

# Induction of HSP27 and HSP70 by constitutive overexpression of Redd1 confers resistance of lung cancer cells to ionizing radiation

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**Abstract.** Redd1 is a stress response protein that functions as a repressor of mTORC1, a central regulator of protein translation, resulting in the inhibition of cell growth and metabolism. However, paradoxically, high Redd1 expression favors cancer progression and generates resistance to cancer therapy. Herein, we revealed that constitutive overexpression of Redd1 induced HSP27 and HSP70 expression in lung cancer cells. The expression of Redd1, HSP27 and HSP70 was highly increased in lung cancer tissues compared with that in normal lung tissues. Inhibition of HSP27 or HSP70 suppressed AKT phosphorylation, which was induced by constitutive overexpression of Redd1 and enhanced the inhibitory effects on viability of Redd1-overexpressing cells. Inhibition of AKT phosphorylation resulted in a decrease of HSP27 and HSP70 expression in Redd1-overexpressing cells. These data indicated that HSPs and AKT in Redd1-overexpressing cells positively regulated the function and expression of each other and were involved in lung cancer cell survival. Knockdown of HSP27, HSP70 or AKT enhanced ionizing radiation (IR) sensitivity, particularly in lung cancer cells in which Redd1 was stably overexpressed. Collectively, constitutive overexpression of Redd1 led to HSP27 and HSP70 induction and AKT activation, which were involved in lung cancer cell survival and resistance to IR,

suggesting that Redd1 may be used as a therapeutic target for lung cancer.

## Introduction

Redd1 (also known as RTP801, DDIT4 or Dig2) is a highly conserved stress response gene that is induced by various stresses, such as hypoxia, ionizing radiation (IR), DNA damage and energy depletion (1-4). Redd1 acts primarily as an inhibitor of mTORC1, a central regulator of protein translation, resulting in the inhibition of cell growth and proliferation (1,5,6). However, paradoxically, high Redd1 expression favors cancer progression and generates resistance to cancer therapy. It was reported that Redd1 overexpression could predict poor prognosis of ovarian cancer (7) and promote the development of drug resistance in myeloma cells and prostate cancer cells (8,9). *In silico* analysis revealed that higher expression levels of Redd1 protein were associated with worse outcomes in acute myeloid leukemia, glioblastoma multiforme, as well as in breast, colon, skin and lung cancer (10). In our previous study, it was demonstrated that constitutive overexpression of Redd1 led to mTORC1 inhibition and to consequent AKT activation which was involved in lung cancer cell survival and resistance to chemotherapeutic drugs (11).

Heat shock proteins (HSPs) are highly conserved molecular chaperones that play essential roles in protein homeostasis, transport processes and signal transduction (12). HSPs protect cells from environmental stress damage by stabilizing the native folding of proteins, and help to sequester severely damaged proteins for degradation. HSPs are classified according to their size, and include HSP90, HSP70, HSP60, HSP40 and HSP27 (13). When proteotoxic damage is present in the cells, the demand on HSPs is increased. In particular, HSP27 and HSP70 are the most strongly induced by anticancer drugs, oxidative stress or IR (14). Overexpressed HSP27 and HSP70 have been revealed to be associated with tumor metastasis, poor prognosis and resistance to chemotherapy (15). Therefore, inhibition of HSP27 and HSP70 has emerged as a novel therapeutic strategy for cancer therapy.

In the present study, we found that constitutive overexpression of Redd1 led to HSP27 and HSP70 induction in lung cancer

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cells. Inhibition of HSP27 or HSP70 suppressed AKT phosphorylation, which was induced by constitutive overexpression of Redd1 and enhanced the inhibitory effects on the viability of Redd1-overexpressing cells. Inhibition of AKT phosphorylation resulted in a decrease of HSP27 and HSP70 expression in Redd1-overexpressing cells. These data indicated that HSPs and AKT in Redd1-overexpressing cells positively and mutually regulated their function and expression, and were involved in lung cancer cell survival. Knockdown of HSP27, HSP70 or AKT enhanced IR sensitivity, particularly in lung cancer cells in which Redd1 was stably overexpressed. Collectively, constitutive overexpression of Redd1 led to AKT activation and HSP27 and HSP70 induction, all of which were involved in lung cancer cell survival and resistance to IR, suggesting that Redd1 may be used as a therapeutic target for lung cancer.

## Materials and methods

**Cell culture, reagents and  $\gamma$ -ionizing radiation.** H1299 lung cancer cells that stably overexpressed vector or Redd1 (11) were maintained in RPMI-1640 medium (Welgene, Inc., Gyeongsangbuk-do, Korea) supplemented with 10% fetal bovine serum (FBS) and 1  $\mu$ g/ml puromycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cisplatin (*cis*-diammineplatinum (II) dichloride) and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Merck KGaA).  $^{137}$ Cesium ( $^{137}$ Cs) was used as a source of  $\gamma$ -radiation (Atomic Energy of Canada Limited, Chalk River, ON, Canada).

**Measurement of cell viability.** Cell viability was assessed by measuring the mitochondrial conversion of MTT. The proportion of converted MTT was calculated by measuring the absorbance at 570 nm. The results are expressed as the percentage reduction in MTT, assuming that the absorbance of the control cells was 100%. The MTT experiments were repeated 3 times.

**Isolation of RNA and reverse transcription PCR analysis.** Total RNA was isolated from cells using TRIzol reagent, according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA primed with oligo dT was prepared from 2  $\mu$ g total RNA using M-MLV Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). The following specific primers were used for PCR: *Redd1* (5'-GAACTCCCACCCAGATCGG-3' and 5'-CGAGGGTCAGCTGGAAGGTG-3'; 468-bp product) (16), *HSP27* (5'-AAGGATGGCGTGGTGGAGATC-3' and 5'-TCGTTGGACTGCGTGCTAG-3'; 194-bp product) (17), *HSP70* (5'-ATGAAGCAC TGGCCTTTCCA-3' and 5'-TTGTTCTGGCTGATGTCC TT-3'; 512-bp product) (18), *HSP90* (5'-TCTGGAAGATCC CCAGACAC-3' and 5'-AGTCATCCCTCAGCCAGAGA-3'; 189-bp product) (19) and  $\beta$ -actin (5'-GGATTCCTATGTGGG CGACAG-3' and 5'-CGCTCGGTGAGGATCTTCATG-3'; 438-bp product) (16).

**Real-time PCR.** Real-time PCR was conducted using TaqMan gene expression assays (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The

following primers/probes were used: *Redd1* (assay ID: Hs01111681\_g1), *HSP27* (assay ID: Hs00356629\_g1) and *HSP70* (assay ID: Hs00271229\_s1). The expression of each gene was normalised to  $\beta$ -actin (assay ID: Hs01060665\_g1). The thermocycling included an initial step at 50°C for 2 min, followed by 10 min at 95°C and 40 cycles of 15 sec at 95°C and 1 min at 60°C. The fold-change of gene expression was determined using the comparative Cq ( $2^{-\Delta\Delta C_q}$ ) method (20).

Redd1, HSP27 and HSP70 mRNA levels in lung cancer were analyzed using a commercially available TissueScan Lung Cancer Tissue qPCR panel (Lung Cancer cDNA array II #HLRT502; Origene Technologies, Inc., Rockville, MD, USA). This contained cDNA from 5 normal lungs, 25 stage I, 6 stage II, 10 stage III, and 2 stage IV lung cancer samples.

**siRNAs and transfections.** Redd1 (cat. no. sc-45806), AKT1/2 (cat. no. sc-43609), Rictor (cat. no. sc-61478) and control (cat. no. sc-37007) siRNAs were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). HSP70 siRNAs (20) were synthesized as follows (Bioneer, Co., Daejeon, Korea): siRNA-HSP70 sense, CGGUUUCUACAUGCAGAGA-dT dT and siRNA-HSP70 antisense, UCUCUGCAUGUAGAA ACCG-dTdT. The transfection experiments were performed using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.).

**Western blot analysis.** Cells were lysed in RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 5 mM EDTA, 100 mM NaF and 1 mM  $\text{Na}_3\text{VO}_4$ ] containing protease inhibitor cocktail (Roche Diagnostics GmbH, Penzberg, Germany) for 30 min at 4°C. Cell lysates were cleared by centrifugation at 12,000  $\times$  g for 20 min at 4°C, and the protein concentrations were measured by Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein samples (15–30  $\mu$ g) were separated using 8–12% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween (TBST) and 5% non-fat dry milk for 1 h at room temperature. The primary antibodies were incubated overnight at 4°C. The following antibodies were used: Redd1 (dilution 1:1,000; cat. no. 10638-1-AP) was obtained from ProteinTech Group, Inc. (Rosemont, IL, USA); AKT (dilution 1:1,000; cat. no. 9272), p-AKT at Ser473 (dilution 1:1,000; cat. no. 9271), HSP27 (dilution 1:2,000; cat. no. 2402), Rictor (dilution 1:1,000; cat. no. 2114), S6 (dilution 1:2,000; cat. no. 2217) and p-S6 at Ser240/244 (dilution 1:2,000; cat. no. 4838) were all obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA); HSP70 (dilution 1:2,000; cat. no. ADI-SPA-812) was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA); HSP90 (dilution 1:2,000; cat. no. sc-69703) was obtained from Santa Cruz Biotechnology, Inc.; and  $\beta$ -actin (dilution 1:3,000; cat. no. A5316) was purchased from Sigma-Aldrich (Merck KGaA). After 3 washes in TBST, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG (dilution 1:3,000; cat. no. sc-2030; Santa Cruz Biotechnology, Inc.) and anti-mouse IgG (dilution 1:3,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) secondary antibodies. The immunoreactive bands were visualized using SuperSignal

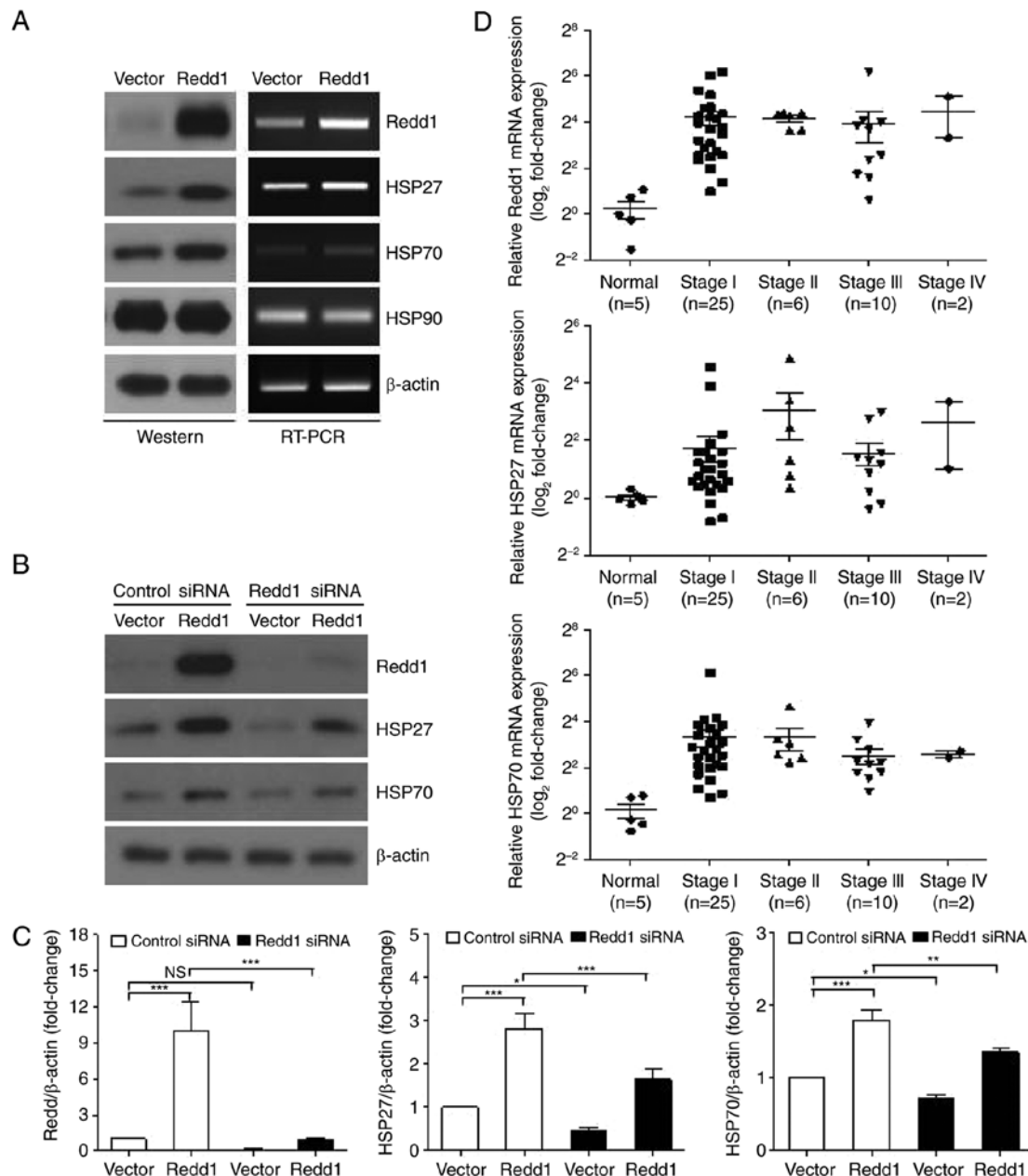


Figure 1. Constitutive overexpression of Redd1 induces HSP27 and HSP70 expression. (A) H1299 cells that stably overexpressed vector or Redd1 were plated at a density of  $5 \times 10^5$  cells/60 mm and incubated for 40 h. The indicated protein and mRNA levels were determined by western blotting and RT-PCR, respectively. β-actin was used as a loading control. (B and C) H1299 cells that stably overexpressed vector or Redd1 were transiently transfected with Redd1 siRNA for 48 h. The indicated protein levels were determined by (B) western blotting. (C) The protein bands were quantitated by ImageJ software and fold intensity with respect to control after normalization was plotted as histogram ( $P < 0.05$ ,  $^{**}P < 0.01$  and  $^{***}P < 0.001$ ; NS, not significant). (D) Redd1, HSP27 and HSP70 mRNA levels were determined by quantitative real-time PCR in normal lung tissues and lung cancer specimens with different pathologic grades (TissueScan lung cancer cDNA array #HLRT502; Origene Technologies). Data are normalized to β-actin expression and presented as the log<sub>2</sub>-fold-change relative to the normal lung tissue.

West Pico Chemiluminescent substrates (Pierce; Thermo Fisher Scientific Inc.). Where indicated, western blot images were quantified using ImageJ software (version 1.52a; NIH; National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis.** The results are expressed as the mean  $\pm$  standard deviation (SD) of 3 independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test of GraphPad Prism software (version 5.0; GraphPad Software Inc., San Diego, CA, USA).  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$  were considered to indicate statistically significant results.

## Results

**Expression of HSP27 and HSP70 is increased in Redd1-overexpressing cells.** We first analyzed proteins with expression levels that had changed in Redd1-overexpressing cells using two-dimensional gel electrophoresis-based proteomic analysis, and we observed increased levels of HSP70 protein (data not shown). Next, we used western blot analysis to further investigate the expression of HSPs in H1299 lung cancer cells that stably overexpressed Redd1. Increased levels of HSP27 and HSP70 protein were observed in Redd1-overexpressing cells (Fig. 1A, left panel). However, constitutive overexpression

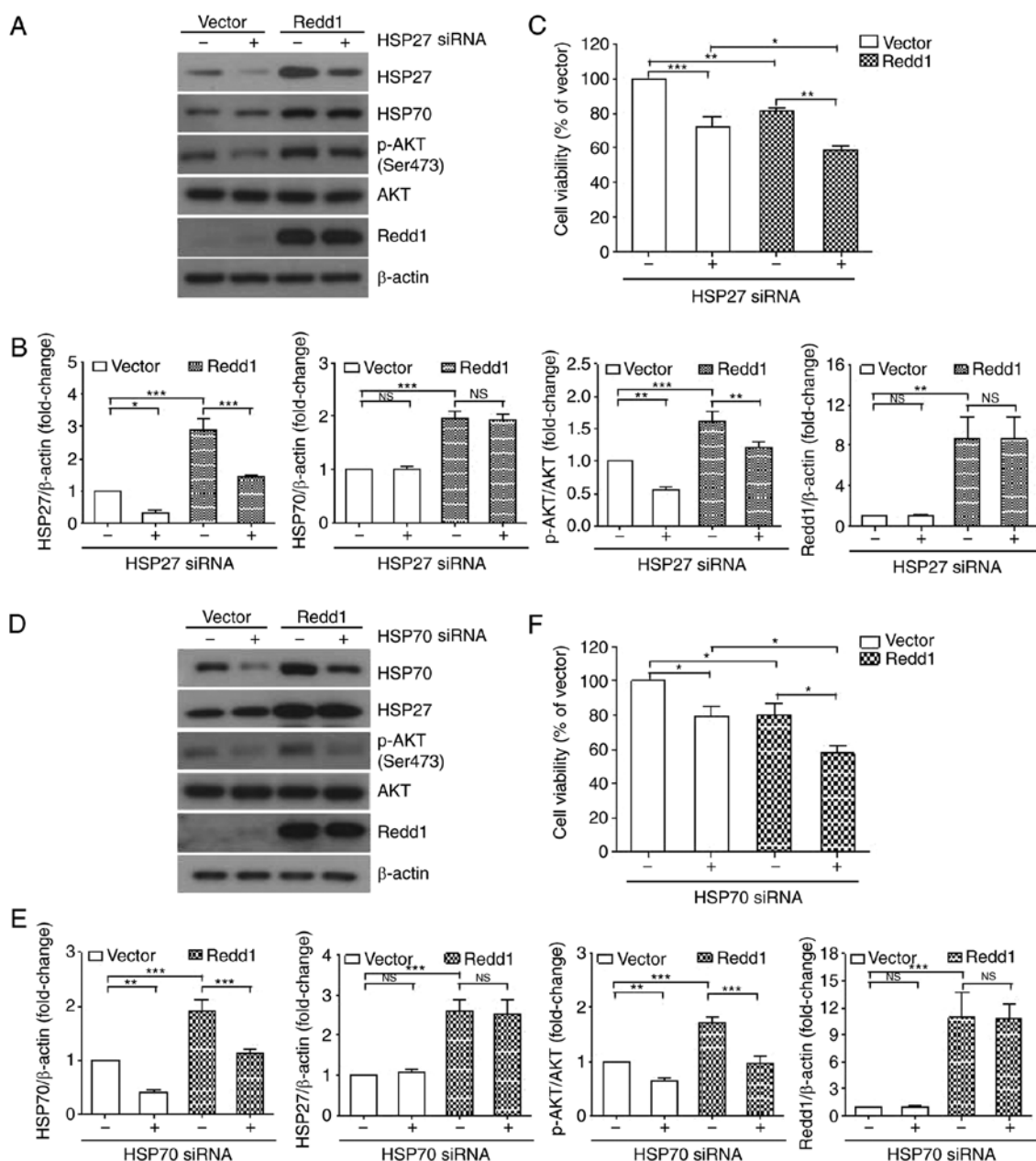


Figure 2. Knockdown of HSP27 or HSP70 inhibits AKT phosphorylation and enhances the inhibitory effects on the viability of Redd1-overexpressing cells. (A-C) H1299 cells that stably overexpressed vector or Redd1 were transiently transfected with HSP27 siRNA for 48 h. (D-F) H1299 cells that stably overexpressed vector or Redd1 were transiently transfected with HSP70 siRNA for 48 h. (A and D) The indicated protein levels were determined by western blot analysis.  $\beta$ -actin was used as a loading control. (B and E) The protein bands were quantitated by ImageJ software and fold intensity with respect to the control after normalization was plotted as a histogram (\* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001; NS, not significant). (C and F) Cell viability was measured using an MTT assay. The data are presented as the mean  $\pm$  SD (\* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001; NS, not significant).

of Redd1 did not change the levels of HSP90 protein (Fig. 1A, left panel). Expression of HSP27 and HSP70 mRNA was also increased in Redd1-overexpressing cells (Fig. 1A, right panel). To further ascertain whether constitutive overexpression of Redd1 induced HSP27 and HSP70 expression, Redd1 siRNAs were transfected in Redd1-overexpressing cells. As revealed in Fig. 1B and C, treatment with Redd1 siRNA reduced Redd1-mediated HSP27 and HSP70 expression. Based on these results, it was indicated that constitutive overexpression of Redd1 induces HSP27 and HSP70 expression.

Clinically, increased expression of HSP27 and HSP70 has been shown in high-grade malignant tumors (21-23). Redd1 expression was significantly increased in non-small lung

cancer tissues compared with that in normal lung tissues (24). To evaluate HSP27, HSP70 and Redd1 mRNA expression in the different stages of lung cancer, we used a commercially available Lung Cancer Tissue qPCR panel. This included cDNAs obtained from 48 patients with histopathologically confirmed lung cancer, representing all stages (stage I,  $n=25$ ; stage II,  $n=6$ ; stage III,  $n=10$ ; stage IV,  $n=2$ ), and normal lung samples ( $n=5$ ). Redd1, HSP27 and HSP70 mRNA levels were higher in lung cancer tissues than in normal lung tissues (Fig. 1D).

*Knockdown of HSP27 or HSP70 inhibits AKT phosphorylation and enhances the inhibitory effects on the viability of Redd1-overexpressing cells.* HSP27 has been reported to control

apoptosis by regulating the AKT signaling pathway (25,26). We previously reported that constitutive overexpression of Redd1 resulted in AKT activation, which was involved in lung cancer cell survival (11). Thus, we examined AKT phosphorylation levels and viability in Redd1-overexpressing cells following treatment with a siRNA against HSP27. As anticipated, treatment with HSP27 siRNA resulted in decreased levels of AKT phosphorylation, which was induced by constitutive overexpression of Redd1 (Fig. 2A and B). Moreover, cell viability was significantly reduced in the HSP27 siRNA-treated, Redd1-overexpressing cells when compared with the control siRNA-treated, Redd1-overexpressing cells (Fig. 2C). However, knockdown of HSP27 did not affect the levels of HSP70 in either the vector or the Redd1-overexpressing cells (Fig. 2A and B).

Next, we examined AKT phosphorylation levels and viability in Redd1-overexpressing cells following treatment with a siRNA against HSP70. HSP70 siRNA inhibited AKT phosphorylation which was induced by constitutive overexpression of Redd1 (Fig. 2D and E), and enhanced the inhibitory effects on the viability of Redd1-overexpressing cells (Fig. 2F). However, knockdown of HSP70 did not affect the levels of HSP27 in either the vector or the Redd1-overexpressing cells (Fig. 2D and E). These data indicated that HSP27 and HSP70 induction by constitutive overexpression of Redd1 was required for AKT phosphorylation, and was involved in lung cancer cell survival.

*Inhibition of AKT phosphorylation reduces HSP27 and HSP70 expression induced by constitutive overexpression of Redd1.* To examine the effects of AKT on HSP27 and HSP70 expression induced by constitutive overexpression of Redd1, we investigated the expression of HSP27 and HSP70 following treatment with AKT1/2 siRNA or perifosine, a selective AKT inhibitor. As revealed in Fig. 3A and B, depletion of Akt by siRNAs or inhibition of AKT by perifosine suppressed Redd1-induced HSP27 and HSP70 expression, indicating that AKT activity was required for HSP27 and HSP70 expression in Redd1-overexpressing cells. Treatment of AKT1/2 siRNA or perifosine also further enhanced the inhibition of mTORC1 activity by Redd1, as judged by the decrease in S6 phosphorylation. We previously reported that Redd1-induced AKT activation was mediated by mTORC2 (11). Thus, we investigated the effects of mTORC2 on Redd1-induced HSP27 and HSP70 expression. The expression of Rictor, a key component of mTORC2, was suppressed by treatment with Rictor siRNA. As revealed in Fig. 3C, Rictor siRNA not only inhibited AKT phosphorylation as previously reported (11), but also decreased HSP27 and HSP70 expression in Redd1-overexpressing cells. Knockdown of Rictor led to further increases in the cisplatin sensitivity of Redd1-overexpressing cells when compared with that of vector-overexpressing cells (Fig. 3D). These data indicated that mTORC2 activity was required for HSP27 and HSP70 expression in Redd1-overexpressing cells.

*Inhibition of HSP27, HSP70 or AKT increases the sensitivity of Redd1-overexpressing cells to IR.* Radiation therapy for lung cancer is effective for destroying cancer cells and shrinking tumors. Thus, H1299 cells that stably overexpressed

vector or Redd1 were exposed to increasing doses of IR for 24 h. The cell viability rate was >80%, despite the exposure of both cells to 5 Gy of IR (Fig. 4A). IR increased the levels of HSP27 and HSP70 expression and AKT phosphorylation in a dose-dependent manner in H1299 cells that stably overexpressed vector or Redd1 (Fig. 4B). Transfection of HSP27, HSP70 or AKT1/2 siRNAs in Redd1-overexpressing cells led to further increases in IR sensitivity when compared with the control siRNA-treated, Redd1-overexpressing cells (Fig. 4C). These data indicated that inhibition of HSP27, HSP70, or AKT enhanced IR sensitivity, particularly in cells in which Redd1 was stably overexpressed.

## Discussion

Redd1 (also known as RTP801, DDIT4 or Dig2) acts as a negative regulator of mechanistic target of rapamycin complex I (mTORC1), which integrates diverse signals to regulate cell growth and metabolism (1,5,6). In the present study, we revealed that constitutive overexpression of Redd1 induced HSP27 and HSP70 expression. Clinically, increased expression of HSP27 and HSP70 has been monitored in high-grade malignant tumors, such as osteosarcoma, leukemia, breast, ovarian and endometrial cancer, and renal cell carcinoma (21-23). Redd1 was significantly increased in non-small cell lung cancer (NSCLC) tissue compared with normal lung tissue (24). In the present study, qRT-PCR human lung cancer tissue cDNA arrays demonstrated that Redd1, HSP27 and HSP70 mRNA expression levels were increased in lung cancer tissue compared with those in normal lung tissue.

Several studies have reported that the resistance to apoptosis and antiproliferative signals observed in cancer cells is actually correlated with HSP27 and HSP70 induction (14). Pre-clinical and patient studies demonstrated that overexpression of HSP27 and HSP70 corresponded with an increased proliferation of malignant cells, and that the inhibition of HSP27 and HSP70 expression and function reduced the proliferation and increased the susceptibility of tumor cells to chemotherapy and radiotherapy (27-29). Disruption of HSP27 or HSP70 enhanced the viability of Redd1-overexpressing H1299 lung cancer cells (Fig. 2). Ionizing radiation (IR) increased HSP27 and HSP70 expression levels in a dose-dependent manner in cells that stably overexpressed vector or Redd1 (Fig. 4B). The inhibition of HSP27 and HSP70 with siRNA in Redd1-overexpressing cells led to further increases in IR sensitivity when compared with the vector-overexpressing cells (Fig. 4C). These data indicated that HSP27 and HSP70 induction, which occurred as a consequence of the constitutive overexpression of Redd1, played a role in lung cancer cell survival and resistance to IR.

Several studies have shown that HSP27 and HSP70 activate an adaptive mechanism to preserve cell survival through AKT signalling (30,31). The AKT signaling pathway was also revealed to regulate the expression of HSP27 and HSP70, which contributed to tumor cell survival (32,33). We also observed that inhibition of HSP27 or HSP70 function suppressed AKT phosphorylation induced by the constitutive overexpression of Redd1, and the inhibition of AKT function suppressed Redd1-mediated HSP27 or HSP70 expression. These data indicated that heat shock proteins (HSPs) and AKT

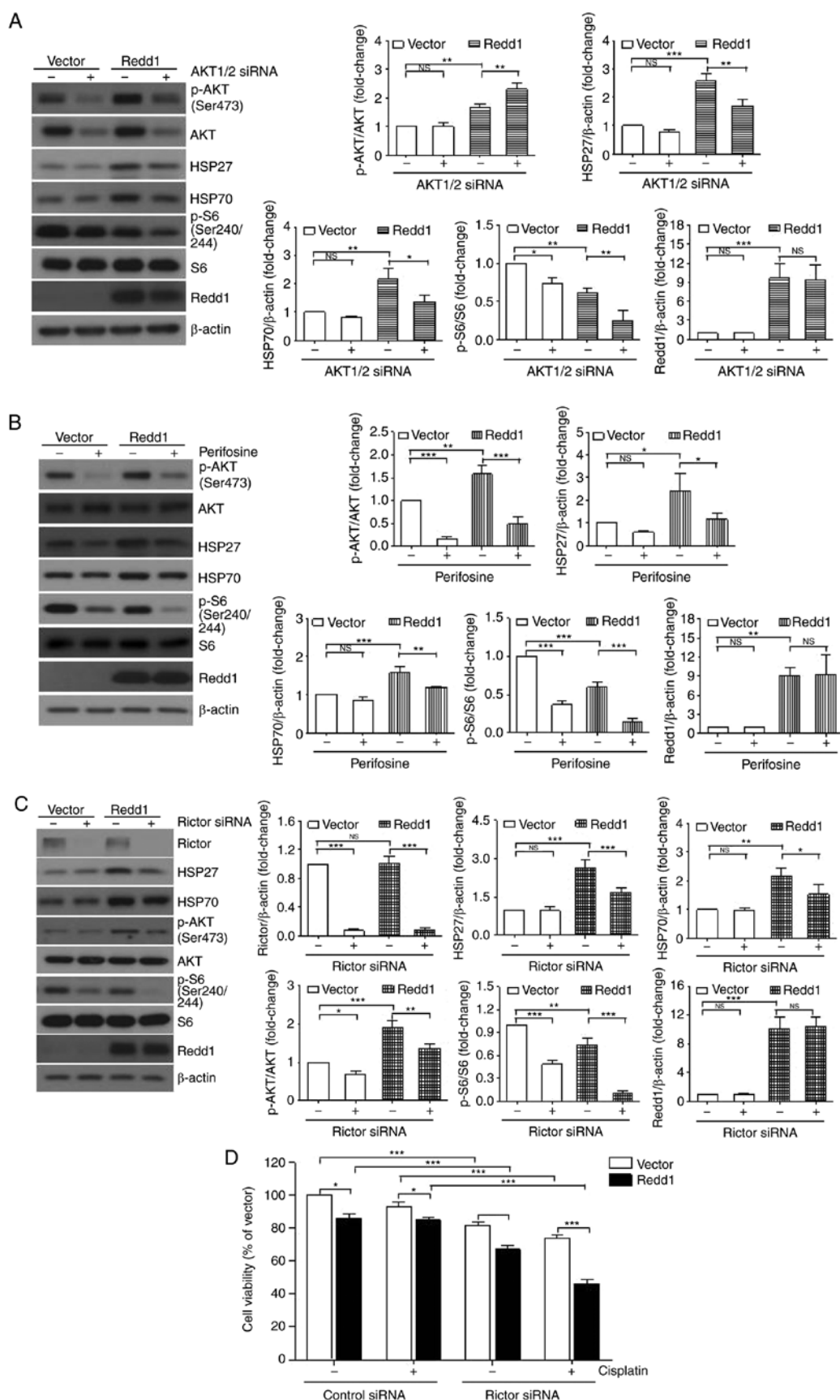


Figure 3. Inhibition of AKT or Rictor reduces HSP27 and HSP70 expression induced by the constitutive overexpression of Redd1. (A) H1299 cells that stably overexpressed vector or Redd1 were transiently transfected with AKT1/2 siRNA for 48 h. (B) H1299 cells that stably overexpressed vector or Redd1 were treated with 5  $\mu$ M perifosine for 48 h. (C) H1299 cells that stably overexpressed vector or Redd1 were transiently transfected with Rictor siRNA for 48 h. (A-C, left panels) The indicated protein levels were determined by western blot analysis. (A-C, right panels) The protein bands were quantitated by ImageJ software and fold intensity with respect to the control after normalization was plotted as a histogram (\* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001; NS, not significant). (D) H1299 cells that stably overexpressed vector or Redd1 were transiently transfected with Rictor siRNA for 16 h and subsequently treated with 5  $\mu$ M cisplatin for 24 h. Cell viability was measured using an MTT assay. The data are presented as the mean  $\pm$  SD (\* $P$ <0.05 and \*\*\* $P$ <0.001; NS, not significant).

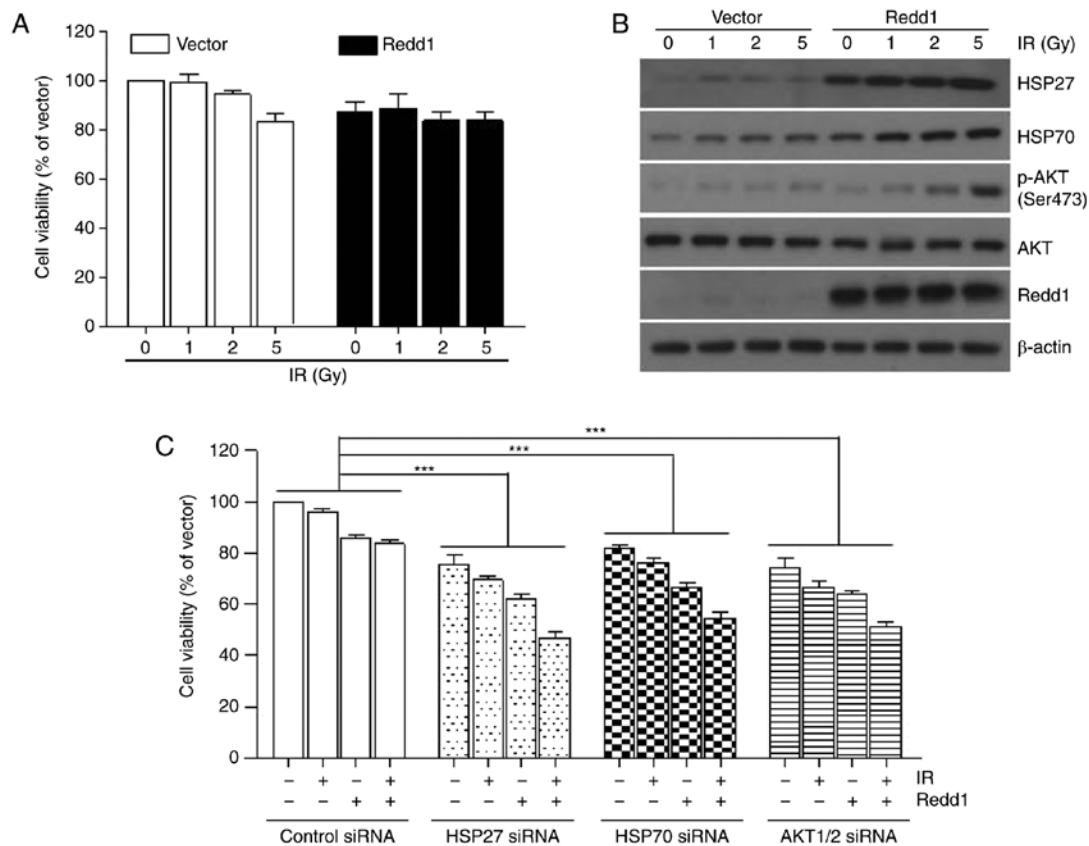


Figure 4. Inhibition of HSP27, HSP70 or AKT1/2 in H1299 cells that stably overexpress Redd1 increases sensitivity to IR. (A and B) H1299 cells that stably overexpressed vector or Redd1 were exposed to the indicated doses of IR for 24 h. (C) H1299 cells that stably overexpressed vector or Redd1 were transiently transfected with control, HSP27, HSP70 or AKT1/2 siRNA for 16 h, and subsequently exposed to 5 Gy of IR for 24 h. (A and C) Cell viability was measured using an MTT assay. The data are presented as the mean  $\pm$  SD (\*\* $P < 0.001$ ). (B) The indicated protein levels were determined by western blot analysis.  $\beta$ -actin was used as a loading control. The blots shown are representative of 2 independent experiments. IR, ionizing radiation.

positively regulated each other in Redd1-overexpressing cells, and that they were involved in lung cancer cell survival.

We previously reported that Redd1-induced AKT activation was mediated by mTORC2 (11). Thus, we investigated the effects of mTORC2 on Redd1-induced HSP27 and HSP70 expression. Inhibition of mTORC2 using Rictor siRNA led to decreases in HSP27 and HSP70 expression and further increases in cisplatin sensitivity in Redd1-overexpressing cells (Fig. 3C and D), suggesting that mTORC2 activity was partially required for HSP27 and HSP70 expression in Redd1-overexpressing cells.

Collectively, constitutive overexpression of Redd1 led to HSP27 and HSP70 induction and AKT activation, all of which were involved in lung cancer cell survival, suggesting that Redd1 may be used as a therapeutic target for lung cancer.

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## Availability of data and materials

The authors declare that the materials included in the manuscript, including all relevant raw data, will be freely available to any researchers who wish to use them for non-commercial purposes, while preserving any necessary confidentiality and anonymity.

## Authors' contributions

HOJ and ICP developed the concept and designed the study. HOJ, SEH, JYK and MRK performed the experiments. YHC, YJH and JKL provided technical support and conceptual advice. HOJ and ICP wrote the manuscript. HOJ, YHC, YJH, JKL and ICP reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that 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 that they have no competing interests.

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