant differences. The statistical software package Excel (Microsoft Corp.) was used for the analysis. The Prism software package (Graph Pad Prism 5.0 for Windows) was used for data collection and presentation for Fig. 3B. The data ranged from 3 to 12 separate experiments and are presented as the means ± SD. Two-way ANOVA followed by a Bonferroni post hoc test were used to determine the significant differences between the various experimental and control groups.

Results

Depletion of CNOT2 affects the proliferation and growth of NSCLC cells. To determine whether CNOT2 has an oncogenic function in NSCLC similar to that in other cancer types as we previously demonstrated (15), we depleted CNOT2 using specific shRNA in the NSCLC cell lines, A549 and H1299

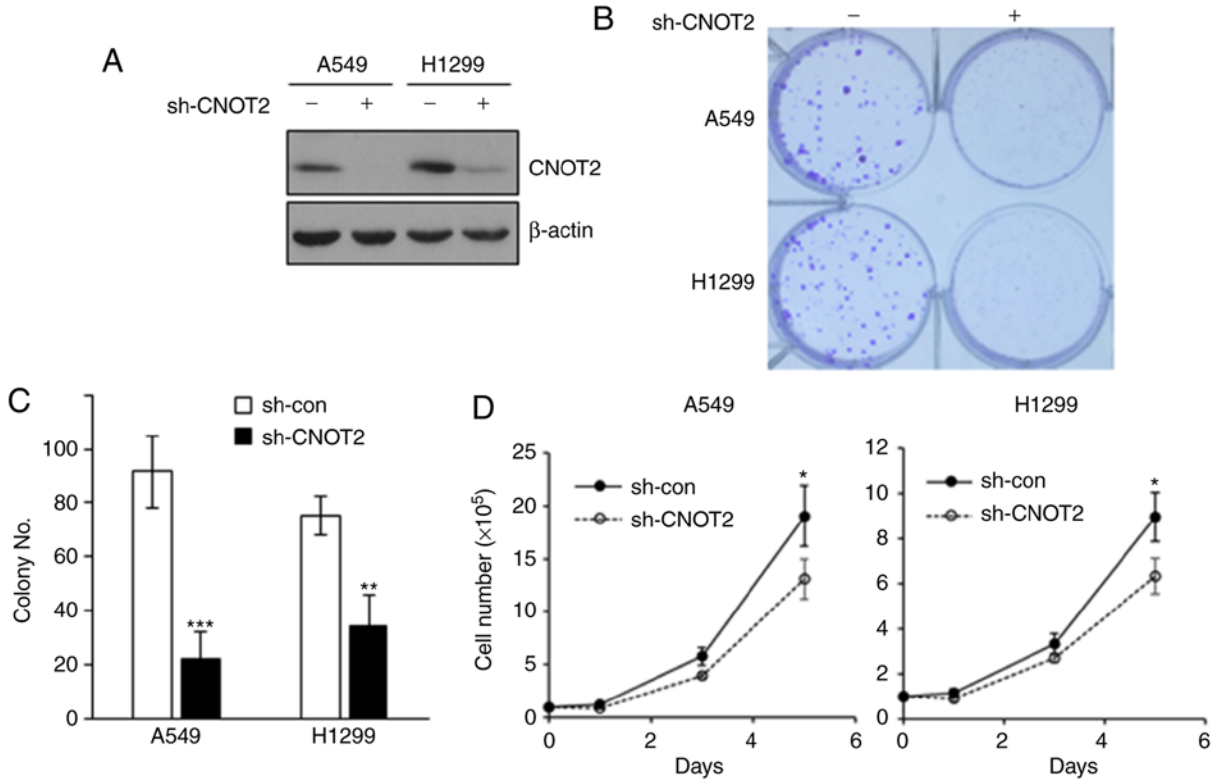

Figure 1. Depletion of CNOT2 partially inhibits the proliferation of NSCLC cells. A549 and H1299 cells were stably transfected with plasmid containing shRNA against CNOT2 or a control. (A) Cell lysates were prepared and subjected to western blot analysis for detecting CNTO2 expression. β-actin was used as an internal loading control. (B) A total of $5x10^2$ cells were seeded in 6-well plates and incubated for 7 days. The plates were then stained with crystal violet. These experiments were repeated 3 times independently. (C) Colonies were counted with a stereomicroscope. Data are presented as the means ± standard deviation (n=3). **P<0.01 and ***P<0.001 vs. sh-con. (D) Cells were seeded at $1x10^5$ cells in 6-well plates and cell numbers were counted at the indicated days. These experiments were repeated 3 times independently. Data are presented as the means ± standard errors of the means (n=3). *P<0.05 vs. sh-con. CNOT2, CCR4-NOT transcription complex subunit 2; NSCLC, non-small cell lung cancer.

(Fig. 1A). We found that the knockdown of CNOT2 resulted in a 50 to 75% decrease in colony number (Fig. 1B and C) and an approximately 40% decrease in the growth rates of NSCLC cells (Fig. 1D). These results indicate that CNOT2 affects the proliferation and growth of NSCLC cells.

Knockdown of CNOT2 markedly increases TRAIL sensitivity in A549 and H1299 cells. To explore whether CNOT2 participates in the regulation of TRAIL sensitivity, the cells in which CNOT2 was downregulated were treated with TRAIL (Figs. 2A and S1). As shown in Figs. 2A and S1B, treatment with TRAIL markedly decreased the survival rates of cells when compared with the control in the A549, H1299 and H596 cells. TRAIL treatment enhanced apoptosis, the main death mechanism promoted by TRAIL, in a dose-dependent manner in the CNOT2-depleted cells (Fig. 2B). These results indicate that CNOT2 participates in the TRAIL-mediated apoptosis of NSCLC cells.

CNOT2 overexpression increases TRAIL resistance in the TRAIL-sensitive cell line, H460. To confirm that CNOT2 participates in TRAIL sensitivity in NSCLC, we established a CNOT2-overexpressing H460 line that is TRAIL-sensitive (Fig. 3A). Treatment of the CNOT2-overexpressing cells with TRAIL increased the cell survival rates by 1.2- to 1.7-fold, as compared with the control cells (Fig. 3B). In addition, the CNOT2-overexpressing cells exhibited decreased apoptosis following treatment with TRAIL (Fig. 3C). These data suggest that the increase in CNOT expression imparts TRAIL resistance on TRAIL-sensitive cells.

CNOT2 depletion downregulates the SHP1/STAT3 signaling pathway. To validate the gene expression profiles and molecular mechanisms involved in CNOT2-mediated TRAIL sensitivity, we analyzed GO pathways in CNOT2-silenced A549 cells using a cDNA microarray. CNOT2 depletion induced dominant changes in the pathways of cell migration, angiogenesis, RNA splicing and cell proliferation (Figs. 4A and S2). In previous studies, it was demonstrated that CNOT2 is highly associated with angiogenesis, migration and metastasis in various cancer types (11,16-18). In this study, we focused on proliferation, which was also altered in CNOT2-depleted lung cancer cells (Fig. 4B). Sixteen genes regulated by CNOT2 depletion were commonly STAT3-related genes, involved in cell proliferation and CNOT2-related genes (Fig. 4C). Consistent with these findings, RT-qPCR analysis confirmed that the inhibition of CNOT2 downregulated the gene expression levels of IL6 and CLCF1 as tumor progression markers and STAT3-related genes (19,20) as well as LIF and ERBB4 as proliferation markers (21,22) in A549 and H1299 cells (Fig. 4D). In the same analysis, IL6ST, IL6R and JAK2, as STAT3-related genes without differences in CNOT2-depleted cells, were confirmed (Fig. 4C and D). Subsequently, STAT3 activity was also verified by measuring the phosphorylation

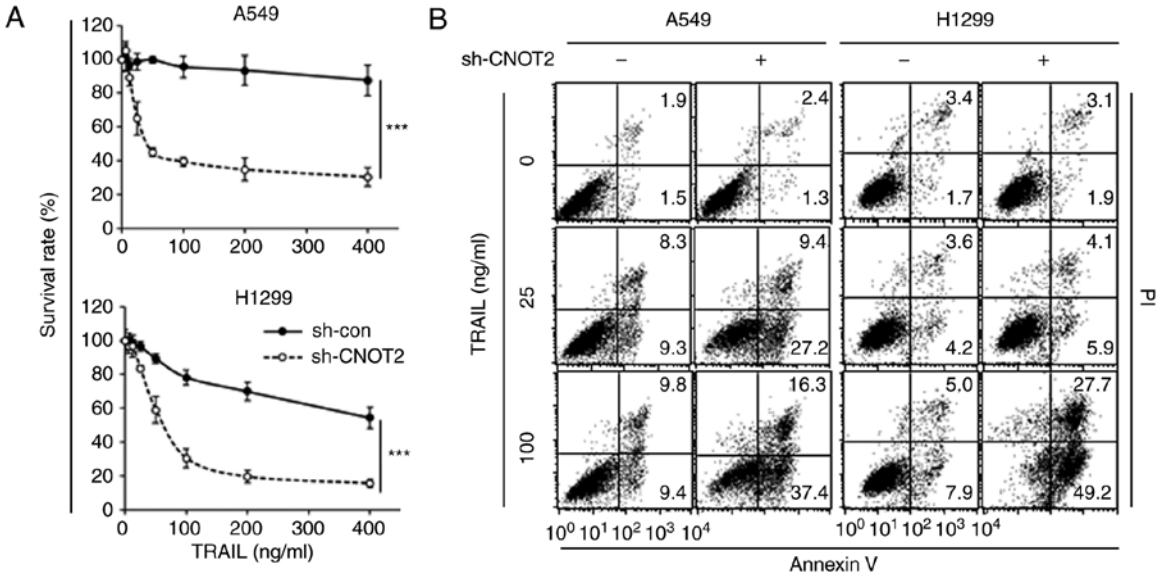

Figure 2. Depletion of CNOT2 increases TRAIL sensitivity via the induction of apoptosis of TRAIL-resistant cells. CNOT2-depleted cells were treated with the indicated concentrations of TRAIL for 48 h. (A) Cytotoxicity was then analyzed using an MTT assay. These experiments were repeated 3 times independently. Data are presented as the means ± standard errors of the means (n=3). ***P<0.001 vs. sh-con (except for 6.25 ng/ml of TRAIL). (B) The apoptosis of each cell was measured by Annexin/PI staining. The horizontal axis represents the Annexin V dye and the vertical axis represents the PI dye, respectively. The experiment was repeated 3 times and the data presented represent all of the results. CNOT2, CCR4-NOT transcription complex subunit 2; TRAIL, TNF-related apoptosis-inducing ligand.

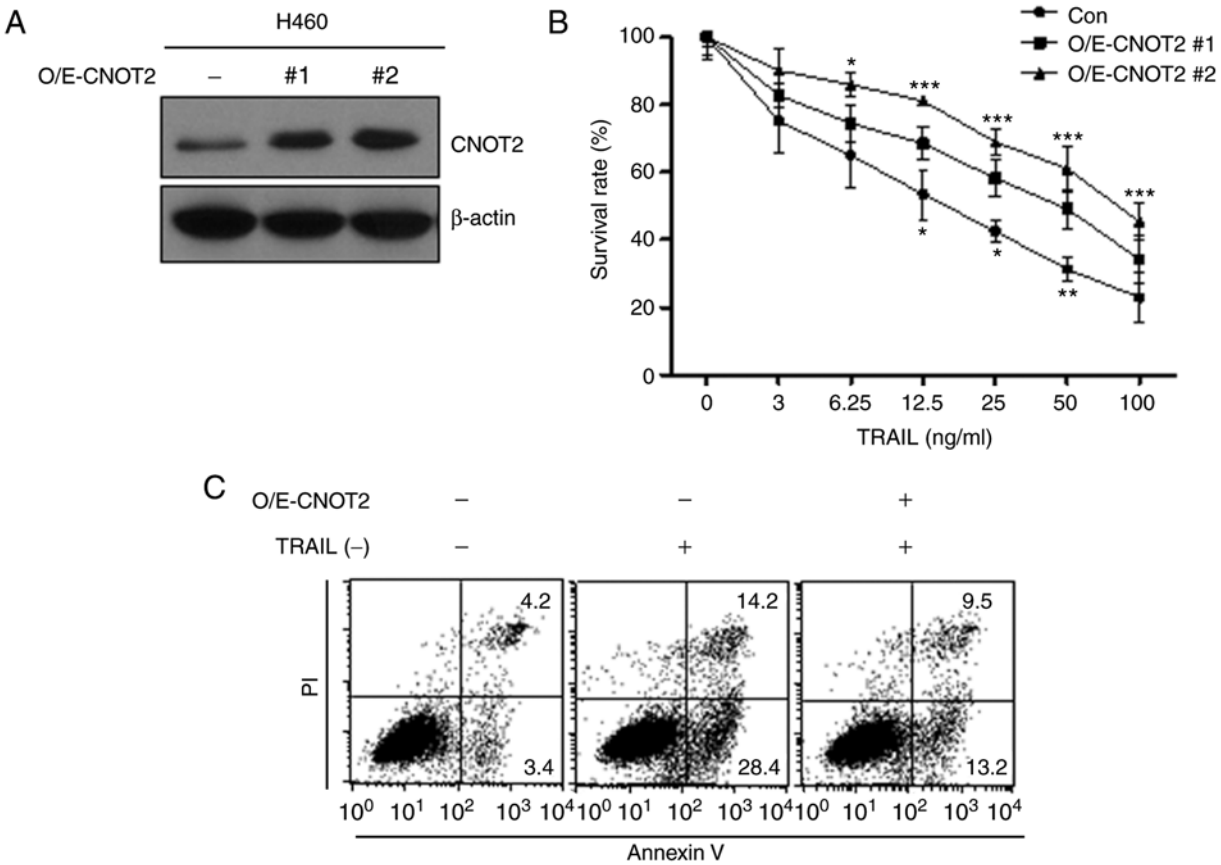

Figure 3. Overexpression of CNOT2 decreases TRAIL sensitivity in H460 cells. H460 cells were transfected with plasmid containing control or CNOT2 and 2 different clones stably expressing CNOT2 were then selected. O/E refers to overexpression. (A) H460 cell lysates were prepared and subjected to western blot analysis for detecting CNTO2 expression. b-actin was used as an internal loading control. (B) Cytotoxicity was then analyzed using an MTT assay. These experiments were repeated 3 times independently. Data are the means ± standard errors of the means (n=3). *P<0.05, **P<0.01 and ***P<0.001 compared to the control group. The upper asterisks indicate the comparison between O/E-CNOT2 #2 vs. Con. The lower asterisks indicate the comparison between O/E-CNOT2 #1 vs. Con. (C) Following treatment with TRAIL for 48 h, apoptosis was measured using Annexin/PI staining. The horizontal axis represents the Annexin V dye and the vertical axis represents the PI dye, respectively. The experiment was repeated 3 times and the data presented represent all of the results. CNOT2, CCR4-NOT transcription complex subunit 2; TRAIL, TNF-related apoptosis-inducing ligand.

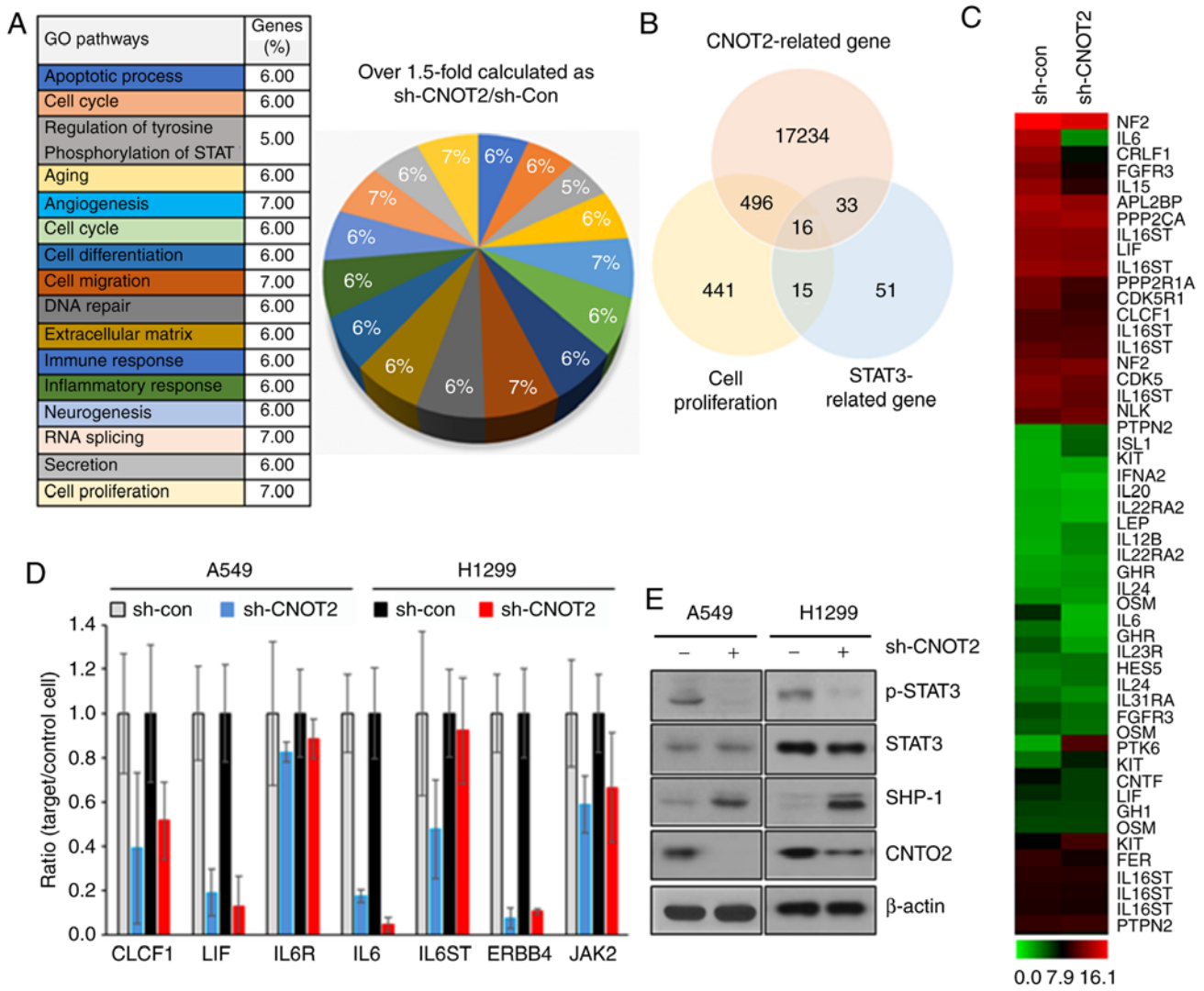

Figure 4. Gene profile in CNOT2-depleted cells and verification by RT-qPCR and western blot analysis. (A) Gene ontology (GO) pathway gene sets were compared to untreated controls in CNOT2-depleted A549 cells. (B) Associations between cell proliferation genes, STAT3-related genes and CNOT2-related genes in CNOT2-depleted A549 cells. (C) Heatmap of top-ranked upregulated or downregulated genes in CNOT2-depleted A549 cells. (D) Effect of CNOT2 depletion on the mRNA levels of CLCF1, LIF, IL6R, IL6, IL6ST, ERBB4 and JAK2 by RT-qPCR. GAPDH was used as a loading control. (E) STAT3 activity in CNOT2-depleted A549 and H1299 cells was analyzed by western blot analysis. Cell lysates were used for detecting phospho-STAT3, STAT, SHP1 and CNOT2. β-actin was used as an internal loading control. CNOT2, CCR4-NOT transcription complex subunit 2; STAT3, signal transducer and activator of transcription 3; CLCF1, cardiotrophin-like cytokines factor 1; LIF, leukemia inhibitory factor; IL6, interleukin 6; IL6R, interleukin 6 receptor; IL6ST, interleukin 6 signal transducer; ERBB4, Erb-B2 receptor tyrosine kinase 4; JAK2, Janus kinase 2; SHP1, Src homology region 2 domain containing phosphatase-1.

level of STAT3 in CNOT2-depleted cells. As shown in Fig. 4E, the phosphorylation of STAT3 was significantly decreased, while STAT3 protein expression was not altered in the A549 or H1299 cells in which CNOT2 expression was silenced. In addition, we found that SHP1 was dramatically upregulated in the same sample (Fig. 4E). To verify whether SHP1 plays an important role in CNOT2-mediated gene regulation, we inhibited the SHP1 gene in CNOT2-depleted cells. The inhibition of SHP1 increased the phosphorylation of STAT3 as compared with the control (Fig. 5A) and recovered the survival rates of CNOT2-depleted cells (Fig. 5B). These results indicate that STAT3 is a main regulator of the CNOT2-mediated signaling pathway.

Inhibition of CNOT2 increases endoplasmic reticulum (ER) stress. Recent studies have indicated that STAT3 and ER stress signaling pathways interact with each other through diverse mechanisms, leading to the control of cellular fate (23,24). Thus, it was hypothesized that STAT3 inactivation by the depletion of CNOT2 may affect the ER stress signaling pathway. To examine this hypothesis, we validated the expression level of ER stress signaling molecules. As shown in Fig. 6, CNOT2 depletion induced the expression of GRP78, CHOP and DR5, ER stress-related molecules, but not that of DR4 in the A549 and H1299 cells. These results suggest that the CNOT2-mediated STAT3 signaling pathway is closely associated with ER stress, but not apoptosis.

Discussion

In the current study, the findings demonstrated that the depletion of CNOT2 induced the TRAIL-mediated apoptosis of various TRAIL-resistant NSCLC cells. These increases in TRAIL sensitivity by CNOT2 depletion were mediated by

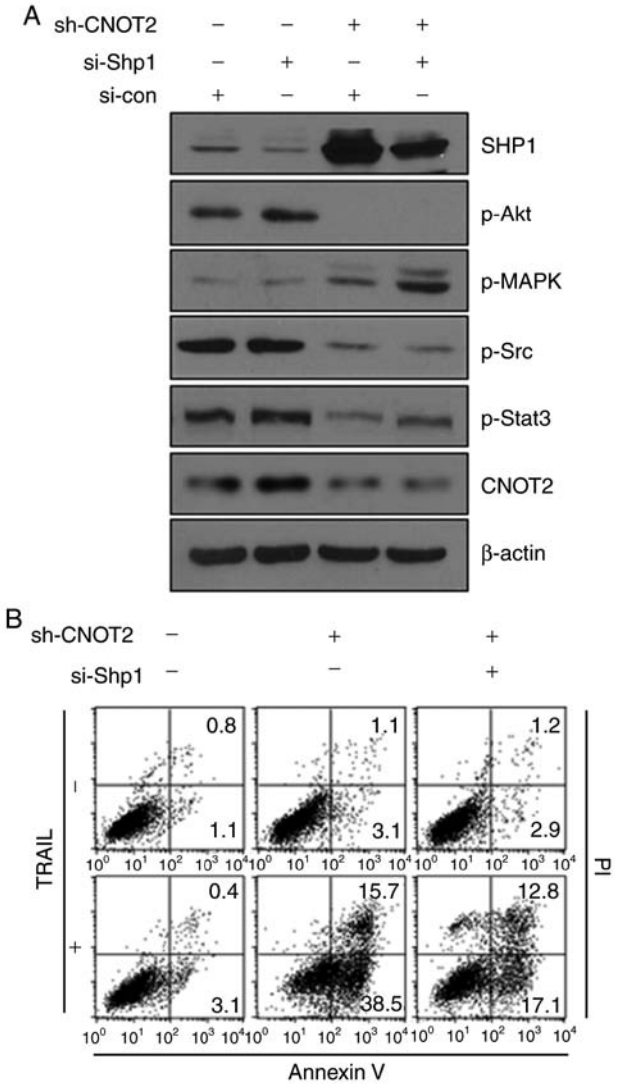

Figure 5. Inhibition of SHP1 in CNOT2-depleted cells restores STAT3 activity and cell viability. A549 cells were transfected with siRNA against CNOT2 or control siRNA to temporarily deplete SHP1. (A) Following lysis, cell lysates were examined for protein expression by western blot analysis. (B) The cells were treated with TRAIL for 48 h and apoptosis was then analyzed by Annexin V/PI staining. All of the experiments were repeated 3 times and the data represent all of the results. SHP1, Src homology region 2 domain containing phosphatase-1; CNOT2, CCR4-NOT transcription complex subunit 2; STAT3, signal transducer and activator of transcription 3; TRAIL, TNF-related apoptosis-inducing ligand.

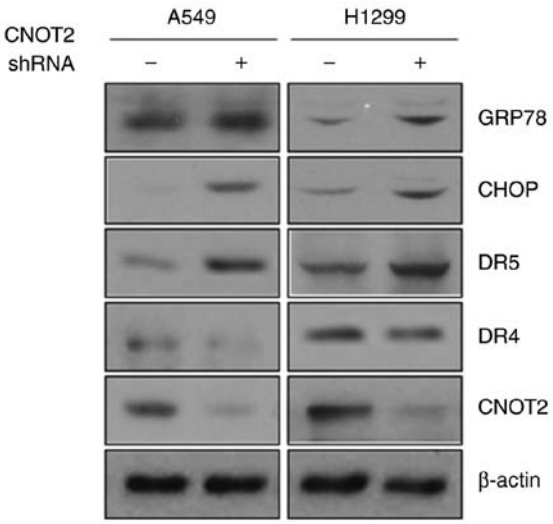

Figure 6. CNOT2 knockdown increases ER stress and partially induces apoptotic signals. A549 and H1299 cells were stably transfected with plasmid containing shRNA against CNOT2 or control. Cell lysates were analyzed according to the expression of protein to ER stress-related molecules, such as GRP78, CHOP, DR4 and DR5 by western blot analysis. CNOT2, CCR4-NOT transcription complex subunit 2; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; DR4, death receptor 4; DR5, death receptor 5; CHOP, C/EBP homologous protein.

the induction of ER stress, leading to the upregulation of DR5. In addition, it was demonstrated that CNOT2 may participate in STAT3 signaling via the regulation of SHP1 expression.

TRAIL is a promising molecule which can be used to kill tumors, even though the majority of tumors present acquired resistance against TRAIL-mediated cell death. Following the binding of TRAIL to death receptors, DR4 and DR5 receptors are clustered by the recruitment of Fas-associated protein with death domain (FADD), which initiates the extrinsic apoptotic signaling pathway (25). Subsequently, FADD recruits caspase-8 to form the death-inducing signaling complex (DISC), leading to the cleavage of caspase-3, -6 and -7 (25-27). The activation of these caspases induces apoptotic phenotypes, such as membrane blebbing, cleavage of proteins and the cytoskeleton, and DNA fragmentation. In addition, recruited caspase-8 activates the intrinsic apoptotic pathway in certain instances, such as for example, the Bax/Bcl-2 pathway (28).

However, tumors, including lung cancer tumors usually have a number of evasive tactics against TRAIL-induced apoptosis. One typical evasive strategy of various tumors is the upregulation of the anti-apoptotic proteins, Bcl-2, Bcl-xL, induced myeloid leukemia cell differentiation protein (Mcl-1), BHRF1 and E1b-19K (5). More than 50% of cancer types possess overexpressed Bcl-2 (29) and XIAP is upregulated in various types of cancer (6). The other evasion mechanism involves the downregulation of pro-apoptotic or apoptosis-inducing proteins, such as p53, caspases, PARP, Bax and the tumor necrosis factor receptor family, including DR4 and DR5 (30-33). For example, the expression levels of caspases, including caspase-8 are decreased in a diverse range of tumor cells (6,34). Mutations in the p53 gene have been reported in up to 25% of tumors (35) and the upregulation of p53 isoforms leading to the inactivation of the canonical p53 function has also been found in tumors (36).

To overcome these evasion strategies against apoptosis, diverse combination therapies with TRAIL and agents to either increase TRAIL activity or sensitize TRAIL-resistant cells have been considered. A number of phytochemicals can modulate the expression of various proteins to increase TRAIL efficiency. For example, decursin enhances TRAIL-induced apoptosis through the upregulation of ER stress-mediated DR5 in lung cancer cells (12). Another approach is to modulate genes regulating apoptosis-related proteins, leading to an increased susceptibility to TRAIL efficiency. The depletion of calcium and integrin-binding protein 1 (CIB1), regulators of oncogenic PI3K/AKT and MET/ERK signaling, with TRAIL enhances the apoptosis of triple-negative breast cancer cells (37).

Similar to CIB1, in the case of proteins with an oncogenic or tumor suppressor function, the modulation of the expression may affect TRAIL-mediated cell death. As previously demonstrated, CNOT2 participates in the regulation of angiogenesis, mobility autophagy and proliferation in diverse tumor types (11,15). Based on these oncogenic functions of CNOT2, it was hypothesized that CNOT2 may participate in TRAIL-resistant mechanisms. Consistently, the depletion of CNOT2 with TRAIL also markedly increased the apoptotic rate of TRAIL-resistant lung cancer cells.

STAT3 is a well-documented oncogenic transcription factor that participates in diverse cellular signaling pathways, including proliferation, apoptosis and cell growth (38,39). In various malignant tumors, STAT3 signaling is constitutively activated, enhancing tumorigenesis (40,41). SHP1, a non-receptor protein tyrosine phosphatase, is a major negative regulator by the dephosphorylation of STAT3 (42). A number of studies have indicated that the constitutive activation of STAT3 in various tumor types is prompted by diminished or abolished SHP1 expression (43,44). Therefore, the induction of SHP1 expression in cancer may be an effective method with which to diminish tumors via the inactivation of STAT3. In this aspect, CNOT2 may be an attractive target which may be used to boost SHP1 expression. Nakase *et al* reported that Sp1, Oct-1, NF-κB and CREB-1 interacted with the core SHP1 P2 promoter in $CD4^+$ T-cells and Jurkat cells (45). Previously, Warner *et al* suggested that CNOT2 depletion affected NF-κB expression (46). These two results indicate that CNOT2 may regulate SHP1 expression through the NF-κB signaling pathway. However, further detailed studies are required to better elucidate the mechanisms through which CNOT2 regulates SHP1.

Recent studies have suggested that the STAT signaling pathway may be connected with ER stress (23,24,47). Based on the findings of this study, under a CNOT2-depleted condition in NSCLC cells, SHP1 and ER stress molecules were concurrently increased. In addition, the inhibition of SHP1 suppressed apoptosis induced by TRAIL and CNOT2 depletion in NLCLC cells. However, this study was not able to obtain any direct evidence to connect STAT3 and ER stress in CNOT2-depleted NSCLC cells (data not shown). Further studies are warranted to address this issue.

Taken together, the depletion of CNOT2 highly induces the TRAIL-mediated apoptosis of various TRAIL-resistant NSCLC cells. These increases in TRAIL sensitivity by CNOT2 depletion are mediated by the induction of ER stress, leading to the upregulation of DR5. In addition, this study demonstrated that CNOT2 may participate in STAT3 signaling via the regulation of SHIP1 expression.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Authors' contributions

EOK was involved in acquisition, analysis and interpretation of the data and development of the methodology. SEK was involved in the acquisition of data. MC contributed to the acquisition and analysis of the data during revision. KJR was involved in the analysis of the data and revised critically for important intellectual content. MY was involved in the analysis and interpretation of the data and the writing of the manuscript, conception and design of the study, and funding. All authors discussed the results, commented on the manuscript, and approved the final submitted and published versions. In addition, all authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no that they have no competing interests.

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