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Depletion of hepatoma-derived growth factor-related protein-3 induces apoptotic sensitization of radioresistant A549 cells via reactive oxygen species-dependent p53 activation

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ABSTRACT

Biomarkers based on functional signaling have the potential to provide greater insight into the pathogenesis of cancer and may offer additional targets for anticancer therapeutics. Here, we identified hepatoma-derived growth factor-related protein-3 (HRP-3) as a radioresistance-related gene and characterized the molecular mechanism by which its encoded protein regulates the radio- and chemoresistant phenotype of lung cancer-derived A549 cells. Knockdown of HRP-3 promoted apoptosis of A549 cells and potentiated the apoptosis-inducing action of radio- and chemotherapy. This increase in apoptosis was associated with a substantial generation of reactive oxygen species (ROS) that was attributable to inhibition of the Nrf2/HO-1 antioxidant pathway and resulted in enhanced ROS-dependent p53 activation and p53-dependent expression of PUMA (p53 upregulated modulator of apoptosis). Therefore, the HRP-3/Nrf2/HO-1/ROS/p53/PUMA cascade is an essential feature of the A549 cell phenotype and a potential radiotherapy target, extending the range of targets in multimodal therapies against lung cancer.

1. Introduction

Hepatoma-derived growth factor (HDGF) was initially purified as a secreted mitogen from the Huh-7 human hepatoma cell lines [1]. HDGF is a nuclear protein with mitogenic activity that functions as a transcriptional repressor by binding to a conserved element in the promoter of target genes [2]. Recently, HDGF has been found to be a potent tumorigenic and prognostic factor in cancers, including hepatocellular carcinoma (HCC), gastric cancer, and non-small-cell lung cancer (NSCLC). For example, overexpression of HDGF is correlated with promotion of lymph node metastases in gastric cancer [3]. Despite the accumulated knowledge about the biochemical functions of HDGF protein, its signaling mechanisms in development, vascular injury, and cancer are still largely unknown.

Currently, five HDGF-related proteins (HRPs) have been identified from different species, including HRP-1 to -4 and lens epithelium-derived growth factor (LEDGF), all of which share a conserved DNA-binding HATH domain at the N-terminus [4]. Studies on HRP expression in mouse and bovine models have reported that both HRP-1 and HRP-4 mRNA expression are limited to the testis, whereas HRP-2 transcripts are more broadly distributed [4,5]. Although HRP-3 mRNA is expressed to a slight extent in the ovary, kidney, spleen, and liver, it is mainly restricted to the brain in humans, suggesting a particular role in the nervous system [6]. Consistent with this, HRP-3 expression in the brain is regulated in a developmental stage-dependent manner in mice and has biological roles beyond growth-promoting activity in the brain [7]. For example, HRP-3 is involved in neuritogenesis of primary cortical neurons through modulation of the neuronal cytoskeleton by interacting with tubulin [8] or heparin sulfate proteoglycans [9]. One recent study has reported an association of HRP-3 with cancer [10]. The authors of this study suggested that HRP-3 plays an essential role in the pathogenesis of HCC based on the significant upregulation of HRP-3 in human HCC tissues compared to non-cancerous tissues. Although HRP-3 signaling has been implicated in the physiology of neuronal cells, its pathological relevance in NSCLC cells and associated molecular mechanisms of action remain largely unknown. In particular, the role of HRP-3 in the

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acquisition of a resistant phenotype against radiation or anticancer agents by these cells remains unclear.

Here, we demonstrate that HRP-3 knockdown using small interfering RNA (siRNA) promoted apoptosis of radio- and chemoresistant A549 cells and significantly sensitized these cells to radiotherapy- and chemotherapy-induced apoptosis. Consistent with this, HRP-3 depletion alone or in combination with radiation markedly increased the generation of reactive oxygen species (ROS) via inhibition of the Nrf2 (nuclear factor erythroid 2 related factor 2)/HO-1 (heme oxygenase 1) pathway. Elevated ROS levels, in turn, contributed to apoptosis by enhancing ROS–dependent p53 activation and expression of PUMA (p53 upregulated modulator of apoptosis). Disruption of HRP-3 thus reveals an important role of the HRP-3/Nrf2/HO-1/ROS/p53/PUMA cascade in promoting apoptosis of A549 cells and suggests that HRP-3 may serve as a novel molecular target for the development of new agents for lung cancer therapy.

2. Materials and methods

2.1. Cell culture and treatment

Human A549 and H460 cells were purchased from the ATCC (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. Cells were irradiated using a ^{137}Cs-ray source (Atomic Energy of Canada, Ltd., Mississauga, Canada) at a dose rate of 3.81 Gy/min. Where indicated, cells were treated with 0.1 μg/mL doxorubicin or 5 ng/mL vinblastine to induce apoptosis. We used 1 mM N-acetyl cysteine (NAC) to scavenge ROS and 10 μM cycloheximide to block de novo protein synthesis. All chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell proliferation

HRP-3-knockdown and control cells were plated on culture dishes at a density of 3.5×10^5 cells/cm^2 for the indicated times. Cell proliferation was determined by directly counting surviving cells using a hemocytometer.

2.3. Clonogenic assay

Cell survival was determined using clonogenic assays, as described previously [11].

2.4. Cell death assay

Cell death was quantified as described previously [12]. Apoptotic cell death was also determined by Western blot analysis of cleaved poly (ADP–ribose) polymerase (PARP) and activated caspase-3. Alterations in cellular morphology were observed by light microscopy.

2.5. ROS assay

Cells were incubated with 10 nM 2′,7′-dichlorofluorescein diacetate (Molecular Probes, Inc., Eugene, OR, USA) for 20 min and analyzed by fluorescence-activated cell sorting (FACS) to detect ROS, as described previously [12].

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously described [12]. The following primer pairs were employed: HRP-3 (426-bp product), 5′-ATGAAGGGCTACCCGCACTG-3′ (sense) and 5′-CCGGGACTGTTTAGAGGATTTC-3′ (antisense); p53 (361-bp product), 5′-GTGGTGCCCTATGAGCCGCC-3′ (sense) and 5′-GCTCACGCCCACGGATCTGA-3′ (antisense); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (305-bp product), 5′-CATCTCTGCCCCCTCTGCTGA-3′ (sense) and 5′-GGATGACCTTGCCCACAGCCT-3′ (antisense). Quantitative real-time PCR was performed in triplicate using a Chromo4 thermocycler and SYBR Premix Ex Taq (Takara Bio, Shiga, Japan). The amplification signal of the target gene was normalized to that of GAPDH in the same reaction.

2.7. Knockdown of HRP-3 by siRNA

The following siRNA oligonucleotides targeting HRP-3 were synthesized by Genolution Pharmaceuticals, Inc (Seoul, Korea): siRNA #1, 5′-CCAGUGAAGGGACCUAACUUU-3′ (sense) and 5′-AGUUAGGUCCCUUCACUGGUU-3′ (antisense); siRNA #2, 5′-GGCCAUGUGUAAAGUUUAAUU-3′ (sense) and 5′-UUAAACUUUACACAUGGCCUU-3′ (antisense). Non-silencing siRNA was used as a negative control. In experiments, cells were transfected with siRNA #2 as described previously [11].

2.8. Western blot analysis

Western blot analyses were performed as described previously [12] using primary antibodies against HRP-3 (Proteintech Group, Inc., Chicago, IL, USA); p53 and Nrf2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); Keap1 (R&D Systems, Inc., Minneapolis, MN, USA); and cleaved-PARP (Asp214), cleaved caspase-3, phospho-p53 (Ser15), PUMA, and HO-1 (Cell Signaling Technology Inc., Beverly, MA, USA). β-Actin (Sigma) was used as a loading control.

2.9. Immunofluorescence confocal microscopy

For staining, cells were incubated for 1 h with 10 μg/ml of rabbit polyclonal anti-HRP-3 primary antibody (Proteintech Group, Inc.) and then incubated for an additional 1 h with fluorescein isothiocyanate-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA), as described previously [11].

2.10. Statistical analysis

Cell culture experiments were repeated at least three times. Statistical differences between groups were assessed by Students *t*-test, and a *p*-value <0.05 was considered significant.

3. Results

3.1. Depletion of HRP-3 induces apoptosis of radio- and chemoresistant A549 cells

Individual treatment of A549 cells with radiation, doxorubicin, or vinblastine induced approximately 17%, 16% and 19% cell death, respectively, compared to the corresponding values of 41%, 38% and 43% in H460 cells (Fig. 1A), suggesting that A549 cells were more tolerant to radiation and the anticancer drugs than H460 cells. To identify genes that contributed to the radioresistant cellular phenotype, we screened lung cancer EST libraries (#1611) for resistance-related genes using an *in silico/in vitro* analysis. Among the radiation-responsive genes identified by these screens was that for HRP-3 gene. Notably, the transcript (Fig. 1B, top) and protein (Fig. 1B, bottom) levels of HRP-3 were much higher in A549 cells than in H460 cells, supporting the association of HRP-3 with the radioresistant phenotype of A549 cells. Immunofluorescence staining analyses revealed that HRP-3 was distributed only in the

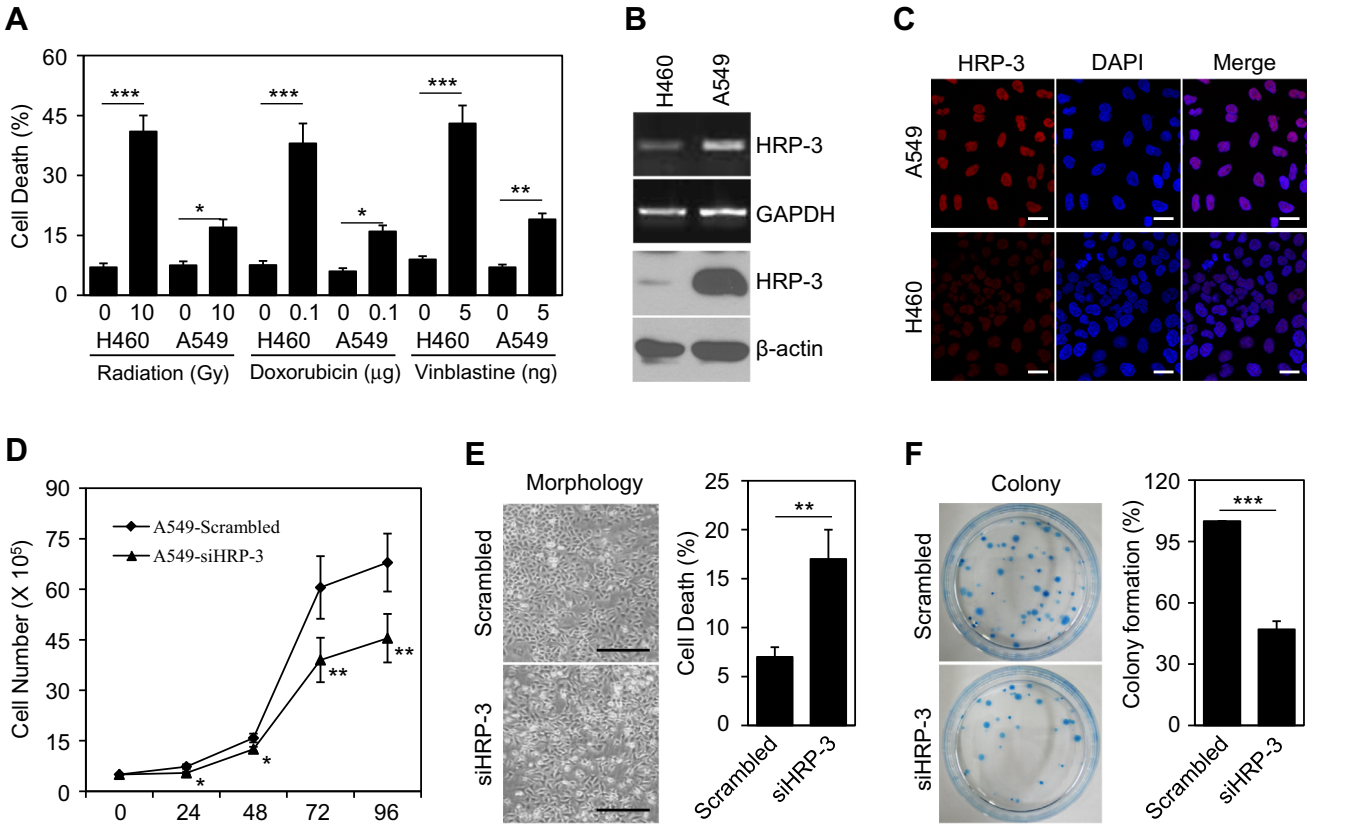

Fig. 1. Association of HRP-3 with the resistant phenotype of A549 cells. (A) H460 and A549 cells were treated with 10 Gy radiation, 0.1 μg/mL doxorubicin, or 5 ng/mL vinblastine for 48 h. Cell death was determined by FACS analysis; data are expressed as means ± SD ($^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0005$ compared with untreated controls). (B) Transcript (top) and protein (bottom) levels of HRP-3 in H460 and A549 cells were determined by RT-PCR (loading control, GAPDH) and Western blotting, respectively. (C) Representative confocal images of HRP-3 localization in H460 and A549 cells (scale bar: 50 μm). (D–F) A549 cells were transfected with or without 100 nM HRP-3 siRNA for 5 h and then cultured for an additional 4 days (D), 2 days (E), or 14 days (F). Proliferation was determined by cell counting; data are expressed as means ± SD ($^{*}p < 0.05$ and $^{**}p < 0.005$ compared with untransfected control) (D). Changes in cell morphology were observed by light microscopy (E, left; scale bar: 1 mm) and cell death was determined by FACS analysis; data are expressed as means ± SD ($^{**}p < 0.005$ compared with untransfected control) (E, right). Colony formation was visualized by trypan blue staining (F, left); data are expressed as means ± SD ($^{***}p < 0.0005$ compared with untransfected control) (F, right).

nucleus of both A549 and H460 cells, a targeting consistent with its presumptive primary role in nuclear signal transduction (Fig. 1C). To examine whether HRP-3 is a key factor in maintaining the resistant phenotype of A549 cells, we knocked downed endogenous HRP-3 by transfecting A549 cells with two different siRNAs. Although both siRNAs markedly decreased HRP-3 expression, siRNA #2 was more effective, as determined by quantitative RT-PCR and Western blotting (Supplemental Fig. 1A and B). Thus, subsequent knockdown experiments were performed using siRNA #2. Depletion of HRP-3 potently reduced proliferation of A549 cells in a time-dependent fashion compared to control cells (Fig. 1D) and induced morphological changes characteristic of apoptosis in approximately 17% of cells compared to 7% in controls (Fig. 1E). In support of these results, the levels of cleaved PARP and active caspase-3, two important apoptotic standards, were elevated in HRP-3-knockdown cells compared to control cells transfected with scrambled siRNA (Supplemental Fig. 1C). In addition, siRNA-mediated HRP-3 knockdown decreased colony formation by approximately 53% compared to control cells (Fig. 1F). Thus, our results suggest that HRP-3 is essential for the maintenance of the radio- and chemoresistant phenotype of A549 cells.

3.2. HRP-3 depletion synergistically induces apoptosis of A549 cells in combination with radiation or anticancer drugs

To examine whether HRP-3 is involved in responses to radiation or anticancer drugs, we treated A549 cells with 10 Gy radiation, 0.1 μg/mL doxorubicin, or 5 ng/mL vinblastine. All stimulations increased HRP-3 protein levels, which reached a peak at about 24 h and then declined to basal levels (Fig. 2A), indicating that HRP-3 is a radiation- and anticancer drug-responsive protein. To better understand the correlation between HRP-3 level and resistant phenotype, we analyzed the effect of HRP-3 loss-of-function in A549 cells exposured to the same stimuli. Although siHRP-3 alone induced cell death compared to control siRNA, stimulation with radiation, doxorubicin, or vinblastine significantly enhanced cell death by approximately 2.3-, 2.4-, and 2.3-fold, respectively, in HRP-3-knockdown cells compared to A549 cells transfected with siHRP-3 alone (Fig. 2B). This combined effect was especially prominent in HRP-3-knockdown A549 cells exposed to 6 Gy radiation, where colony formation was decreased by approximately 74% compared to A549 cells transfected with siHRP-3 alone (Fig. 2C); clonogenic survival assays showed that HRP-3-knockdown cells were also more sensitive than control cells to exposure to single doses of radiation ranging from 0 Gy to 6 Gy (Fig. 2D). In line with these results, combined radiation treatment and HRP-3 depletion caused a significant increase in cleaved PARP and active caspase-3 levels, although each treatment alone induced a slight increase in both cleaved PARP and active caspase-3 levels compared to control (Fig. 2E). The levels of these two apoptotic markers also consistently paralleled cell death patterns in A549 cells stimulated with anticancer drugs and/or siHRP-3 (Fig. 2F). Therefore, our data suggest that HRP-3 is a key regulator of the radio- and chemosensitivity of A549 cells.

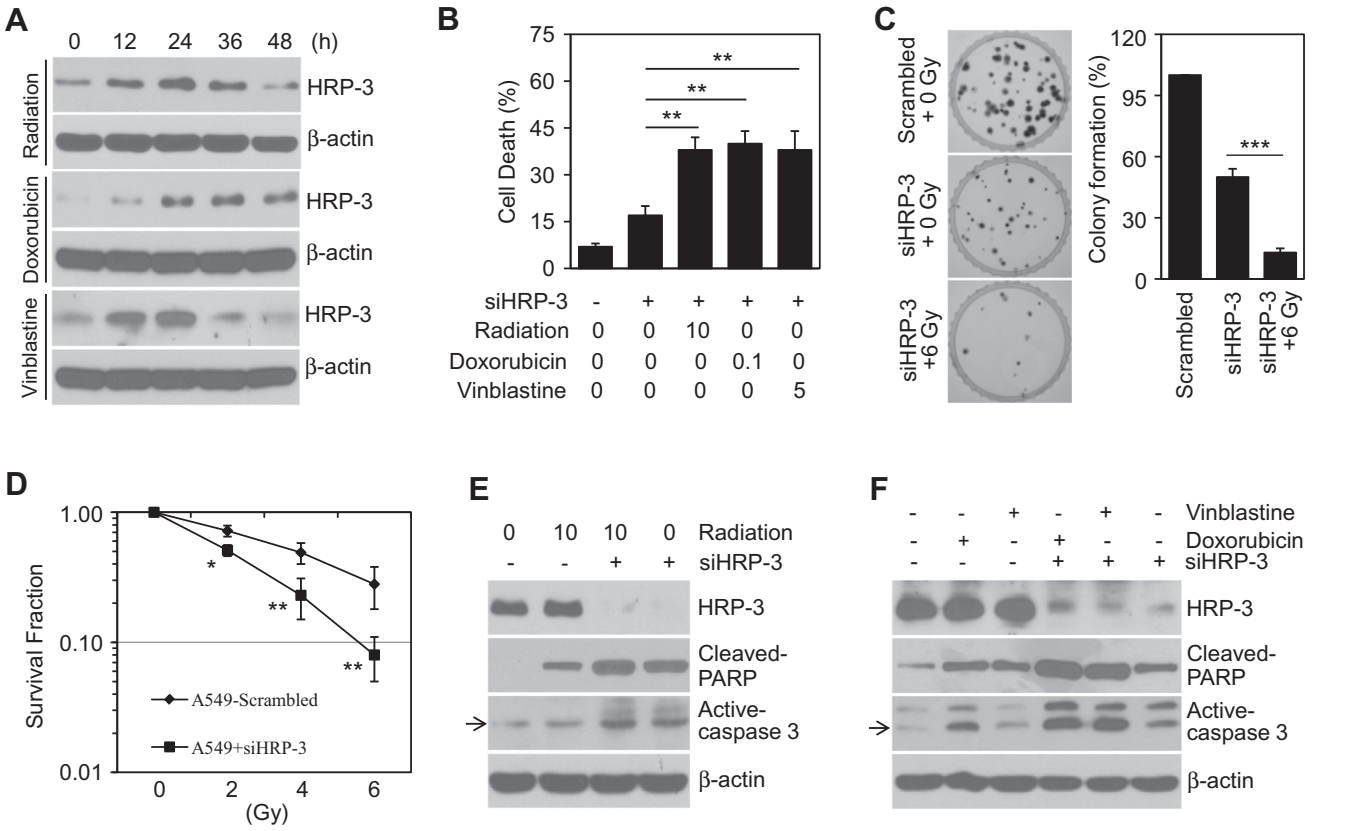

Fig. 2. Synergistic effect of HRP-3 depletion on cell death in A549 cells treated with radiation or anticancer drugs. (A) A549 cells were treated with 10 Gy radiation, 0.1 μg/mL doxorubicin, or 5 ng/mL vinblastine for the indicated times. HRP-3 protein levels were determined by Western blotting. (B) A549 cells were transfected without or with 100 nM HRP-3 siRNA and then treated as in (A) for 48 h. Cell death was determined by FACS analysis; data are expressed as means ± SD (**$p < 0.005$ compared with cells transfected with siHRP-3 alone). (C and D) A549 cells were transfected as in (B) and then treated with single doses of radiation ranging from 0 to 6 Gy for 14 days. Representative image of colony formation, visualized with trypan blue staining (C, left); data are expressed as means ± SD (***$p < 0.0005$ compared with cells transfected with siHRP-3 alone) (C, right). Survival fraction is presented as a survival curve; data are expressed as means ± SD (*$p < 0.05$ and **$p < 0.005$ compared with untransfected control) (D). (E and F) A549 cells were transfected as in (B) and then treated with 10 Gy radiation (E) or anticancer drugs (0.1 μg/mL doxorubicin, 5 ng/mL vinblastine) (F) for 48 h. Expression of cell death markers was determined by Western blotting.

3.3. HRP-3 regulates ROS generation via the Nrf2/HO-1 pathway in A549 cells

To identify the mechanisms by which HRP-3 depletion induced cytotoxicity in A549 cells, we compared the levels of proteins involved in ROS production between H460 and A549 cells. Interestingly, highly HRP-3-expressing A549 cells maintained higher levels of antioxidant coordinators, notably the transcription factor Nrf2 and its target HO-1, compared to those of H460 cells; however, protein levels of Keap-1, a repressor of Nrf2 activation, were similar in the two cell lines (Fig. 3A). To determine whether HRP-3 directly regulated Nrf2 and HO-1 expressions, we depleted endogenous HRP-3 with siRNA and examined the levels of Nrf2 and HO-1. Depletion of HRP-3 markedly reduced Nrf2 and HO-1 protein levels without altering Keap-1 expression (Fig. 3B), indicating that Nrf2 and HO-1 are downstream targets of HRP-3. Because Nrf2/HO-1 signaling is a key antioxidant pathway that acts as a primary cellular defense system in response to oxidative stress [13], we examined the effect of HRP-3 on ROS generation using RNA interference. siRNA-mediated HRP-3 knockdown in A549 cells induced a significant (~1.6-fold) increase in ROS generation compared to control cells, an increase that was reduced to below basal levels by treatment with the ROS scavenger NAC (Fig. 3C). Treatment of A549 cells with radiation alone induced approximately a 1.8-fold increase in ROS production, compared with untreated control; combining radiation with HRP-3 depletion significantly enhanced this effect, increasing ROS production by approximately 3.4-fold compared with controls (Fig. 3D). Thus, our results suggest that HRP-3 normally acts to positively regulate ROS scavenging via Nrf2/HO-1 signaling.

3.4. HRP-3 is essential for regulating ROS-dependent, p53-induced cell death

To further dissect the signaling pathway involved in HRP-3 depletion-induced apoptosis, we focused on the tumor suppressor protein p53, a transcription factor activated by redox imbalance and DNA damage [14]. Transfection of A549 cells with HRP-3 siRNA led to an increase in the expression and activation of p53 and also induced expression of PUMA, a key mediator of p53-dependent cell death, compared to controls (Fig. 4A). To confirm the effect of ROS on p53-mediated cell death, we assessed apoptosis and monitored the levels of the above proteins after treatment with NAC. As shown in Fig. 4B, the induction of cleaved PARP by HRP-3 knockdown was significantly decreased by NAC treatment, suggesting a reduced commitment to apoptosis. Consistent with this notion, p53 and PUMA levels were also dramatically decreased by NAC treatment in siHRP-3-transfected A549 cells (Fig. 4B), suggesting that p53 and PUMA accumulation is downstream of ROS signaling. To determine whether HRP-3 depletion-mediated upregulation of p53 protein is due to a posttranslational increase in p53 stability, we treated control and HRP-3-knockdown A549 cells with cycloheximide for 1, 2, and 3 h and analyzed p53 protein levels. The p53 exhibited a linear elimination over time, as shown by

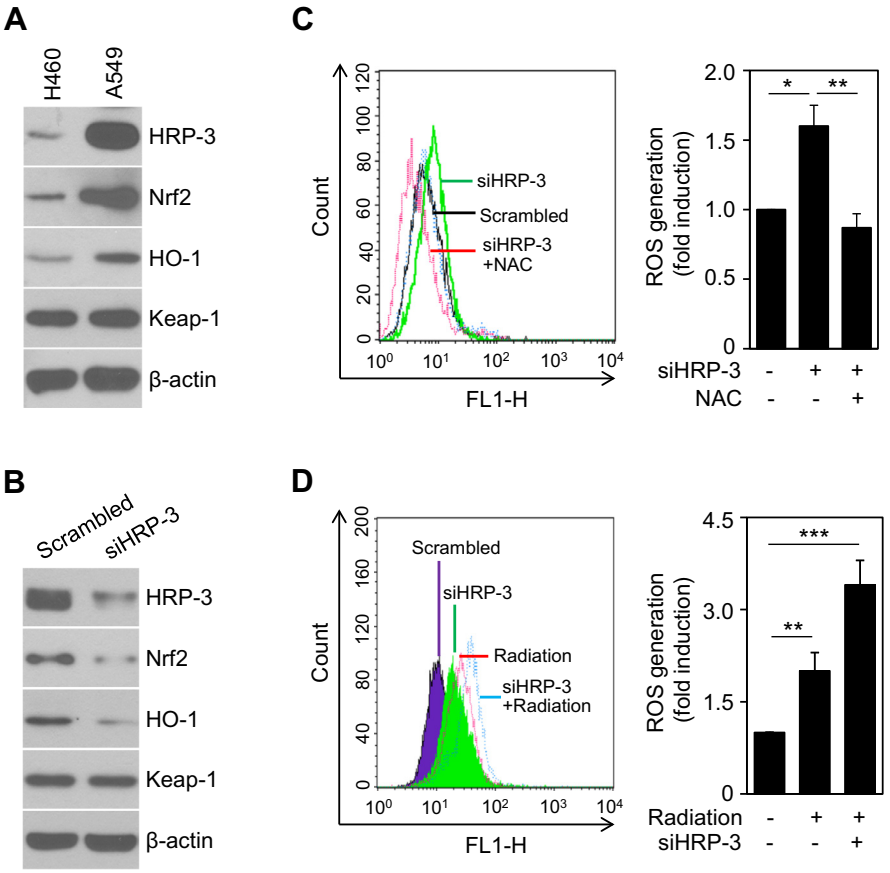

Fig. 3. HRP-3 knockdown induces ROS generation via inhibition of the Nrf2/HO-1 pathway. (A) Levels of ROS-related proteins in H460 and A549 cells were determined by Western blotting. (B) A549 cells were transfected without or with 100 nM HRP-3 siRNA for 48 h. Levels of ROS-related proteins were determined by Western blotting. (C) A549 cells were transfected as in (B) and incubated without or with 1 mM NAC for 48 h. ROS production was assessed by FACS analysis; data are expressed as means ± SD (*$p < 0.05$ compared with control; **$p < 0.005$ compared with cells transfected with siHRP-3 alone). (D) A549 cells were transfected as in (B) without or with exposure to 10 Gy radiation. ROS production was assessed by FACS analysis; data are expressed as means ± SD (**$p < 0.005$ compared with control; ***$p < 0.0005$ compared with cells treated with radiation alone).

Western blotting (Fig. 4C, left) and quantitative band-intensity analysis of Western blots (Fig. 4C, right), and its half-life was short (<1 h) in control cells and was prolonged (>2 h) in HRP-3-knockdown cells. Neither cycloheximide nor HRP-3 knockdown affected the levels of p53 transcript (Fig. 4C, left). The half-life of phosphorylated p53 paralleled that of p53 protein in control A549 cells, but was significantly prolonged (>3 h) in HRP-3-knockdown cells (Fig. 4C), demonstrating that HRP-3 is involved in regulating p53 activation. Finally, to establish the role of p53 in apoptosis induction, we treated control and siHRP-3-transfected A549 cells with radiation. During the process of apoptotic cell death, p53 activation and PUMA were significantly induced in radiation-treated, HRP-3-knockdown A549 cells compared with untreated or radiation-only treated control cells (Fig. 4D), confirming that p53 activated in response to ROS contributes to apoptotic commitment through its downstream target PUMA.

4. Discussion

Lung cancer is the leading cause of cancer deaths worldwide. The 5-year survival rate for patients with stage I NSCLC is roughly 60%, but patients with advanced stage NSCLC show very poor survival rates despite aggressive multimodality treatments [15]. Because NSCLC patients may receive additional benefit from adjuvant radiotherapy or chemotherapy following curative surgical attempts, these adjuvant therapies represent standard practice for the care of cancer patients. However, many patients suffer undesirable and potentially treatment-limiting side effects, despite the benefits of such therapies. Thus, identification and application of novel biomarkers based on an individual patient's molecular profile is an unmet need in the therapeutic management of cancer patients. To this end, we screened for molecules with a significant correlation with the radio- and chemoresistant phenotype of NSCLC A549 cells and identified HRP-3.

Although HDGF, a member of the HRP family, is thought to play a role in the development and progression of numerous human cancers, including colorectal and liver cancers [16–18], little is known concerning the role of HRP-3 in cancer beyond a single study that demonstrated an important role for HRP-3 in HCC pathogenesis [10]. This study reported that HRP-3 is highly upregulated in human HCC tissues compared to normal tissue and showed that knockdown of HRP-3 sensitized HCC cells to anoikis via activation of the extracellular signal-regulated kinase pathway. These observations suggested that HRP-3 could be a target for therapeutic agents designed to enhance sensitivity to chemotherapy in human HCC. In the present study, we identified HRP-3 as a radioresistence- and chemoresistance-related gene in NSCLC A549 cells harboring wild-type p53 and characterized the HRP-3-dependent signaling mechanism that regulates the resistant phenotype of these cells. Specifically, we found that depletion of HRP-3 promoted cell death by inducing a substantial increase in intracellular ROS levels, clearly demonstrating that HRP-3 normally acts through suppression of ROS to play an essential role in regulating

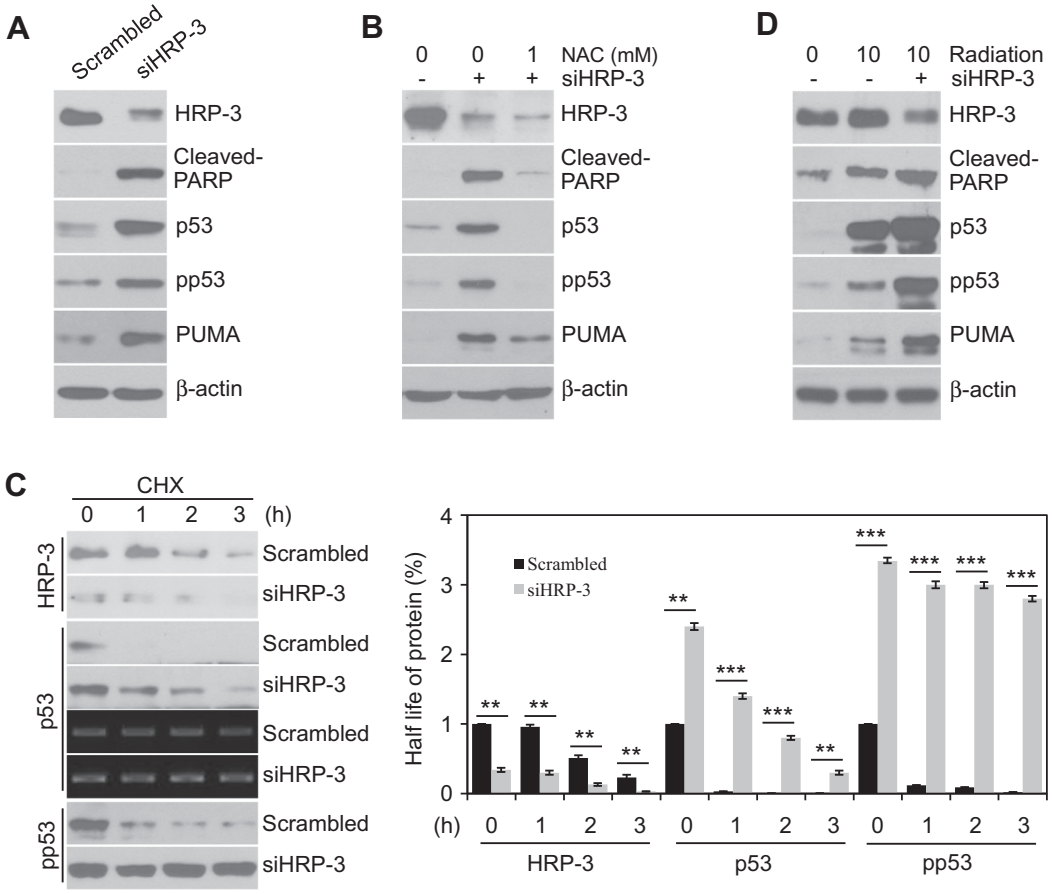

Fig. 4. HRP-3 knockdown promotes ROS-dependent activation of p53. (A and B) A549 cells were transfected without or with 100 nM HRP-3 siRNA for 48 h in the absence (A) or presence (B) of 1 mM NAC. Levels of apoptosis-related proteins were detected by Western blotting. (C) A549 cells were transfected as in (A) and then treated with 10 μM cycloheximide for the indicated times. Protein levels of HRP-3, p53, and pp53 were determined by Western blotting and p53 mRNA was detected by RT-PCR (left). The half-life of proteins is presented graphically; data are expressed as means ± SD ($^{**}p < 0.005$ and $^{***}p < 0.0005$ compared with untransfected control) (right). (D) A549 cells were transfected as in (A) without or with exposure to 10 Gy radiation for 48 h. Levels of apoptosis-related proteins were detected by Western blotting.

the proliferation of A549 cells (Fig. 3). Notably, ROS are mutagenic and may thus promote cancer. Our experiments further showed that the increase in ROS levels in HRP-3-knockdown A549 cells was caused by down-regulation of the Nrf2 transcription factor, which directly affects the homeostasis of ROS by regulating antioxidant defense systems via several mechanisms [19]. On the basis of differential expression patterns and RNAi experiments, we concluded that Keap-1, which participates in the degradation of Nrf2, was not involved in regulating the HRP-3-dependent ROS signaling mechanism in A549 cells. However, HO1, a downstream target gene of Nrf2 encoding the antioxidant enzyme heme oxygenase 1, was markedly decreased in HRP-3-knockdown A549 cells, implicating the Nrf2/HO-1 pathway in HRP-3-depletion-mediated intracellular ROS accumulation and cell death. ROS-mediated DNA damage generated by anticancer agents or radiation has also been previously shown to activate p53 and upregulate its target PUMA to play a critical role in enhancing the cytotoxicity of several cancer cell types [20]. Consistent with these previous results, the accumulation of ROS induced by HRP-3 knockdown prolonged the half-life of p53 protein and sustained the active form of p53 in A549 cells. This maintained active p53 status up-regulated the expression of PUMA, a pro-apoptotic mediator of p53, in A549 cells, suggesting that p53 could act through PUMA signaling to produce a loss of mitochondrial membrane potential and promote apoptosis via the intrinsic pathway. Notably, however, signaling pathways downstream of HRP-3 in p53-null or -mutant types of lung cancer cells remain completely unknown and warrant further investigation.

In conclusion, these studies provide the first demonstration that the HRP-3/Nrf2/HO-1/ROS/p53/PUMA cascade is an essential component of intracellular signaling associated with NSCLC pathogenesis. Importantly, our findings establish that the expression of HRP-3 governs both radioresistance and chemoresistance in NSCLC cells, suggesting that HRP-3 could serve as a prognostic biomarker and molecular target for multimodal therapies against NSCLC.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.086.

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