Inhibition of Notch and HIF enhances the antitumor effect of radiation in Notch expressing lung cancer

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Abstract

Background Notch receptor has an important role in both development and cancer. We have previously reported that Notch3 is up-regulated by radiation in non-small cell lung cancer (NSCLC) cell lines and that the Notch pathway inhibitor γ secretase inhibitor (GSI), when combined with radiation therapy, significantly suppressed the growth of lung cancer cells. However, little is known about the mechanism of Notch up-regulation induced by radiation. Notch expression has been reported to be activated through hypoxic inducible factor (HIF) under hypoxia, so we hypothesized that HIF would be involved in radiation-induced Notch activation in NSCLC.

Methods The changes in expression of HIF and Notch after radiation were examined using Western blotting in two Notch expressing NSCLC cell lines. Notch expression was evaluated after HIF-1α suppression by siRNA. The cytotoxic effect of YC-1, GSI and radiation was examined using MTT assay *in vitro* and xenograft model.

Result We found that radiation induced protein expression of HIF-1α at 2- 6 h and up regulated protein expression of Notch3 at 24 h after radiation under hypoxia. Specific suppression of HIF-1α down-regulated radiation-induced Notch3 activation, which suggests that Notch pathway is activated though HIF-1α after radiation. An antitumor effect of YC-1, HIF-1 inhibitor, was evident under hypoxia only when radiation was simultaneously applied. Moreover, GSI and YC-1

had a synergistic antitumor effect *in vitro*. Combination of GSI and YC-1 showed the greatest radiosensitivity *in vivo*.

Conclusion Radiation-induced up-regulation of Notch pathway and HIF-1α might provide therapeutic targets for more effective radiation therapy.

Key words: Notch; HIF-1; hypoxia; radiosensitivity; lung cancer

Introduction

Lung cancer has high incidence in many solid cancers and is the most common cause of cancer-related death worldwide [1]. Although several treatments are available for the advanced stage, they are palliative and the cure rate remains low for patients with non-small cell lung cancer (NSCLC). Therefore, new therapeutic strategies are required to improve the poor prognosis of patients with NSCLC.

The Notch receptor is a single-pass transmembrane protein that regulates cell-fate determination in multi-cellular organisms. There are four Notch receptors (Notch1-4), which binding two families of ligands, Jagged (Jagged1-2) and Delta-like (Dll-1, -3 and -4) [2, 3]. Upon ligand binding, the Notch receptor undergoes a number of proteolytic cleavages. The final cleavage by the γ secretase complex results in release of the Notch intracellular domain (NICD), which forms a nuclear complex with transcription factor and induces expression of target genes such as the Hairy and enhancer of split (*Hes*) and Hairy/enhancer of split related with YRPW (*Hey*) gene family [4]. Several studies have highlighted the aberrant activation of Notch pathways in tumorigenesis of many cancers [5- 8]. We have demonstrated that Notch3 is expressed in ~40% of NSCLC tumors and that suppression of the NICD3 by dominant negative or γ secretase inhibitor (GSI) inhibits growth of lung cancer both *in vitro* and *in vivo* [9, 10].

Resistance to radiation is one of the major reasons for radiotherapy failure in NSCLC. We

reported that Notch3 and its downstream effector Hey1 were up-regulated after radiation in NSCLC cell lines. GSI, when combined with radiation therapy, significantly suppressed the growth of lung cancer cells compared with either GSI or radiation alone, by a mechanism that possibly included GSI suppressing radiation-induced Notch activation [11]. However, this radiation-induced Notch3 activation mechanism has not been clarified. In this study, in an attempt to clarify this mechanism we focused on Hypoxic inducible factor (HIF)-1. HIF-1 is a heterodimeric transcription factor composed of HIF-1α and HIF-1β. It is stabilized by hypoxia and affects many processes such as glycolysis, mitosis, and angiogenesis [12-15]. Moreover, hypoxia activates Notch responsive promoters and increases expression of NICD, which interacts with HIF-1α [16]. In this study, we hypothesized that HIF-1α is involved in radiation-induced Notch activation in NSCLC and, therefore, a HIF inhibitor (YC-1) would have an antitumor effect when combined with radiation therapy.

Methods

Cell lines and inhibitors

The NSCLC cell line H460 was obtained from American Type Culture Collection. HCC2429 was established as previously described [17]. H460 and HCC2429 have been shown previously to have high Notch3 expression [18]. Cell lines were maintained in RPMI supplemented with 10%

fetal calf serum at 37°C in a humid environment in 5% CO_2. γ secretase inhibitor XX (GSIXX) was obtained from Calbiochem and HIF-inhibitor, YC-1, was obtained from AG Scientific.

Hypoxic treatment

Cells were incubated in a personal multi-gas incubator (ASTEC Co ltd, JAPAN). During incubation, a humidified environment at 37°C was maintained and the atmosphere was maintained at 5% CO_2 and 1% O_2. All *in vitro* experiments were performed under hypoxia.

Antibodies and Western blotting analysis

HIF-1α (1:500; BD Biosciences) was detected using a mouse polyclonal antibody and Notch3 was detected using a rabbit polyclonal antibody (1:500; Allele Biotechnology and Pharmaceuticals, Inc.) . All analyses were stained with Ponceau S and it was confirmed that protein loading was equal. The band intensity was demonstrated by quantitative densitometric analysis using NIH Image Ver1.62 software (NIH, Bethesda, MD, USA). Standardization was performed with actin measured in the same blots with anti-actin antibody (A-2066, Sigma-Aldrich Corp). Standardization was performed with actin measured in the same blots with anti-actin antibody. The number under each blot represents ratios of treated/untreated (control) protein expression

siRNA transfection

The HIF-1α siRNA sequence is ON-TARGET plus SMART pool L-004018-00 and Notch3 siRNA sequence is ON-TARGET plus SMART pool L-011093-00-0005 obtained from Thermo Fisher Scientific. Cells were transfected with 100 nmol/L siRNA in Opti-MEM medium (Invitrogen) using 100 pmol Lipofectamine 2000 (Invitrogen). Non-specific siRNAs against the target sequence were used as controls.

MTT proliferation assay and CI isobologram for the combination of radiation, GSI and YC-1 when cells were irradiated

To analyze antitumor efficacy of the combination of YC-1 and radiation, the cells were treated with radiation at 8 Gy and with an increasing dose of YC-1 and radiation simultaneously, or increasing dose of YC-1 at 24 h before or after radiation. To analyze the combined effect of GSI and radiation, GSI was administered 24 h after radiation at 8 Gy. Treated cells were then incubated for 24 h under hypoxia. The MTT assay was performed according to the manufacturer's recommendation. The absorption of light was determined at 560 nm using a microplate reader in the MTT assay (Varioskan Flash; Thermo Fisher Scientific)**.** In the CI isobologram analysis. Cells were irradiated and YC-1 given simultaneously; GSI was

administered 24 h after radiation and YC-1. GSI and YC-1 were administered at a constant concentration ratio of 1:1, which was determined by IC_{50} data (Figure 3a and 3c). Treated cells were then incubated for 24 h under hypoxia. The MTT assay was performed and the combinational cytotoxicity of GSI and YC-1 at each radiation dose was analyzed by a Fa-CI isobologram plot using the computer software Compusyn (Biosoft,Cambridge, Great Shelford, UK) [19].

Clonogenic assay

Cells were exposed to radiation at 4, 8 and 12 Gy, treated with increasing doses of YC-1 and incubated under hypoxia for 48 h. Then, cells were fixed and stained for 1 min with 0.5% crystal violet in methanol. After staining, colonies were counted three times by the naked eye. Survival fraction was calculated as (mean colony counts)/(cells inoculated) x (plating efficiency (PE)). PE was defined as (mean colony count)/(cells inoculated for non-irradiated controls) [20].

***In vivo* tumorigenicity**

All animal husbandry and experiments were performed under protocol approved by the Institutional Animal Care Committee at Hokkaido University School of Medicine. H460 at 1.0×10^6 cells were diluted in 100 μL of PBS and injected subcutaneously into the right posterior legs of

athymic, 5-week-old, female nude mice (nu+/nu+). When the tumors were palpable, the mice were randomly assigned to a group. Each group consisted of five mice. The mice in the radiation group received 8 Gy of radiation on days 1 and 8. The radiation was administered to the tumor only, with the remainder of the body shielded with lead. In the GSI group, 200 μg/kg GSIXX was administered i.p. on days 2, 3, 4 and 9, 10, 11, as previously described [11,18]. In the YC-1 group, 15 μg/g YC-1 was administered by i.p. on day 1 and 8, as previously described [21]. The tumors were then measured every 2 days using a digital caliper. Tumor volume (TV) was determined using the formula: TV = (length) × (width) × (height)/2 [20]. Tumor growth rate (%TV) on day X was calculated as: (TV on day X/TV on day 1) ×100, as previously described [18]. Some tumors were resected on day 15 for Western blotting analysis and immunohistochemistry.

Immunohistochemical analysis The detailed experimental methods were shown in Supplementary figure legend 1.

Statistical analysis

Statistical analysis was carried out using Microsoft Excel software and Jump (Pro 12.0.0, SAS institute; Japan). Significance between the control and observation arms of both *in vitro* and *in vivo* assays was determined using the Student t test, ANOVA test and Turkey-Kramer test.

Statistical significance was established at $p<0.05$.

Results

Radiation enhanced activation of Notch pathway under hypoxia in lung cancer

To investigate the effect of radiation on Notch and HIF pathway under hypoxia, the expression of Notch receptors, HIF-1α, were assessed after cells were irradiated at varying doses under hypoxia. In both H460 and HCC2429, the expression of HIF-1α was induced when the cells were incubated for 2 h and enhanced at 6h under hypoxia (0 Gy). However, its expression was slightly increased, but not significantly changed by radiation compared with control (0 Gy) (Figure 1a). Moreover, VEGF, which was a target gene of HIF, was up-regulated by radiation, suggesting that HIF downstream gene was involved (Supplementary Figure S1a). The expression of NICD3 was up-regulated at 24 h after radiation under hypoxia compared with control (0 Gy) (Figure 1b), whereas the expression of other Notch receptors (Notch1, 2 ,4) was not affected (Supplementary Figure S2). Notch target genes, Hey1 mRNA was enhanced, but not Hes1 protein by radiation (Supplementary Figure S2b, c).

Specific suppression of HIF-1α by siRNA ameliorated radiation-induced Notch activation under hypoxia

Next, to examine whether HIF-1α was involved in radiation-induced Notch expression under hypoxia, specific suppression of HIF-1α using siRNA was performed before cells were irradiated and the effect of specific suppression of HIF-1α on radiation-induced Notch pathway was evaluated. HIF-1α was inhibited by siRNA at 6 h after radiation (Figure 2a). HIF-1α suppression did not inhibit NICD3 under hypoxia without radiation (0 Gy), while NICD3 activation (Figure 2b) and Hey1 mRNA (Supplementary Figure S3b) was down-regulated by HIF-1α suppression at 24 h after radiation under hypoxia. HIF-1α inhibition did not affect other Notch receptors (Supplementary Figure S3a).

Concurrent treatment with YC-1 and radiation enhanced growth inhibition of lung cancer *in vitro*

HIF inhibitors are reported to be radiosensitizers [22, 23] and the efficacy of YC-1, a HIF inhibitor, with radiation depends on the treatment schedule [24]. We showed that colony formation was not suppressed by radiation at 4Gy in clonogenic assay and more than 8 Gy was needed in suppression of colony formation in Figure 3b. Therefore, in this study, we performed radiation experiment at 8 Gy when cells were treated with combination. To determine whether combining YC-1 and radiation can inhibit the growth of NSCLC cell lines, and which treatment schedule enhances radiosensitivity, H460 and HCC2429 were treated with radiation at 8 Gy and YC-1 at

varying doses: cell viability was determined using the MTT assay. When YC-1 was administered simultaneously with radiation under hypoxia, there was a significant decrease in IC_{50} of YC-1 compared with treatment with YC-1 alone (0 Gy) in both cell lines. In contrast, sequential treatment had no significant impact on IC_{50} of YC-1 compared with treatment with YC-1 alone (0 Gy) (Figure 3a). To confirm these results, we performed a clonogenic assay, which showed that this concurrent treatment schedule resulted in greater suppression in colony formations compared with radiation only (Figure 3b).

Sequential treatment with GSI after radiation enhanced growth inhibition of lung cancer *in vitro*

We have previously reported that the antitumor effect of GSI and radiation was more effective when GSI was administered 24 h after radiation under normoxia [11]. To evaluate whether this sequential treatment schedule under hypoxia also inhibited cell proliferation, H460 and HCC2429 were treated with GSIXX at various doses after 8 Gy radiation and their cell viability determined using the MTT assay. Sequential treatment reduced the IC_{50} of GSIXX compared with treatment with GSIXX alone (0 Gy) (Figure 3c).

Combination of GSI and YC-1 had a synergistic antitumor effect when cells were

irradiated under hypoxia *in vitro*

To investigate the combined antitumor effect of GSI, YC-1 and radiation, the cells were treated with GSI 24 h after simultaneous treatment with YC-1 and 8 Gy radiation, doses based on our previous and current findings. The Fa-CI plot of isobologram analysis for GSI and YC-1 at control and 8 Gy radiation revealed that the CI value was below 1. The resulting CI theorem of Chou-Talalay offers quantitative definition for synergistic effect (CI<1) in drug combination. This suggests that the combination of GSI and YC-1 had a synergistic effect at control and 8 Gy radiation (Figure 3d).

Administration of GSI and YC-1 ameliorated radiation-induced NICD3 up-regulation

We have already reported that radiation-induced Notch activation has a potential radioprotective role and GSI prevented this activation under normoxia [11]. Next, we examined the effect of the combination of GSI and YC-1 on the expression of radiation-induced Notch and HIF pathway under hypoxia using Western blotting analysis. Cells were treated with 8 Gy radiation and YC-1 (30 μM in H460 and 50 μM in HCC2429) simultaneously. GSIXX (30 μM in H460 and 50 μM in HCC2429) was administered 24 h after radiation and YC-1. These cells were incubated for another 24 h under hypoxia and harvested. When cells were irradiated under hypoxia, GSI or YC-1 down-regulated radiation-induced NICD3 activation in both cell lines, and the combination

of GSI and YC-1 significantly prevented radiation-induced NICD3 activation compared with single treatment (Figure 4a). YC-1 administration inhibited HIF-1α more than GSI treatment, with and without radiation in both cell lines (Figure 4b). Furthermore, specific suppression of HIF-1α and NICD3 using siRNAs was performed. As the same case with GSI and YC-1 admission, specific suppression of both HIF-1α and NICD3 significantly impaired radiation-induced NICD3 activation (Figure 4c).

Combination of GSI, and YC-1 enhanced radiosensitivity *in vivo*

To examine whether the combination of GSI and YC-1 enhances radiosensitivity *in vivo*, we utilized a xenograft model. The scheduling of GSI, YC-1 and radiation was as outlined in Figure 5a based on our *in vitro* findings and our previous reports [11, 21]. As a result, we noted a significant delay in tumor growth with the combination of GSI, YC-1 and radiation compared with other treatments (Figure 5b). No body weight loss was encountered (data not shown), indicating that all treatments, including the combination therapy, was well tolerated.

Combination of GSI, YC-1 and radiation has the greatest suppression of NICD3 and HIF-1α

Some tumors were resected on day 15 for molecular analysis. GSI or YC-1 down-regulated NICD3 expression slightly without radiation compared with control (Figure 5c). The expression of

NICD3 was enhanced in tumors treated with radiation, and this activation was mitigated significantly by the addition of GSI and YC-1 compared with single treatment, which is consistent with *in vitro* findings (Figure 5c). Next, immunohistochemical analysis for the expression of Notch3 protein showed that the radiation-induced Notch3 up-regulation of tumors was significantly suppressed more strongly when GSI and YC-1 were administered ($p<0.05$) (Figure 5d). The expression of HIF-1α protein also showed that additional treatment with YC-1 or a combination of GSI and YC-1 suppressed radiation-induced HIF-1α up-regulation ($p<0.05$) (Figure 5e).

Discussion

It is important to understand the mechanisms of radiation resistance and to develop new strategies to improve radiation-induced tumor cytotoxicity. We have previously reported that NICD3 is up-regulated after radiation in NSCLC cell lines and that GSI, when combined with radiation therapy, significantly suppressed NICD3 expression and the growth of lung cancer cells [11], suggesting that activation of this oncogenic pathway is involved in radiation resistance. However, the mechanism of NICD3 up-regulation and resistance to radiation is not fully understood.

Hypoxia is a major cause of radioresistance [15, 25] and HIF also has an important role in

hypoxia-related resistance to radiation [14, 26]. The association between Notch pathway and HIF-1α has been reported to be important for the invasion, proliferation and metastasis of several cancers under hypoxia [27- 30]. HIF-1α activates Notch pathway by interacting with and stabilizing NICD in glioblastoma and medulloblastoma under hypoxia [31]. However, no reports have examined whether the relationship between Notch pathway and HIF affects radiation treatment. Our results showed that specific suppression of HIF-1α by siRNA did not influence Notch pathway in the absence of radiation, but prevented radiation-induced Notch pathway activation under hypoxia, indicating that Notch pathway is activated through HIF-1α on radiation treatment.

However in this study, we did not detect direct binding between NICD3 and HIF-1α when cells were treated with radiation in immunoprecipitation assay (data not shown). HIF-1α activates genes related to angiogenic factors, glycolytic enzymes and glucose transporters [32- 34]. It has been reported that VEGF, which is a target gene for HIF signaling increases expression of DLL4, which is a ligand of Notch pathway, and, in turn, up-regulates Notch expression, leading to productive Notch signaling [35-37]. Moreover, Snail and Slug, which induced epithelial mesenchymal transition (EMT), were reported to be involved by the interaction of Notch and HIF under hypoxia (29. 30). These indicate that alternative mechanism, such as VEGF or EMT may modulate the radiation-induced Notch pathway activation.

Solid tumor environment contains hypoxic lesions which are a major cause of radioresistance. Therefore, we considered that this experimental design could mimick actual cancer environment and overcome resistance of radiotherapy under hypoxia. We found that the treatment schedule was important in the efficacy of combination therapy, which agreed with our previous data and other reports [11, 24]. In clinical settings, we may have to consider the treatment schedule as following: the administration of radiation and HIF-1 inhibitor followed by GSI in patients with NSCLC.

In summary, our data provided evidence that HIF-1α is associated with radiation-dependent NICD3 activation and that addition of GSI and HIF inhibitor enhanced the cytotoxicity of radiation in lung cancer. Although further studies are needed to confirm our hypothesis, this study provides compelling evidence that combining HIF inhibitor, GSI and radiation represents a rational strategy for the treatment of patients with NSCLC.

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Figure Legends

Figure 1. Radiation up-regulated Notch pathway under hypoxia.

H460 or HCC2429 was seeded at 1.0 x 10^6 cells per 6-well plate and incubated overnight under hypoxia. These cells were irradiated and then incubated under hypoxia and each protein was harvested after radiation. The numbers under actin blot represents the time after radiation. Standardization was performed with actin measured in the same blots with anti-actin antibody. The number under each blot represents ratios of treated/untreated (control) protein expression. The data shown are representative results from three different experiments.

(a) The expression of HIF-1α under hypoxia was induced at 2-6 h under hypoxia in H460 and HCC2429 cells.

(b) Radiation enhanced the expression of NICD3 at 24 h after radiation (4, 8, 12 Gy) under hypoxia compared with control (0 Gy) in both cell lines. (n=3). The numbers under actin blot represents the time after radiation. 20s: 20 seconds.

Figure 2. Specific suppression of HIF-1α by siRNA inhibited radiation-induced Notch3 activation under hypoxia.

H460 or HCC2429 was seeded at 1.0 x 10^6 cells per 6-well plate the day before transfection. Cells were transfected with 100 nmol/L HIF-1α siRNA in Opti-MEM medium using

100 pmol Lipofectamine 2000. Medium was exchanged after 6 h and cells were treated with radiation at 8 Gy. These cells were then incubated under hypoxia and harvested at 6 h and 24 h after radiation. The data shown are representative results from three different experiments. C: control; H: siRNA targeting HIF-1α.

(a) The efficiency of siRNA transfection was measured with Western blotting analysis. HIF-1α expression was suppressed by siRNA at 6 h after siRNA transfection.

(b) NICD3 was not inhibited by HIF-1α suppression without radiation treatment (0 Gy) under hypoxia. Radiation-induced NICD3 activation was down-regulated by HIF-1α suppression in a dose-dependent manner (4, 8, 12 Gy) at 24 h after radiation. Standardization was performed with actin measured in the same blots with anti-actin antibody. The number under each blot represents ratios of treated/untreated (control) protein expression.

Figure 3. The combination of YC-1 and radiation or GSI and radiation suppressed proliferation of lung cancer under hypoxia.

(a) Comparison of IC_{50} values of YC-1 with the different treatment schedules in the MTT proliferation assay. H460 at 6000 cells per well and HCC2429 at 10,000 cells per well were seeded into a 96-well plate and incubated overnight. The cells were treated with YC-1 and radiation simultaneously, YC-1 24 h after radiation or radiation 24 h after YC-1. Treated cells

were then incubated for 24 h under hypoxia and the MTT assay was performed. When YC-1 was simultaneously administered with radiation, IC_{50} of the combination was less than that of YC-1 alone in both H460 and HCC2429. Means and 95% confidence intervals from three independent experiments are shown (n=3). On the other hand, there was no difference in IC_{50} between YC-1 alone and in sequential combined with radiation. YC-1/RT: concurrent treatment schedule. YC-1 →RT: radiation after YC-1 administration. RT→YC-1: YC-1 administration after radiation.

(b) Clonogenic assay of the concurrent combination of radiation and YC-1. H460 at 30,000 cells per well and HCC2429 at 50,000 cells per well were plated using a 6-well plate. Cells were exposed to radiation, YC-1 was administered, and the cells were incubated under hypoxia for 48 h. The concurrent treatment schedule resulted in greater suppression in colony formation compared with radiation only.

(c) Comparison of IC_{50} values in GSIXX after radiation in the MTT proliferation assay. H460 at 6000 cells and HCC2429 at 10,000 cells per well per well were seeded into a 96-well plate and incubated overnight. Plated cells were treated with radiation at 8 Gy; GSIXX at varying doses was administered 24 h after radiation. Treated cells were then incubated for 24 h under hypoxia and the MTT assay was performed. The IC_{50} was less for combined therapy than for GSIXX alone in both cell lines.

(d) The Fa-CI plot of isobologram analysis for GSI and YC-1 at each radiation dose. H460 at

6000 cells per well and HCC2429 at 10,000 cells per well were seeded into a 96-well plate and incubated overnight. Cells were treated with 8 Gy radiation and YC-1 simultaneously, then GSI 24 h after radiation and YC-1. YC-1 and GSI were administered at a constant concentration ratio (1:1), which was determined on the basis of IC_{50} data of GSIXX and YC-1 (Figure 3A and Figure 3B). Treated cells were then incubated for 24 h under hypoxia and the MTT assay was performed. The CI value tended to be below 1, indicating synergistic effect at each radiation dose. The resulting CI theorem of Chou-Talalay offers quantitative definition for additive effect (CI=1), Synergistic (CI<1), and antagonist effect (CI>1) in drug combination.

Figure 4. Administration of GSI and YC-1 ameliorated radiation-induced NICD3 up-regulation under hypoxia.

H460 and HCC2429, 1 x 10^6 cells, were irradiated at 8 Gy per dose and YC-1 (30 μM in H460 and 50 μM in HCC2429) was administered simultaneously. GSIXX (30 μM in H460 and 50 μM in HCC2429) was administered 24 h after radiation and YC-1. These cells were incubated for another 24 h under hypoxia and harvested. The data shown are representative results from three different experiments. Standardization was performed with actin measured in the same blots with anti-actin antibody. The number under each blot represents ratios of treated/untreated (control) protein expression.

(a) NICD3 expression was down-regulated slightly by GSI, YC-1 or the combination under hypoxia without radiation. Combination of GSI and YC-1 showed greatest inhibition of radiation-induced NICD3 up-regulation compared with single treatment.

(b) HIF-1α expression was not up-regulated by radiation compared with control (0 Gy). YC-1 administration inhibited HIF-1α with and without radiation in both cell lines.

(c) H460 was seeded at 1.0 x 10^6 cells per 6-well plate the day before transfection and were transfected with HIF-1α siRNA or Non-specific siRNAs and treated with radiation at 8Gy. These cells were incubated for 24 h and transfected with NICD3 siRNA or Non-specific siRNAs. The cells were harvested at 24 h after transfection with NICD3 siRNA. Specific suppression of both HIF-1α and NICD3 using siRNAs significantly prevented radiation-induced NICD3 activation.

Figure 5. The combination of GSI and YC-1 enhanced radiosensitivity and suppressed radiation-induced Notch3 and HIF-1α expression *in vivo*.

(a) Treatment schedule *in vivo*. H460, 1 x 10^6 cells, was inoculated subcutaneously into the right posterior legs of nude mice. Treatment was initiated when tumors were palpable. Mice were treated with 200 μg/kg GSIXX injected intraperitoneally 3 days per week after 8 Gy of radiation and 15 μg/g YC-1 once a week. Tumor size was measured every 2 days.

(b) Radiation, GSI and YC-1 showed significant delay of tumor growth, compared with control or

other treatment. Results are the mean ± s.d. n=5.

RT= Radiation (8 Gy)

(c) The expression of NICD3 was examined by Western blotting analysis. GSI slightly inhibited NICD3 expression compared with control under hypoxia without radiation. NICD3 expression was up-regulated in tumors treated with radiation. This up-regulation was mitigated, compared with single treatment, by the addition of GSI and YC-1. The number under each blot represents ratios of treated/untreated (control) protein expression.

(d) Notch3 positive tumor cells were counted under high magnification (400x) in three random and non-overlapping fields (100 tumor cells per field). Notch3 expression was up-regulated in tumors treated with radiation (81%) compared with control (62%). This up-regulation was impaired by the addition of GSI and YC-1 (47%). Results are the mean ± s.d. n=3.

(e) HIF-1α positive tumor cells were counted under high magnification (400x) in three random and non-overlapfiping fields (100 tumor cells per field). HIF-1α expression tended to be up-regulated with radiation (80%) compared with control (74%). YC-1 or GSI and YC-1 inhibited radiation-induced HIF-1α up-regulation, respectively (72% and 68%). Results are the mean ± s.d. n=3.

Supplementary Figure Legends

Supplementary Figure Legend 1

Immunohistochemical analysis

Paraffin-embedded sections of each tumor resected on day 15 were deparaffinized with xylene and rehydrated with graded concentrations of ethanol. For antigen retrieval, sections were placed in citrate buffer and heated in a pressure cooker. Endogenous peroxidase was then blocked by immersing the sections in 3% hydrogen peroxidase-methanol for 10 min. After antigen retrieval in citrate buffer, slides were incubated overnight at 4^{o}C with Notch3 antibody (1:2500) and HIF antibody (1:100) (Santa Cruz Biotechnology). Immunohistochemistry was performed and the staining intensity and proportion of positive cells was evaluated. The intensity score was graded from 0 to 3 (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining). The proportion score was determined as 1 ($\leq$25 of tumor cells) or 2 (>25% of tumor cells). The intensity score and proportion score were multiplied together for a total score, which was 0 (negative), 1 (low), 2 (moderate) or 3 (strong). Scores of 2 or 3 were considered a positive immunohistochemistry result. Notch3 and HIF-1α positive tumor cells were counted under high magnification (400x) in three random and non-overlapping fields (100 tumor cells per field, total of 300 tumor cells per specimen).

Supplementary Figure S1

H460 or HCC2429 was seeded at 1.0 x 10^6 cells per 6-well plate and incubated overnight. These cells were irradiated and then incubated under hypoxia and each protein was harvested after radiation. For real-time PCR, total RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA was reverse transcribed into cDNA by using TaqMan reverse transcript reagents with random hexamers obtained from Applied Biosystems. Expression of Hey1 and GAPDH mRNA was determined by quantitative real-time polymerase chain reaction (RT-PCR) with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions, using Hey1 and GAPDH reagents obtained from Applied Biosystems. All PCR amplifications were performed using a MicroAmp optical 96-well reaction plate with TaqMan Fast Universal PCR Master Mix and with TaqMan Gene Expression Assay (Applied Biosystems).

(a) Radiation enhanced the expression of VEGF at 24 h under hypoxia compared with control (0 Gy) in both cell lines.

(b) The expression of Hes1 was not affected by radiation.

(c) Hey1 mRNA was accumulated at 24 h after radiation under hypoxia compared with control.

Supplementary Figure S2

H460 or HCC2429 was seeded at 1.0 x 10^6 cells per 6-well plate and incubated overnight. These

cells were irradiated at 8 Gy and then incubated under hypoxia and each protein was harvested after radiation. Radiation did not activate the expression of other Notch receptors protein.

Supplementary Figure S3

H460 or HCC2429 was seeded at 1.0 x 10^6 cells per 6-well plate the day before transfection. Cells were transfected with 100 nmol/L siRNA using 100 pmol Lipofectamine 2000. Medium was exchanged after 6 h and cells were treated with radiation at 8 Gy. These cells were incubated under hypoxia and harvested at 6 h and 24 h after radiation.

(a) Specific suppression of HIF-1α by siRNA did not affect other Notch receptors and Hes1.

(b) HIF-1α suppression did not inhibit Hey1 mRNA under hypoxia without radiation (0 Gy), while Hey1 mRNA was down-regulated by HIF-1α suppression at 24 h after radiation under hypoxia.

C: control; H: siRNA targeting HIF-1α

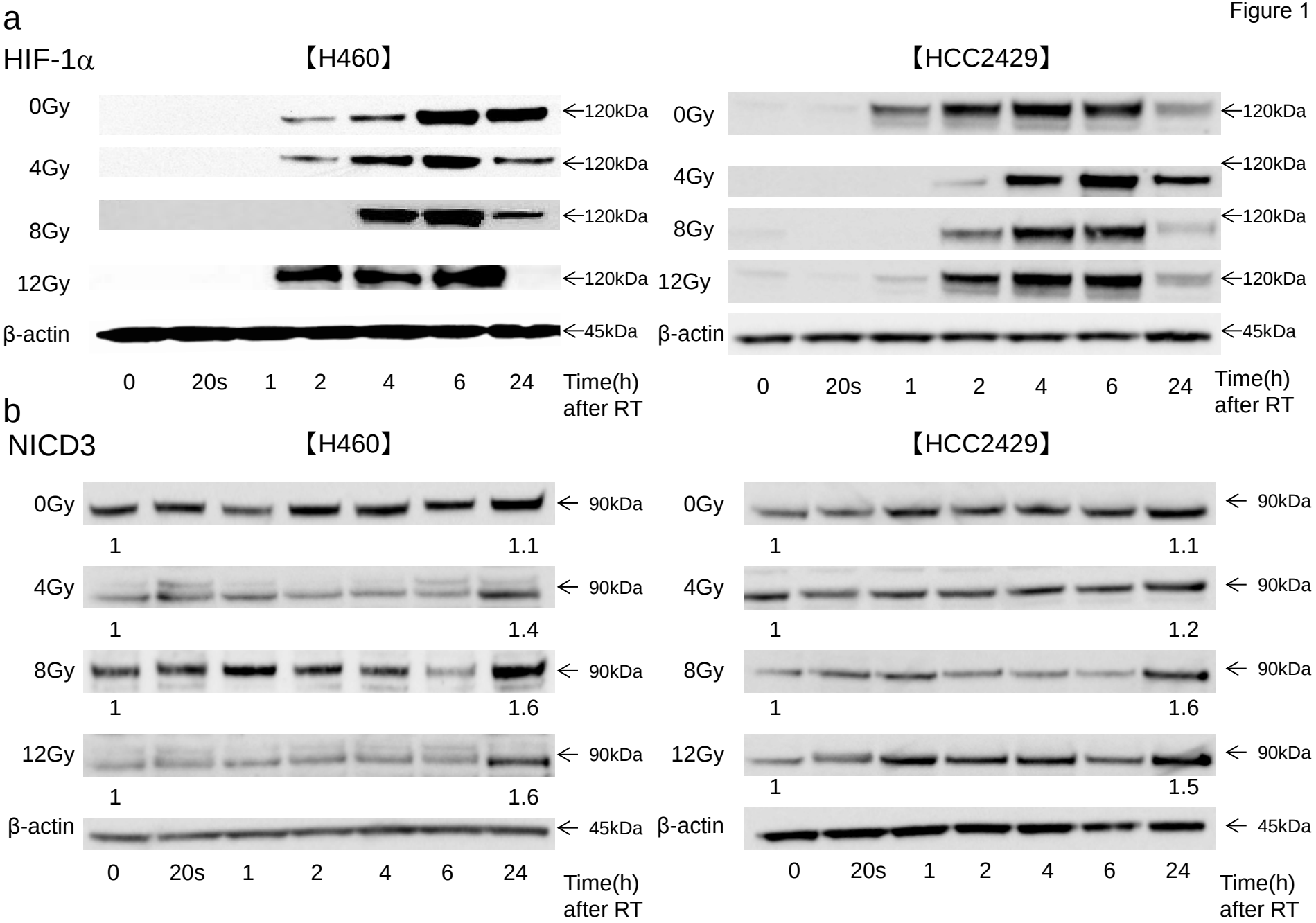

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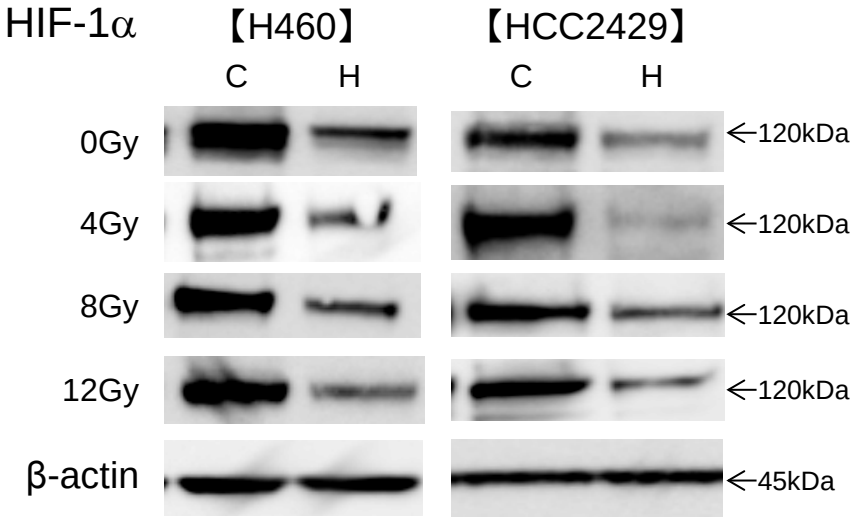
Figure2

a


HIF-1α
【H460】
【HCC2429】
C
H
C
H
0Gy
4Gy
8Gy
12Gy
β-actin
←120kDa
←120kDa
←120kDa
←120kDa
←45kDa

b

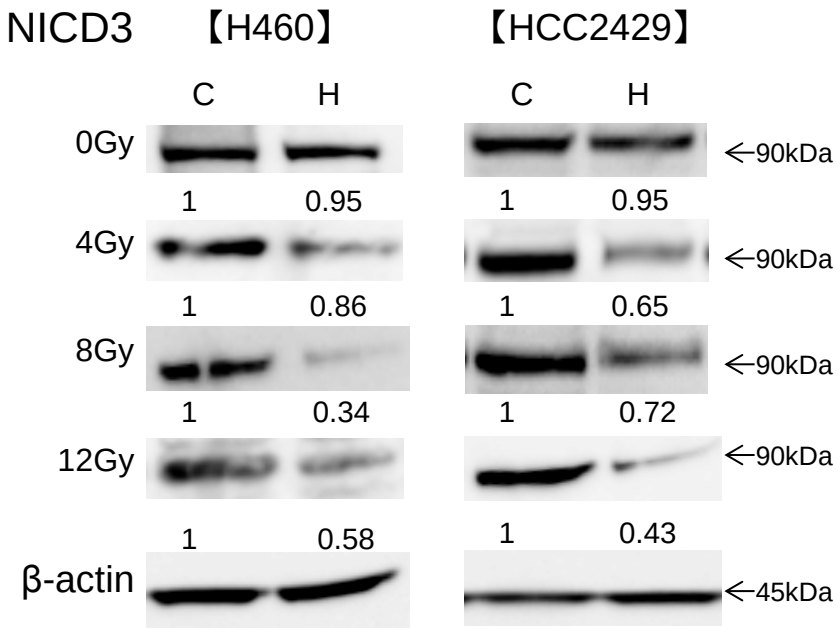

NICD3
【H460】
【HCC2429】
C
H
C
H
0Gy
1
0.95
1
0.95
4Gy
1
0.86
1
0.65
8Gy
1
0.34
1
0.72
12Gy
1
0.58
1
0.43
β-actin
←90kDa
←90kDa
←90kDa
←90kDa
←45kDa

Figure 3

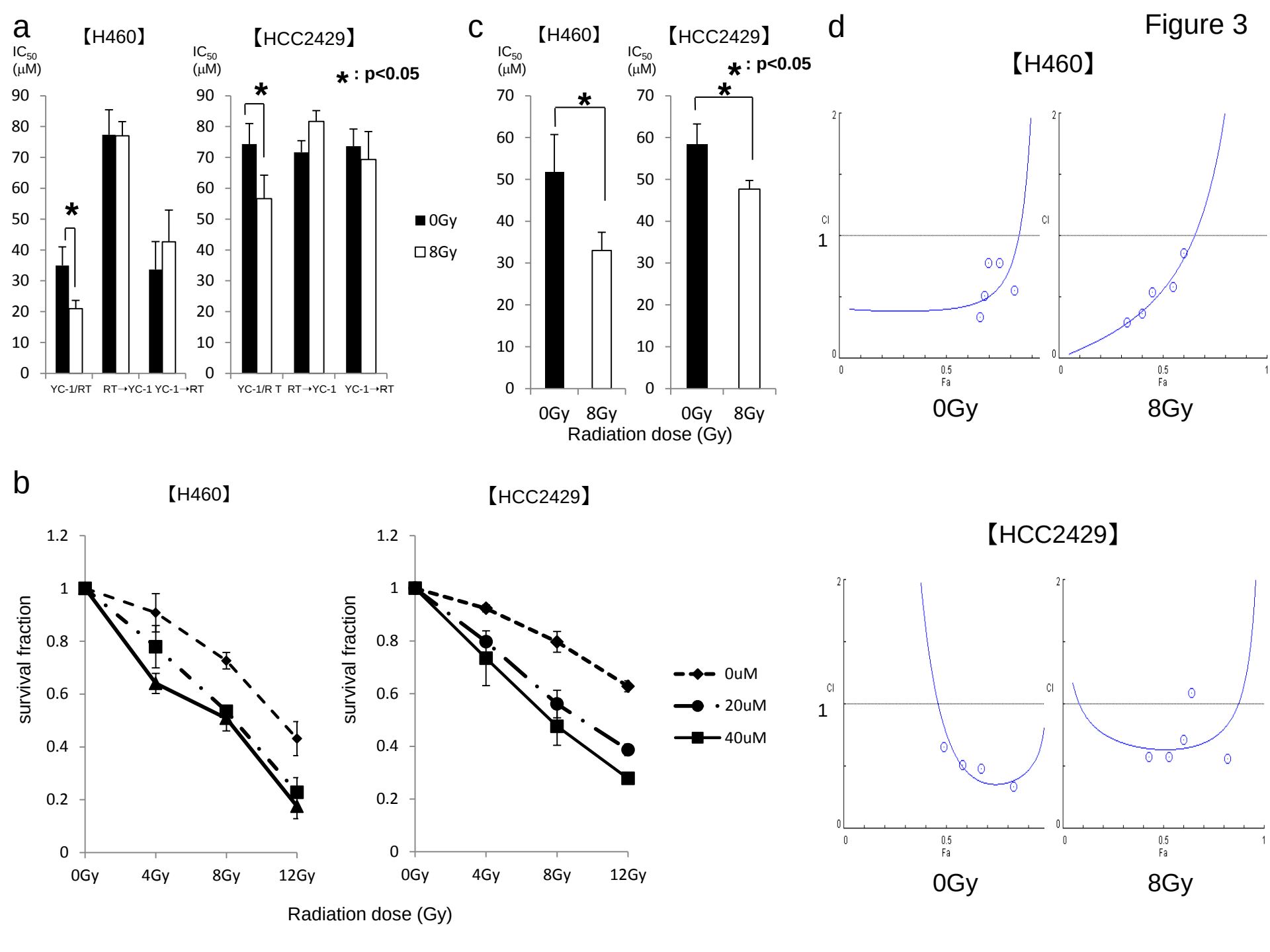
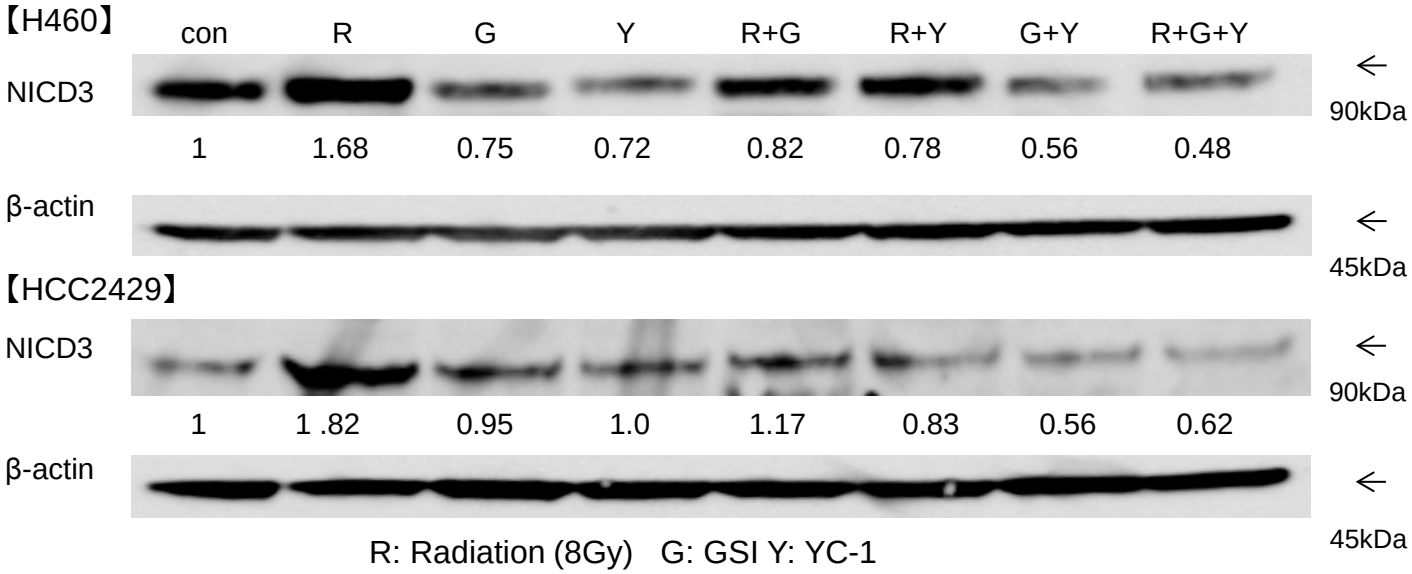

a
【H460】
【HCC2429】
IC50 (μM)
* : p<0.05
0Gy
8Gy
YC-1/RT
RT→YC-1
YC-1→RT
c
【H460】
【HCC2429】
IC50 (μM)
* : p<0.05
0Gy
8Gy
Radiation dose (Gy)
d
【H460】
CI
Fa
0Gy
8Gy
【HCC2429】
CI
Fa
0Gy
8Gy
b
【H460】
【HCC2429】
survival fraction
0Gy
4Gy
8Gy
12Gy
0uM
20uM
40uM
Radiation dose (Gy)

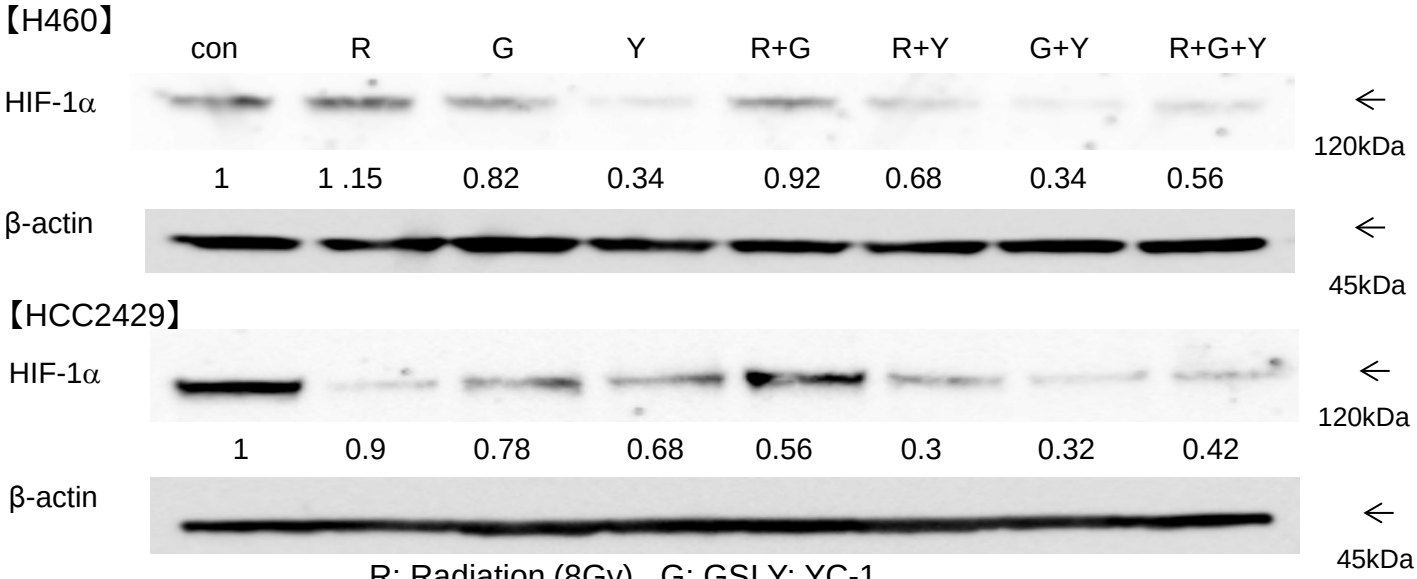
Figure 4

a



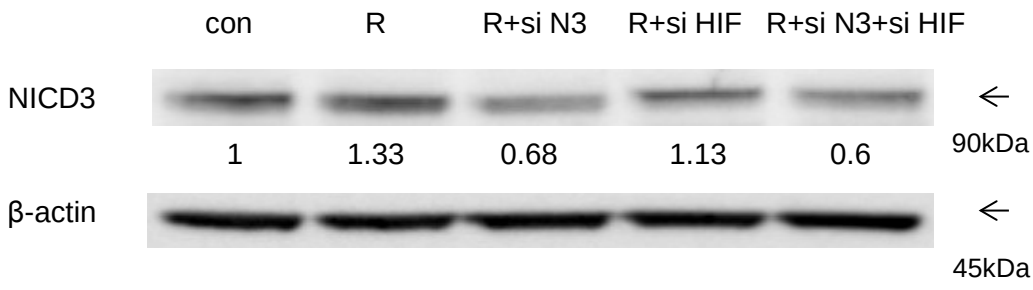
R: Radiation (8Gy)　G: GSI Y: YC-1

b



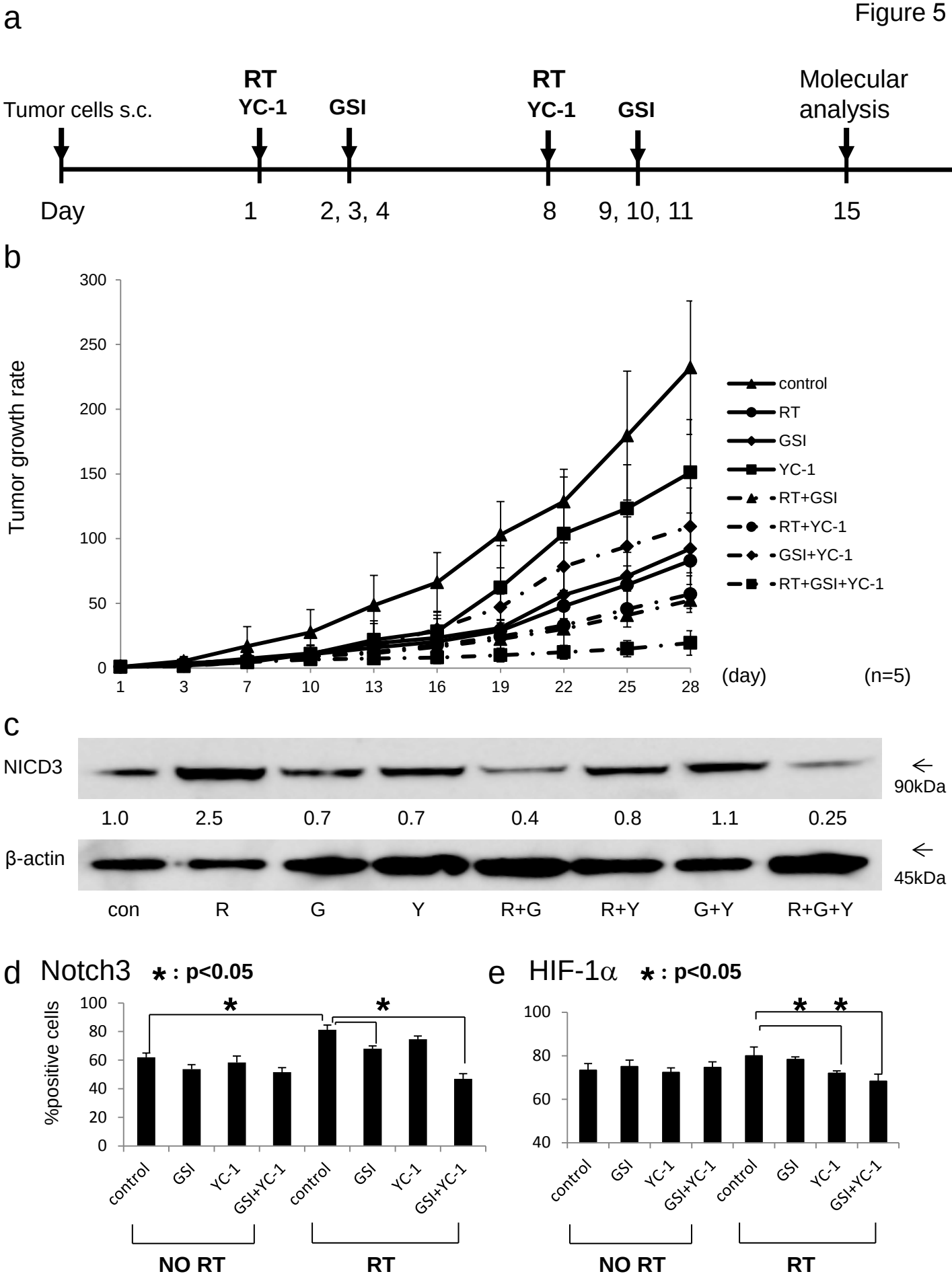
R: Radiation (8Gy)　G: GSI Y: YC-1

c

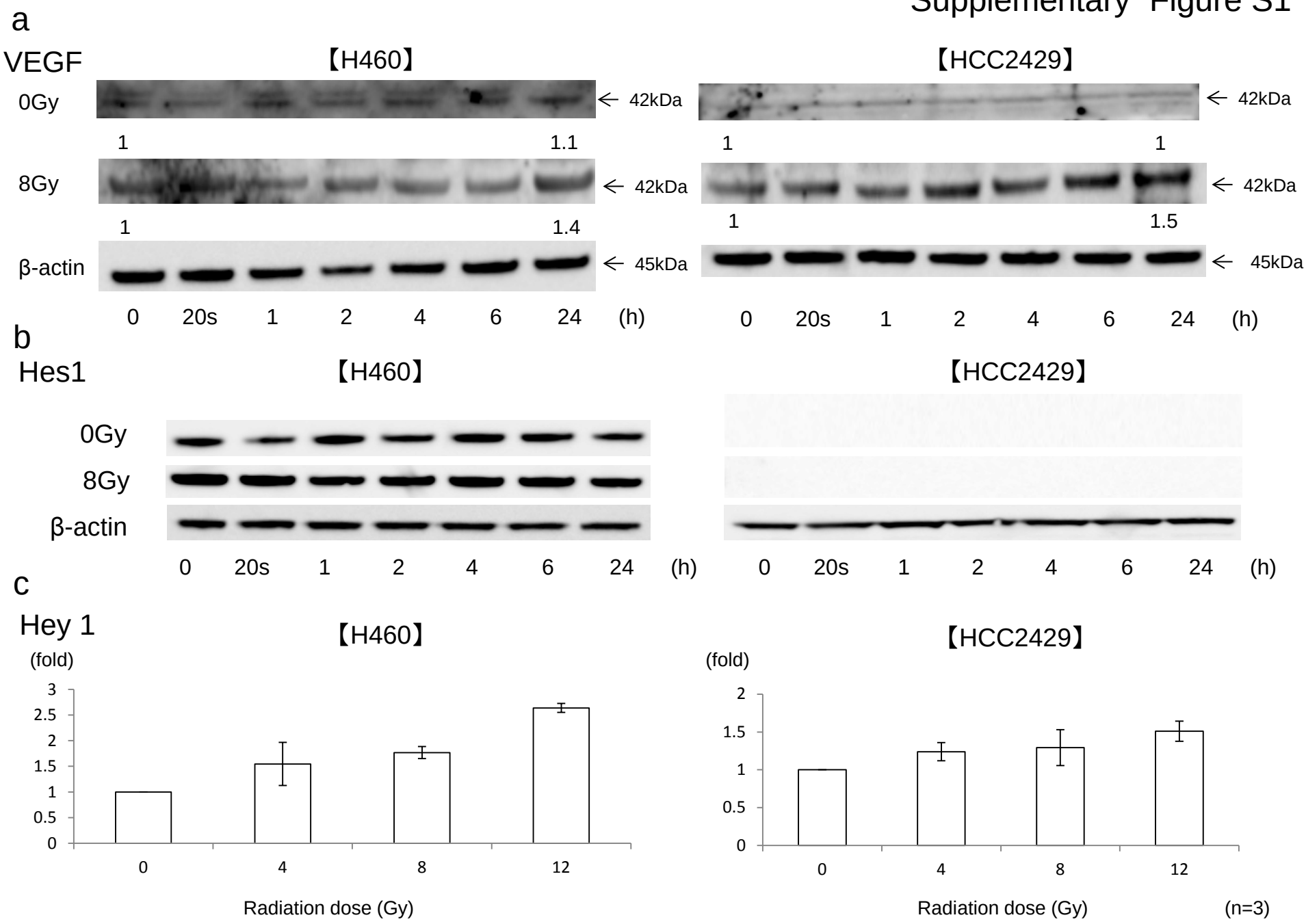


R: Radiation (8Gy)　si N3 : siRNA targeting NICD3　si HIF: siRNA targeting HIF-1a

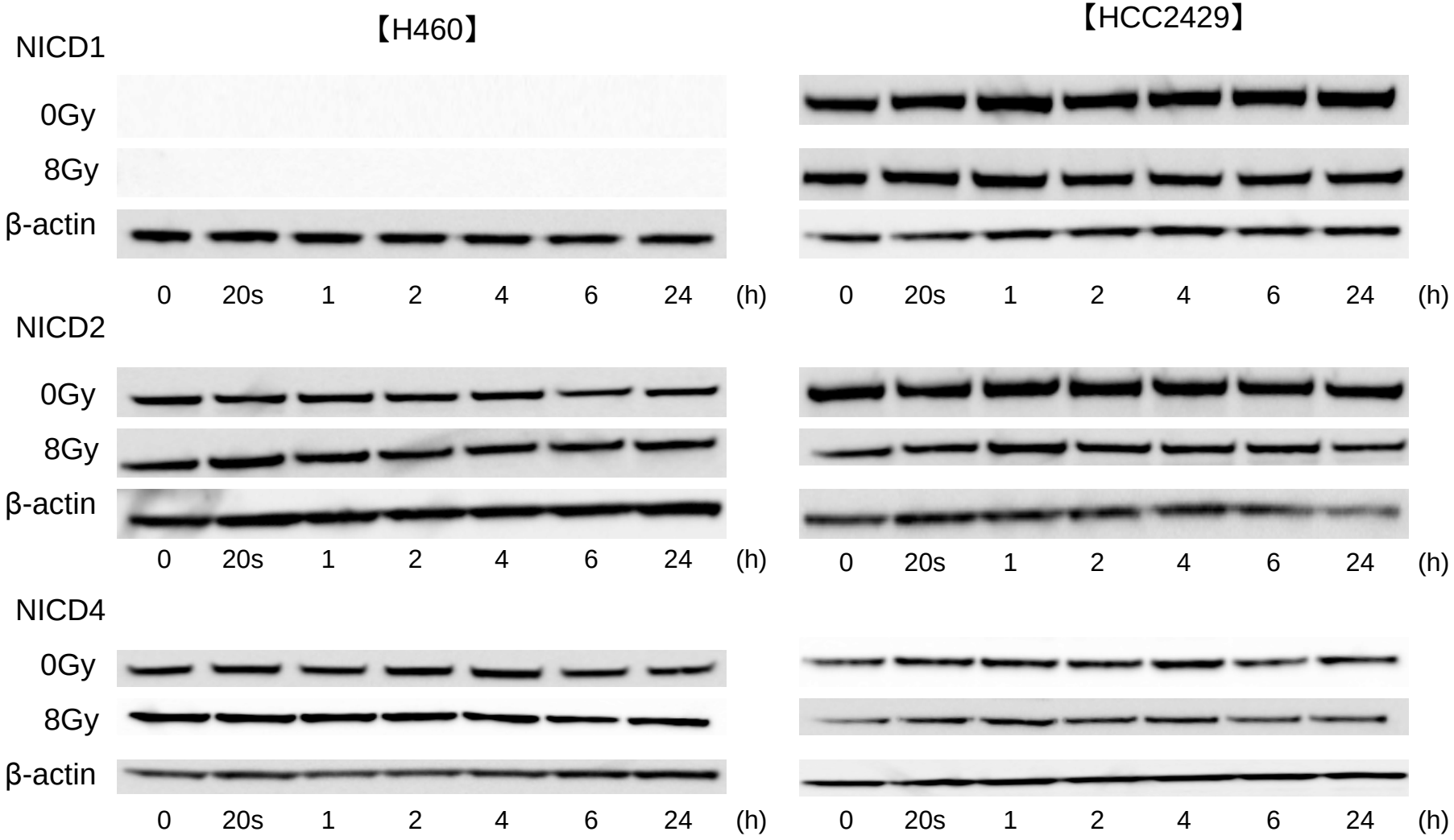
Figure 5


a
RT
YC-1
GSI
RT
YC-1
GSI
Molecular analysis
Tumor cells s.c.
Day
1
2, 3, 4
8
9, 10, 11
15
b
Tumor growth rate
300
250
200
150
100
50
0
1
3
7
10
13
16
19
22
25
28
(day)
(n=5)
control
RT
GSI
YC-1
RT+GSI
RT+YC-1
GSI+YC-1
RT+GSI+YC-1
c
NICD3
90kDa
1.0
2.5
0.7
0.7
0.4
0.8
1.1
0.25
β-actin
45kDa
con
R
G
Y
R+G
R+Y
G+Y
R+G+Y
d
Notch3
* : p<0.05
%positive cells
100
80
60
40
20
0
control
GSI
YC-1
GSI+YC-1
control
GSI
YC-1
GSI+YC-1
NO RT
RT
e
HIF-1α
* : p<0.05
100
80
60
40
control
GSI
YC-1
GSI+YC-1
control
GSI
YC-1
GSI+YC-1
NO RT
RT

Supplementary Figure S1

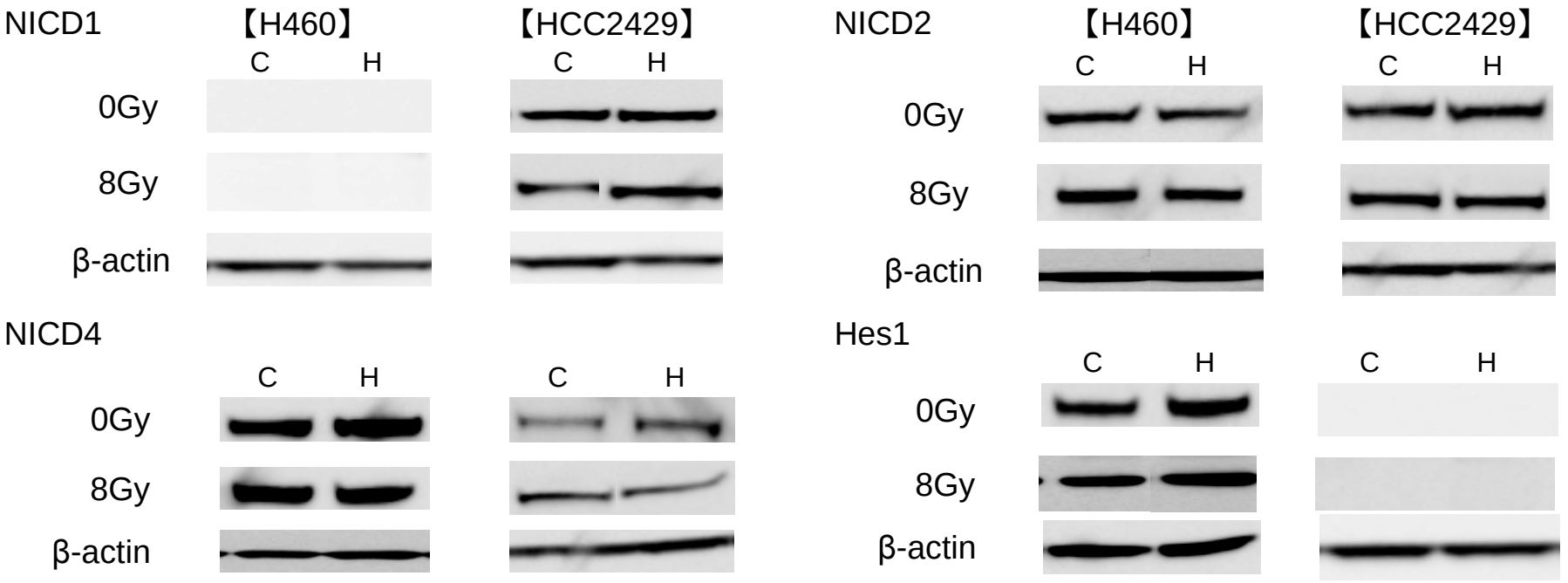


Supplementary Figure S2



Supplementary Figure S3

a



b