Therapeutic Discovery

Pharmacologic Inactivation of Kinase Suppressor of Ras1 Sensitizes Epidermal Growth Factor Receptor and Oncogenic Ras-Dependent Tumors to Ionizing Radiation Treatment

Hongyan Xiao¹, Qingbei Zhang¹, Jikun Shen¹, Vytas Bindokas⁴, and H. Rosie Xing¹,²,³

Abstract

Selective enhancement of tumor response to radiation therapy is a highly attractive objective, but it has not been met clinically. Gain-of-function Ras (gf) signaling via hyperactivation of receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR), or via oncogenic mutation of Ras is shown to confer radioresistance and requires the engagement of the Raf/MEK/ERK pathway. However, upstream mediators of such interaction in cancer cells that could be targeted for radiosensitization have not been identified and characterized. Here, we provide original observations both in vitro and in vivo that kinase suppressor of Ras1 (KSR1) is a new target for reversing gf Ras-mediated radioresistance. We employed EGFR-dependent A431 squamous cell carcinoma (SCC) and genetically defined the molecular function of KSR1 in irradiation-induced Raf/MEK/ERK activation. In vitro KSR1 inactivation via genetic inhibition of its expression or kinase function abrogated ionizing radiation-induced activation of the Raf/MEK/ERK cascade, enhanced the cytotoxic effect of radiation, and achieved radiosensitization associated with inhibition of DNA damage repair and enhancement of clonogenic death. In vivo pharmacologic inactivation of KSR1 by KSR1 AS-ODN infusion leads to radiosensitization in EGFR-dependent A431 SCC and in oncogenic K-Ras-driven A549 human non-small cell lung carcinoma. These observations collectively establish KSR1 as a novel target for radiosensitization and show the feasibility of using KSR1 AS-ODN as a radiosensitizer for treating gf Ras-dependent human malignancies. Identification of such mediators of gf Ras signaling in response to irradiation holds promises for improving the therapeutic efficacy of radiation therapy and our ability to eradicate tumor.

Introduction

Selective enhancement of tumor response to radiation therapy is a highly attractive objective, yet it has not been met clinically. The presence of overexpressed or activated oncogenes such as epidermal growth factor receptor (EGFR; ref. 1) or Ras (2, 3) increases cellular resistance to radiation via promoting cancer cell survival and proliferation (4–7). Antagonists of EGFR radiosensitize human cancer cells apparently by suppressing radiation-induced DNA damage repair (8–12). In cancer cells, the Raf/MEK/ERK module is coupled to gain-of function (gf) Ras signaling in response to radiation (5, 7). There is emerging molecular evidence linking this signaling cascade with checkpoint pathway (13–15), mismatch repair process (16), and DNA damage repair (5, 17). However, upstream mediators that mediate the interaction between EGFR/Ras and Raf/MEK/ERK remain poorly characterized.

Recent studies have identified and characterized evolutionarily conserved kinase suppressor of Ras (KSR1) as a specific mediator of gf Ras signaling. Since its discovery in 1995 (18–20), significant effort has been devoted to elucidate the precise biochemical mechanisms of this molecule in regulation of Raf/MEK/ERK activation. To date, both the kinase property and scaffold function of KSR1 have been equally shown. In the scaffold model, KSR1 coordinates optimal signaling through the Raf/MEK/ERK module without directly increasing c-Raf-1 activation (21–23). In contrast, investigations supporting KSR1 as a functional kinase show that KSR1, immunopurified to homogeneity, phosphorylates and activates c-Raf-1 (24–30). Although genetic studies from ksr1-deficient Caenorhabditis elegans and mice show that ksr1 is dispensable for normal development (20, 31, 32), we show the obligate function of KSR1 in mediating gf Ras-signaling of oncogenesis and the effectiveness of...
KSR1 antisense phosphorothioate oligonucleotide (AS-ODN) as a tumor-specific agent for the treatment of gf Ras-dependent human malignancies (31, 33).

Here, we provide original observations supporting KSR1 as a novel target for reversing gf Ras-mediated radioresistance. We molecularly order KSR1 as an obligatory mediator that couples the Raf/MEK/ERK module to EGFR-mediated radioprotective response in A431 cancer cells. In vitro, KSR1 inactivation via genetic inhibition of its expression or kinase function abrogates ionizing radiation–induced activation of the Raf/MEK/ERK2 cascade and enhances radiosensitivity by suppressing the efficacy of DNA damage repair leading to more robust clonogenic/reproductive death. In vivo pharmacologic inactivation of KSR1 function via infusion of KSR1 AS-ODN prior to irradiation treatment leads to radiosensitization in EGFR-dependent A431 squamous cell carcinoma (SCC) and in oncogenic K-Ras-driven A549 human non–small cell lung carcinoma (NSCLC) in nude mice. These observations show the feasibility of using KSR1 AS-ODN as a radiosensitizer for treating gf Ras-dependent human malignancies. KSR1 AS-ODN is currently under development for the treatment of human pancreatic cancer.

Materials and Methods

Culture of A549, A431, and A431-Rev-Tet-Off-KSR1 cell lines

The human cervical squamous carcinoma A431 cells and NSCLC A549 cells used for this study were obtained from the American Type Culture Collection, and no authentication was done. They were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37°C in 5% CO2. A431-Tet-Off-KSR1 cell lines stably expressing inducible serum, penicillin, and streptomycin at 37°C in 5% CO2. A431-Tet-Off-KSR1 cell lines stably expressing inducible serum, penicillin, and streptomycin at 37°C in 5% CO2. A431-Tet-Off-KSR1 cell lines stably expressing inducible serum, penicillin, and streptomycin at 37°C in 5% CO2. A431-Tet-Off-KSR1 cell lines stably expressing inducible serum, penicillin, and streptomycin at 37°C in 5% CO2.

A431-Tet-Off-KSR1 cell lines stably expressing inducible AS-ODN, and ionizing radiation in vitro

The EGFR1 inhibitor AG1478 was purchased from Calbiochem. Oligofectamine was from Invitrogen. Human KSR AS-ODN (5′-CTTTCCTTACTAGTGTCG-3′) was generated as phosphorothioate against nucleotides 214 to 231 of the unique CA1 domain (amino acids 42-82) of KSR1 by Genelink Inc. as previously described (33). Control ODN (5′-ACGTCACGCACGCACACTAT-3′) was prepared similarly (33).

For drug treatment, AG1478 was freshly diluted from 10 mmol/L stock solutions to the indicated concentrations. For irradiation experiments, parental A431 and vector-infected A431-pTRE cells were plated at 3.2 × 10^5 cells/cm^2 of plate area. A431-KSR-S or KSR-AS and DN-KSR cells were plated at 2.5 × 10^4 or 1.5 × 10^4 cells/cm^2 of plate area, respectively. On day 4, 80% to 90% confluent culture was replaced with serum-free medium supplemented with 0.2% human albumin and incubated for an additional 24 hours. Before irradiation, cells were treated with indicated doses of AG1478 for 1 hour. Thereafter cultures were exposed to graded doses of ionizing radiation using a ^6^Co source. Cells were harvested at indicated time points, frozen on dry ice, and stored at -80°C until further processing for biochemical analysis. For in vitro treatment with KSR1 AS-ODN, KSR1 AS-ODN was dissolved in sterile water and delivered to cells by oligofectamine when cells were 30% to 40% confluent as we had previously described (33). Forty-eight hours after treatment, cells were irradiated as above.

Clonogenic survival assay

Cells treated with either EGFR inhibitor, or KSR1 AS-ODN, or control ODN and irradiated as above were incubated for 24 hours. Cells were then harvested and plated for assessment of clonogenic survival. Three dishes were set up for each experiment point. For cells treated with AG1478, it was also added to the culture medium throughout the course of the clonogenic assay. Cells were maintained in culture for 14 days, stained with crystal violet, and colonies containing more than 50 cells were counted to determine surviving fractions (34). Dose-modifying factor (DMF) was calculated at 1 log kill (SF = 0.1).

In vitro two-stage KSR1 activity assay and Raf activity assay

Flag-tagged KSR1 proteins or endogenous c-Raf-1 were immunoprecipitated and purified to homogeneity from freshly harvested cell lysates as previously described (35). KSR1 kinase activity was measured by an in vitro two-stage assay in which the entire Raf/MEK/ERK/Elk-1 cascade was reconstituted (30). Elk-1 phosphorylation was used as the assay readout. For studies examining the activity of Raf-1, phosphorylation of the kinase-inactive MAP/ERK kinase 1 (MEK1; K97M-MKK1) was used as the assay readout. Elk-1 and MEK1 phosphorylation was visualized by Western blotting analysis using the rabbit anti-phospho-Elk-1 (Ser^385^) antibody or the anti-phospho-MEK1 (Ser^217^/221^) antibody (BioLabs, respectively).

Western blot analysis

Total cell lysates were prepared in NP-40 buffer as described (30, 33). Western blot was done according to the manufacturer’s protocols with the following antibodies: monoclonal anti-Flag M2 and anti-ß-tubulin antibodies from Sigma; polyclonal anti-p44/42 mitogen-activated protein kinase (MAPK), monoclonal anti-phospho-p44/42 MAPK (Thr^202^/Tyr^204^), polyclonal anti-phospho-MEK1/2 (Ser^217^/Ser^221^), and polyclonal anti-phospho-Akt (Ser^473^) antibodies from Cell Signaling; polyclonal anti-c-Raf-1 antibody from Upstate Biotechnology Inc.; and monoclonal anti-KSR antibody from BD Biosciences.
Histopathology and immunohistochemistry

Freshly dissected xenograft tumors were formalin fixed, paraffin embedded, and serially cut into 5-μm sections. One slide of each sample was stained with H&E for morphologic observation. Another slide of each tumor was immunostained for the expression of cell proliferation marker proliferating cell nuclear antigen (PCNA) or Ki67. Briefly, antigen retrieved with heated citric buffer was placed on deparaffinized slides followed by blocking with 5% goat serum. Thereafter, sections were incubated with mouse anti-PCNA (1:50; FC10, Santa Cruz) or with rabbit anti-human Ki67 (1:50; DAKO), followed by incubation with a biotinylated goat anti-mouse IgG or a goat anti-rabbit IgG (1:600; Vector) and horseradish peroxidase–streptavidin (1:200; Invitrogen). Immunostaining was developed with diaminobenzidine, counterstained with Meyer’s hematoxylin.

Determination of EGFR and ERK1/2 phosphorylation by enzyme-linked immunosorbent assay

Cell lysates were prepared as described above. EGFR, extracellular signal-regulated kinase 1 (ERK1), and ERK2 phosphorylation was then measured by using an enzyme-linked immunosorbent assay (ELISA) method (R&D Systems) according to the manufacturer’s instructions.

Confocal microscopic visualization and quantification of γH2AX-foci

γH2AX focus assay was applied for determination of residual DNA double-strand breaks (DNA-DSB). Cells grown on glass slides were irradiated with 2 or 5 Gy and allowed to recover for 24 hours to allow repair of irradiation-induced DNA-DSB (32). Cells were then fixed with 2% formaldehyde, permeabilized with 0.2% Triton X-100 in PBS/1% bovine serum albumin (BSA), and blocked with PBS/3% BSA. Thereafter, cells were incubated with anti-phospho-H2AX monoclonal antibody (1:100; JBW301, Upstate) and then with an Alexa Fluor 488–conjugated anti-mouse IgG secondary antibody (1:400; Molecular Probes). To conduct quantitative comparison of γH2AX foci formation, all confocal images for different cell lines and at different time points were acquired with identical illumination and camera settings.

The number of foci per nucleus was counted using images captured with a confocal fluorescence microscope and 50 cells were counted (Axioplan 2, Zeiss). Confocal image stacks were captured to record foci in all optical slices of cell nuclei. Foci content per cell nucleus was quantified by a custom macro written in ImageJ (Rasband, W.S., ImageJ, NIH, Bethesda, Maryland, http://rsb.info.nih.gov/ij/, 1997-2010). The macro used a spot filter based on difference of means, similar to the difference of Gaussians method. Mean radii were optimized for the data, extracting spots and leveling the background, and those values were kept constant for all analyses. The macro then allowed regions of interest to be placed around individual cell nuclei in three-dimensional image stacks, those cells were sequentially cropped out, and the numbers of spots were counted in each cell using the three-dimensional objects counter plugin (by F. Cordelier and J. Jackson; http://rsb.info.nih.gov/ij/plugins/track/objects.html). Finally, foci centroids were overlaid onto the original image to verify fidelity of counts.

Fluorescent-activated cell sorting (FACS) analysis of cell cycle and γH2AX-foci kinetics

For cell cycle distribution studies, cell pellets collected from exponentially growing monolayers were fixed with 100% ethanol, treated with RNase A (0.1 μg/mL), and stained with propidium iodide (0.05 mg/mL). The proportion of cells in the different phases of the cell cycle was calculated from the experimental fluorescence histograms. For determining γH2AX foci formation and disappearance kinetics, after irradiation treatment at indicated time points, cells were fixed, blocked with rabbit serum, and stained with the anti-phospho-H2AX monoclonal antibody (1:300) as above. Mean values of γH2AX (MV) were calculated according the formula MV = Mx - Mc, where Mx was recorded as the mean γH2AX fluorescence at indicated time points after treatment and Mc was recorded as the mean γH2AX fluorescence in the untreated control.

Measurement of DNA damage by the comet assay

Irradiation-induced DNA-DSB in A431-KSR cell lines was measured by the neutral comet assay using the Trevigen’s CometAssay Kit (R&D). Cells were irradiated with 5 Gy ionizing radiation and harvested at 0, 1, and 24 hours post-irradiation. Slides were viewed using 40× objective of a Zeiss Axioskop fluorescent microscope and images were taken and imported into ImageJ for further analysis. For each sample, the tail lengths (in micrometers) of 50 comets (cells) were analyzed. The tail length of the comet was quantified as the distance from the center of the cell nucleus to the tip of the tail in pixel units, and the tail length was expressed as a mean ± SE from 50 comets.

Tumor induction and treatment with KSR AS-ODN

For tumor induction, 5 × 10⁶ cultured tumor cells were transplanted s.c. into the right lateral flank of 6- to 8-week-old female athymic mice (Germantown, NY). Tumor growth was measured by calipers, and tumor volume was calculated as described (36). To determine the radiosensitizing effect of KSR-AS ODN in vivo, infusion with 5 mg/kg/day AS-ODN prepared using the 7-day Alzet osmotic minipumps was initiated 4 days prior to irradiation. The following definitions were used in analyzing the treatment effects: tumor growth delay was defined as the median time in days for irradiated tumors to reach a size of 500 mm³ minus the time for untreated control tumor to reach the same size; normalized growth delay...
was defined as the time in days for tumors in mice treated with the combination of KSR1 AS-ODN and radiation to reach 500 mm$^3$ minus the time in days for tumors in mice treated with radiation alone to reach the same size; enhancement ratio was defined as normalized tumor growth delay in mice treated with the combination divided by the tumor growth delay in mice treated with radiation alone.

**Statistical analysis**

All data were evaluated by Student’s $t$ test (two-tailed) with $P < 0.05$ considered significant.

**Results**

**Ionizing radiation induces coordinated activation of EGFR, KSR1, and the RAF-MEK-ERK pathway**

Because we and others had previously shown that KSR1 specifically mediates of Ras signaling through the Raf/MEK/ERK module via its kinase or scaffold functions (18, 19, 23, 26, 28, 30, 33, 37, 38), we first established irradiation dose-dependence of EGFR/Raf/MEK/ERK transduction.

We and others had previously shown that KSR1 activates c-Raf-1 by trans-phosphorylation c-Raf-1 in response to physiologic dose of EGF stimulation (1 ng/mL; refs. 26, 30, 35). Similarly, IR exposure dose-dependently increased the overall level of threonine phosphorylation of c-Raf-1 that paralleled the detected IR-stimulated c-Raf-1 activity (Supplementary Fig. S1B). To determine whether IR-induced c-Raf-1 activation requires KSR1 activity, we immmunopurified equal amounts of endogenous c-Raf-1 (Fig. 2B, bottom) either from KSR-S or from KSR-AS expressing A431 cells in which endogenous KSR1 expression was inhibited over 80% (33), and determined c-Raf-1 activity upon 15 Gy IR exposure. Substantial IR-stimulated c-Raf-1 activity was detected at an earlier time point (Fig. 2B, lane 7 versus lane 3) and peaked to a similar level (Fig. 2B, lane 8 versus lane 4) in A431 KSR-S cells compared with pTRE control cells. In contrast, inhibition of KSR1 expression by KSR-AS almost completely abolished c-Raf-1 activity (Fig. 2B, lane 12 versus lanes 4 and 8). Similar inhibitory effects on c-Raf-1 activation were also found in A431 cells expressing the kinase inactive DN-KSR (not shown). Further, KSR1 expression status didn’t change either the EGFR expression or its phosphorylation induced by IR (data not shown), indicating that IR-stimulated KSR1 signaling was downstream of EGFR.

**Genetic and pharmacologic inactivation of KSR1 radiosensitizes A431 cells in vitro by enhancing reproductive cell death**

The lethal effect of ionizing radiation is achieved through induction of DNA DSB damages that lead to either apoptosis or reproductive death (clonogenic death). Emerging genetic evidence shows the interaction of Ras/Raf/ERK signaling with the checkpoint response pathway to enhance reproductive survival postirradiation (15). To examine the requirement of KSR1 in mediating reproductive survival postirradiation, clonogenic survival curves were constructed after exposure to graded doses of radiation (1–12 Gy) using genetically engineered A431 cells expressing different forms of KSR1. As shown in Fig. 3A, inactivation of
KSR1 upon expression of KSR-AS or DN-KSR decreased the efficacy of sublethal damage repair as measured by the elimination of low-dose shoulder $D_0$ on the radiation dose survival curve. Further, inactivation of KSR1 resulted in significantly enhanced intrinsic radiosensitivity, as measured by a reduction in $D_0$ (Fig. 3A, $P < 0.05$). The dose-modifying ratios at one Log kill ($D_{0,1}$) were $2.44 \pm 0.28$ Gy and $2.34 \pm 0.23$ Gy for KSR-AS and DN-KSR cells, respectively, indicating significant radiosensitization (Table 1). Moreover, no significant differences in radiation-induced apoptosis (data not shown) or cell cycle distribution (Supplementary Fig. S2) were observed in A431-KSR1 cell lines. Thus, the ability of KSR1 to regulate radiosensitivity in A431 cells is likely achieved via regulating the efficiency of repairing DNA-DSB.

Subsequently, we characterized the radiosensitizing potential of KSR1 AS-ODN. Treatment of A431 cells with a control-ODN (CTL-ODN) had no apparent effect on the clonogenic survival and radiosensitivity (not shown). In contrast, treatment with 200 nmol/L KSR1 AS-ODN effectively eliminated $D_0$ and reduced $D_0$ (Fig. 3B). The dose modifying ratio was $1.8 \pm 0.11$ Gy indicating significant radiosensitization (Table 2). It is worth noting that to discriminate the antitumor and radiosensitizing properties of KSR1 AS-ODN, we chose doses for KSR1 AS-ODN treatment that were effective in inhibiting endogenous KSR1 expression ($78.2 \pm 1.4\%$, not shown, Materials and Methods) while having minimal antiproliferative effect (<20%). Further, irradiation had no significant effect on AS-ODN treatment-induced KSR1 downregulation (no irradiation, $78.2 \pm 1.4\%$; 24 hours after irradiation, $75.8 \pm 6.6\%$).
These results indicate that it is feasible to target KSR1 using a nontoxic dose of KSR1 AS-ODN to enhance radiosensitivity in vitro.

**KSR1 signaling is epistatic to EGFR in response to IR**

A431 cells overexpress EGFR (39–41), and inhibition of EGFR function in this model leads to radiosensitization in vitro and in vivo (10, 12). To molecularly order EGFR and KSR1 signaling in mediating clonogenic survival response to IR, we inactivated EGFR signaling using a pharmacologic kinase inhibitor of EGFR AG1478. These experiments were done with a concentration of AG1478 at 50 nmol/L, a dose that had no significant growth inhibitory effect (Materials and Methods). Although inhibition of EGFR function significantly enhanced A431-pTRE cells to IR-induced reproductive cell death, overexpression of functional KSR1 (KSR-S) efficiently overrode EGFR inhibition (Fig. 3C). However, radioresistance in A431-KSR-S cells could be overcome upon increasing the concentrations of AG1478 to ≥200 nmol/L (Supplementary Fig. S3). Further, the observed radiosensitizing effect of AG1478 was independent of its effect on apoptosis (Supplementary Fig. S4 and not shown). Therefore, KSR1 acted downstream of EGFR in response to IR. To confirm the specificity of AG1478 in eliciting EGFR-specific radiosensitizing effect that requires KSR1 signaling, we treated the oncogenic K-Ras–dependent human A549 NSCLC cell line with AG1478 at the nongrowth inhibitory dose of 200 nmol/L (Materials and Methods). As shown in Fig. 3Di, whereas EGFR activation was effectively inhibited upon AG1478 treatment (Fig. 3Dii), radiosensitization was not detected (Fig. 3Di). These results collectively show that reproductive survival in A431 cells after IR is mediated by EGFR that requires KSR1 signaling.

**KSR1 regulates the efficiency of irradiation-induced double-strand DNA damage repair**

Emerging evidence has shown a direct link between EGFR signaling and DNA damage repair response (8–12). We next examined the properties of γH2AX foci, a marker for nonrepaired DNA-DSB (42, 43), to assess the involvement of DNA repair mechanism in KSR1 inhibition-elicited radiosensitization. KSR1 status had no apparent effect on the kinetics of γH2AX foci formation that peaked at 30 minutes to 1 hour after IR treatment (Fig. 4A). However, disruption of KSR1 function led to an elevation of baseline γH2AX phosphorylation in A431-KSR-AS and A431-DN-KSR cells measured by FACS (Figs. 4A, visualized and quantified by confocal microscopy (Fig. 4B and C, Supplementary Fig. S5; TRE, 0.75 ± 0.12; KSR-AS, 4.1 ± 1.1; DN-KSR, 3.2 ± 0.8; P < 0.05). In addition, there was a reduction in the magnitude of IR-induced γH2AX phosphorylation upon KSR1 inactivation (Fig. 4A and D, Supplementary Fig. S5).

In pTRE and KSR-S cells, majority of DNA-DSBs were efficiently repaired at 12 hours post-IR (Fig. 4A), and the number of γH2AX foci per nucleus returned to near baseline levels at 24 hours post-IR (Fig. 4B and C). In contrast, inhibition of KSR1 function led to a decreased

![Figure 2](https://example.com/f2.png)
rate of DNA-DSB repair measured by the half-life \( (t_{1/2}) \) of \( \gamma H2AX \) foci processing (Fig. 4A) and a prolonged \( \gamma H2AX \) foci presence (Fig. 4C; KSR-AS, 4.1 ± 1.1 at baseline versus 8.4 ± 0.3 at 24 hours; DN-KSR, 3.2 ± 0.8 at baseline versus 6.6 ± 1.2 at 24 hours; \( P < 0.05 \)). Further, the observed increase in the \( t_{1/2} \) of \( \gamma H2AX \) was associated with a reduction in \( D0 \), thus with radiosensitization (Fig. 3). These observations were consistent with few recent reports on the relationship between the kinetic properties of \( \gamma H2AX \) and radiosensitivity (44–46). To provide additional confirmation of KSR1 involvement in DNA-DSB repair, we conducted comet assay in which DNA-DSB were estimated as the mean tail length of the comet of 50 cells analyzed (Materials and Methods). Although radiation induced a comparable amount of DNA-DSB in all cell lines (Fig. 4D, 1 h), the baseline levels (Fig. 4D, 0 h) as well as remaining unrepaired DNA-DSB (Fig. 4D, 24 h) were significantly different.

**Figure 3.** Genetic and pharmacologic inactivation of KSR1 radiosensitizes A431 cells in vitro to the lethal effect of ionizing radiation. A and B, clonogenic survival assays were conducted in A431-pTRE-KSR-Tet-Off cell lines (A) or in A431 cells that received KSR1 AS-ODN treatment (B). Radiation dose curves were constructed as in Materials and Methods. Dq, shoulder region; \( D0 \), the slope of the survival curve and a quantitative measure of intrinsic radiosensitivity; \( D0.1 \), radiation dose yields \( 1 \) log scale kill.

C, radiation clonogenic response in A431-pTRE and A431-KSR-S cells treated with 50 nmol/L of AG1478. D, radiation clonogenic response in A549 NSCLC cells treated with 200 nmol/L of AG1478 (i) and determination of EGFR expression and IR-induced EGFR activation by ELISA (ii). Error bars, mean ± SE. Six to nine replicates were included for each experimental point to construct clonogenic survival curves.
higher in A431-KSR-AS and DN-KSR cell lines. These observations collectively, for the first time, show a strong association between KSR1-mediated radioresistance and the efficiency of DNA damage repair.

KSR1 AS-ODN treatment radiosensitizes gp Ras-dependent tumors

To elucidate whether KSR1 inactivation might have a similar radiosensitizing effect in vivo, A431 xenografts were established s.c. (Materials and Methods). KSR1 AS-ODN infusion (5 mg/kg/day), given for four days prior to IR, was sufficient to inhibit tumor KSR1 expression while having no significant effect on its own to inhibit tumor growth (76% inhibition on average compared with control-ODN treated; not shown). The 12 Gy IR dose was chosen as it resulted in measurable tumor growth delay without causing permanent eradication of tumors (Fig. 5A and not shown). We observed 10-day and 22-day tumor growth delays (to reach 500 mm3), respectively, for radiation alone and for the combination treatment (Fig. 5A). The normalized growth delay of the combination treatment was 12 days, and thus the in vivo dose enhancement ratio measured by tumor growth delay was 1.83 where a ratio >1.0 suggests synergistic interactions (Materials and Methods). Furthermore, comparing tumor response using a clinically stratification criteria, we observed continued tumor growth in all animals without receiving any treatment intervention. In comparison, whereas 52% of the IR-treated continued to grow (Fig. 5A, Table 3), KSR1 AS-ODN treatment significantly improved the radiation treatment response by reducing the incidence of tumor progression rate to 32% and by increasing tumor regression response to 37%.

We next examined histologic changes in A431 tumors harvested on day 32 post-IR. As shown in Fig. 5B, sham-operated control tumors contained abundant mitotic active tumor cells (arrows) and exhibited little squamous terminal differentiation (Fig. 5Bi, *). A single dose of 12 Gy ionizing treatment significantly increased squamous terminal differentiation as evident by increased horn pearls formation and reduction of mitotic figures in comparable viable tumor areas (Fig. 5Bii). KSR1 AS-ODN treatment prior to IR further accelerated squamous terminal differentiation, presented as a reduction in the layers of spinous cell, substitution of super basal-like keratinocytes with superficial-like keratinocytes, and a further inhibition of proliferating activity (Fig. 5Biii).

To confirm the therapeutic effectiveness of KSR1 AS-ODN in enhancing the efficacy of IR in other gp Ras-dependent human tumors, we examined the radiosensitizing effect of KSR1 AS-ODN using the K-Ras-driven A549 model in which we previously showed the effectiveness of KSR1 AS-ODN in inhibiting tumor growth and spontaneous lung metastases formation (33). A noncurative 10 Gy radiation dose was chosen (not shown). Similar to what we had observed in A431 tumors (Fig. 5A and B), KSR1 AS-ODN treatment significantly sensitized A549 tumors to IR by inhibiting tumor progression (Fig. 5C and Table 4, reduced to 26% in the combination treatment), and by increasing tumor regression (increased to 26% in the combination treatment) and stable disease responses (increased to 48% in the combination treatment).

We also examined histologic changes in A459 tumors harvested on day 54 post-IR. We compared the immunohistochemistry staining of PCNA and Ki-67, markers for cell proliferation in comparable representative peripheral

### Table 1. Radiosensitizing effect on pTRE-KSR-Tet-Off cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>SF2 (%)</th>
<th>PE (%)</th>
<th>D_0 (Gy)</th>
<th>Dq (Gy)</th>
<th>D_0.1 (Gy)</th>
<th>Dose ratios (D = 0.1)</th>
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<tr>
<td>pTRE</td>
<td>39.9</td>
<td>11.0</td>
<td>1.77 ± 0.14</td>
<td>2.26 ± 0.44</td>
<td>6.27 ± 0.50</td>
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<tr>
<td>KSR-S</td>
<td>96.3</td>
<td>13.2</td>
<td>1.90 ± 0.32</td>
<td>3.64 ± 0.24</td>
<td>7.93 ± 0.45</td>
<td>0.79 ± 0.13</td>
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<tr>
<td>KSR-AS</td>
<td>23.6</td>
<td>16.8</td>
<td>1.21 ± 0.18</td>
<td>0.22 ± 0.38</td>
<td>2.56 ± 0.49</td>
<td>2.44 ± 0.28</td>
</tr>
<tr>
<td>DN-KSR</td>
<td>29.9</td>
<td>16.8</td>
<td>1.54 ± 0.16</td>
<td>0.46 ± 0.66</td>
<td>2.67 ± 0.49</td>
<td>2.34 ± 0.23</td>
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Abbreviations: SF2, survival fraction at 2Gy IR; PE, plating efficiency; Dq, shoulder region; D_0, the slope of the survival curve and a quantitative measure of intrinsic radiosensitivity; D_0.1, radiation dose yields 1 log scale kill.

### Table 2. Radiosensitizing effect on A431 cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>SF2 (%)</th>
<th>D_0 (Gy)</th>
<th>Dq (Gy)</th>
<th>D_0.1 (Gy)</th>
<th>Dose ratios (Surv. = 0.1)</th>
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<tbody>
<tr>
<td>AS-ODN</td>
<td>25</td>
<td>1.23 ± 0.24</td>
<td>0.39 ± 0.11</td>
<td>3.20 ± 0.62</td>
<td></td>
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<tr>
<td>CTL-ODN</td>
<td>31</td>
<td>1.08 ± 0.05</td>
<td>3.33 ± 0.16</td>
<td>5.76 ± 0.27</td>
<td>1.80 ± 0.11</td>
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</table>
Figure 4. KSR1 regulates the efficiency of radiation-induced double-strand DNA damage repair. A431-pTRE-KSR-Tet-Off cell lines were irradiated with 5 Gy of IR and cells were collected at the indicated time points for the quantification of γH2AX phosphorylation by FACS (A) or for the visualization of γH2AX foci formation by confocal microscopy (B). A, FACS analysis. T_{1/2}, the half-life of γH2AX foci disappearance; D0, the measure of intrinsic radiosensitivity, determined as in Fig. 3 Bi. B, confocal microscopic visualization of γH2AX foci. Magnification, ×40. C, quantification of number of γH2AX repair foci per nucleus. Fifty nuclei were scored for each experimental point (Materials and Methods). Error bars, mean ± SE; * significantly different from TRE cell line (P < 0.05). D, measurement of irradiation-induced DNA damage by the neutral comet assay (Materials and Methods). The tail length of the comet was measured in 50 cells for each experimental point (Materials and Methods). Error bars, mean ± SE; *, significantly different from TRE cell line (P < 0.05).
viable areas of tumors. As shown in Fig. 5D, majority of tumor cells in sham-treated control tumors exhibited varied degree of brown nuclear Ki-67 staining (Fig. 5Di). Although both the number of Ki-67–positive cells and the intensity of Ki-67 immunoreactivity were significantly reduced in irradiated tumors (Fig. 5Dii), the proliferative activity was further decreased upon KSR1 AS-ODN infusion prior to IR (Fig. 5Diii). Synergistic antiproliferative effect of AS-ODN and IR combination treatment was confirmed by PCNA immunostaining (not shown).

These findings collectively show that KSR1 mediates gf Ras signaling of radioresistance due to hyperactivation of the growth factor receptor signaling (Fig. 5A and B, A431 model) or due to oncogenic activation of Ras (Fig. 5C and D, A549 model). We showed that KSR1 AS-ODN treatment led to in vivo radiosensitization and significantly improved the ability of radiation to achieve tumor control, the goal of cancer treatment. Therefore, KSR AS-ODN represents a novel and effective radiosensitizer.

Discussion

Gain-of-function Ras signaling confers radioresistance (1–3). Although experimental evidence shows the engagement of the Raf/MEK/ERK pathway to gf Ras-mediated radiation response, upstream regulators that specifically modulate such interactions in cancer cells and potentially could be targeted for radiosensitization have not been identified. Here, we provide original observations both in vitro and in vivo that KSR1 is such a novel target for reversing gf Ras-mediated radioresistance.

Here, we molecularly ordered and genetically defined KSR1 signaling as an integral component of the EGFR/Ras-mediated radio-response (Figs. 1B and 2A) that couples the Raf/MEK/ERK module to EGFR/Ras signaling (Fig. 2). Additionally, IR-stimulated KSR1 activation required the kinase function of KSR1 (Fig. 2A), similar to what has been observed in KSR1 activity stimulated by EGF (26, 30, 35) or tumor necrosis factor (28, 29). Inactivation of KSR1 via either expression of
the kinase-inactive KSR1 or inhibition of its endogenous gene expression abrogated IR-induced Raf-1 activity (Fig. 2B) and the subsequent activation of MEK1/2 and ERK2 (Fig. 2A). We also observed for the first time that ERK2, not ERK1, was coupled to the KSR1/Raf/MEK module upon radiation exposure (Fig. 1A). Thus, ERK2 may represent a radiosensitization target for the MAPK pathway and merits further investigation.

A number of mechanisms have been proposed for the reported radiosensitizing effect of inhibitors of EGFR and oncogenic Ras. Only until recently experimental evidence has begun to illustrate a direct interaction of the Raf/MEK/ERK signaling module (5, 13, 14, 16, 47, 48), as well as EGFR signaling with DNA damage repair response (8–12). Although nuclear-localized EGFR direct interacts with DNA damage repair enzyme DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Raf-ERK signaling may regulate G2-M checkpoint and DNA damage repair through induction of critical genes participating in these processes, including GADD45 (14), XRCC1, and ERCC1 (5). Additionally, c-Raf-1 can associate with both 14-3-3 and Cdc25, connecting mitogenic signaling to cell-cycle checkpoint regulation (13). Moreover, BRAF mutation is linked to the proficiency of colorectal tumors in repairing mismatched bases in DNA (16, 47). Furthermore, the regulatory feedback interaction between ATM and ERK signaling is critical for efficient homologous recombiant repair of radiation-induced DNA-DSB and ATM activation (17). In this report, we show KSR1 to be a novel tumor-specific target for radiosensitization in vivo and showed the effectiveness of using subtherapeutic doses of KSR1 AS-ODN as a nontoxic radiosensitizer for enhancing the therapeutic efficacy of radiotherapy in treating of Ras-dependent human xenograft tumors. The potent in vivo radiosensitization effects were attained through inhibition of tumor growth progression and enhancing the effectiveness of IR to achieve tumor stabilization and more critically, tumor control through regression (Fig. 5). Future studies will be conducted to derive the most effective treatment schedule and doses of this combination that optimally leverage both the potent antitumor and radiosensitizing properties of KSR1 AS-ODN. Moreover, because KSR1 resides downstream of EGFR and Ras, it is expected to also mediate Raf/MEK/ERK engagement to the activation of receptor tyrosine kinases.

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<th>Table 3. Tumor growth and treatment response in A431 tumors</th>
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<td>Sham (n = 14)</td>
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<td>IR (n = 21)</td>
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<td>IR+AS (n = 19)</td>
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<th>Table 4. Tumor growth and treatment response in A549 tumors</th>
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<td>Sham (n = 11)</td>
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<td>IR+AS (n = 31)</td>
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other than EGFR/ErbBs. Therefore, targeting KSR1 would represent an effective approach in preventing the development of radioresistance as a result of switching from EGFR/ErbB signaling to an alternative receptor tyrosine kinase in cancer cells.

In summary, we present a body of novel experimental evidence showing the obligate role and emerging mechanistic understanding of KSR1 in modulating the anti-tumor effect of ionizing radiation in g.f Ras-dependent tumor models and the radiosensitizing benefit of adding KSR1 AS-ODN to the single-dose IR regimen. Furthermore, the differential requirement of KSR1 signaling in normal and cancer cells (31–33), and our showing of the effectiveness of KSR1 AS-ODN in radiosensitization have provided an attractive and nontoxic radiosensitization approach via specific attenuation of g.f Ras signaling.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Retraction: Pharmacologic Inactivation of Kinase Suppressor of Ras1 Sensitizes Epidermal Growth Factor Receptor and Oncogenic Ras-Dependent Tumors to Ionizing Radiation Treatment

The article titled "Pharmacologic Inactivation of Kinase Suppressor of Ras1 Sensitizes Epidermal Growth Factor Receptor and Oncogenic Ras-Dependent Tumors to Ionizing Radiation Treatment," which was published in the October 2010 issue of Molecular Cancer Therapeutics (1), is being retracted by the AACR Publications Department, following an investigation conducted by the University of Chicago and a subsequent finding of research misconduct by the Office of Research Integrity (ORI) of the U.S. Department of Health and Human Services.

The finding of misconduct impacts the main conclusions of this article, specifically, that images used in the article "had been among a set of manipulated images produced while at another institution, which had been found to be false by that institution. ORI found that respondent falsely reported these images in Figs. 1D, 2A, and Supplementary Fig. S1B and S1C’ (2).

The ORI report (2) lists the following falsifications:

1. Falsely labeled immunoblots in Figs. 1D and 2A as follows:
   a. Figure 1D (bottom panel), representing the total ERK levels in extracts from cells exposed to 15 Gy of gamma radiation for 0–120 minutes, by using results from an unrelated experiment for MAPK levels in extracts from cells exposed to 2, 12, or 20 Gy of gamma irradiation for 1, 5, 20, or 60 minutes
   b. Figure 2A (KSR1 panel), representing a control Flag-KSR1 immunoblot for extracts of cells transfected with control (TRE), wild-type KSR (KSR-S), or dominant negative inactive KSR (DN-KSR) exposed to no radiation or gamma irradiation for 5 minutes, by using results form an unrelated experiment for KSR-transfected cells (KSR-S) irradiated with 0, 2, 5, 20, 15, 20 Gy irradiation
   c. Figure 2A (ERK panel), representing a control ERK immunoblot for extracts of cells transfected with control (TRE), wild-type KSR (KSR-S), or dominant negative inactive KSR (DN-KSR) exposed to no radiation or gamma irradiation for 5 minutes, by using results from an unrelated experiment for KSR-transfected cells (KSR-S) irradiated with 0, 2, 5, 10, 15, 20 Gy irradiation

2. Falsified images in Figs. 1D, 2A, and Supplementary Fig. S1B and S1C by duplicating bands within the figures as follows:
   a. Figure 1D (top panel) for an immunoblot for p-ERK in A431 cells, by using the same bands to represent cells treated with ionizing radiation for 5 and 10 minutes with the bands for 60 and 90 minutes
   b. Figure 2A (top) for an in vitro kinase assay for p-GST-Elk-1, by duplicating lanes 2 and 5 to represent the control plasmid (TRE) at 5 minutes postradiation (lane 2) and the dominant negative inactive KSR (DN-KSR) NT lane (lane 5)
   c. Supplementary Figure S1B (middle panel) for an in vitro kinase assay for p-GST-MEK, by using the same bands to represent cells exposed to 5 and 20 Gy ionizing radiation
   d. Supplementary Figure S1C (top panel) for an immunoblot for p-MEK1/2, by using the same bands to represent cells exposed to 2 and 20 Gy ionizing radiation

The AACR follows guidelines developed by the Committee on Publication Ethics (COPE). In cases such as this, where there is clear evidence that findings in the publication are unreliable, it is recommended that the article be retracted (3). Thus, it is our responsibility to correct the published record by retracting this article.
References


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Pharmacologic Inactivation of Kinase Suppressor of Ras1 Sensitizes Epidermal Growth Factor Receptor and Oncogenic Ras-Dependent Tumors to Ionizing Radiation Treatment

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