

A novel radioresistant mechanism of galectin-1 mediated by H-Ras-dependent pathways in cervical cancer cells

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Galectin-1 is a lectin recognized by galactoside-containing glycoproteins, and is involved in cancer progression and metastasis. The role of galectin-1 in radiosensitivity has not previously been investigated. Therefore, this study tests whether galectin-1 is involved in the radiosensitivity mediated by the H-Ras signaling pathway using cervical carcinoma cell lines. A knockdown of galectin-1 expression in HeLa cells decreased clonogenic survival following irradiation. The clonogenic survival increased in both HeLa and C33A cells with galectin-1 overexpression. The overexpression or knockdown of galectin-1 did not alter radiosensitivity, whereas H-Ras was silenced in both cell lines. Whereas K-Ras was knocked down, galectin-1 restored the radiosensitivity in HeLa cells and C33A cells. The knockdown of galectin-1 increased the high-dose radiation-induced cell death of HeLa cells transfected by constitutively active H-Ras. The knockdown of galectin-1 inhibited the radiation-induced phosphorylation of Raf-1 and ERK in HeLa cells. Overexpression of galectin-1 enhanced the phosphorylation of Raf-1 and ERK in C33A cells following irradiation. Galectin-1 decreased the DNA damage detected using comet assay and γ -H2AX in both cells following irradiation. These findings suggest that galectin-1 mediates radioresistance through the H-Ras-dependent pathway involved in DNA damage repair.

Cell Death and Disease (2012) 3, e251; doi:10.1038/cddis.2011.120; published online 12 January 2012

Subject Category: Cancer

Cancer of the uterine cervix is prevalent in developing countries. Radiotherapy has a significant role in definitive and adjuvant therapy. Local recurrence in patients following radiotherapy presents a challenge because salvaging previously radioresistant tumors using either radiotherapy or surgery with concern for normal tissue complications is difficult. The analysis of our prior study reveals that the overall incidence of local recurrence is 13% following definitive radiotherapy.¹ Clinical trials targeting EGFR²⁻⁴ or COX-2⁵⁻⁷ failed to improve the treatment outcome; severe side effects were even noted. Hence, the reconsideration of other molecular targets involving radioresistance will be a future goal.

Galectins are proteins with galactoside-binding glycoproteins. Clinical evidence of galectin involvement in cancer progression is increasing.⁸ Galectin-1 is associated with a poor prognosis in head and neck cancer⁹⁻¹² and prostate cancer.¹³ Because radiotherapy is among the major therapies of head and neck, prostate, and cervical cancer, investigating the role of galectin-1 in radiation response is valuable.

Based on clinical evidence, two major mechanisms of radioresistance exist, and galectin-1 may involve both. The first factor is DNA repair. Parliament and Murray¹⁴ reviewed the

clinical evidence of numerous DNA repair genes involving radioresistance. H-Ras involves DNA repair¹⁵⁻¹⁸ and radioresistance.^{15,19-21} In addition, galectin-1 can interact with H-Ras to promote downstream signals.^{22,23} The second factor is tumor hypoxia. Hypoxia is a radioresistance-influencing factor for patients with cervical cancer.^{24,25} Coincidentally, hypoxia is associated with galectin-1 expression.^{9,10,26} Furthermore, galectin-1 is associated with the progression of cervical cancer.²⁷ Overall, this study explored the association between galectin-1 and radioresistance in cervical cancer cells. The results show that galectin-1 mediates resistance to radiation-induced cell death through H-Ras, but not K-Ras.

Results

Varying expressions of galectin-1 in different cervical cancer cell lines. To evaluate the role of galectin-1 in the radiation response of cervical cancer cells, this study first assessed the galectin-1 expression in different cervical cells. We noted a high expression of galectin-1 in HeLa cells and an extremely weak expression of galectin-1 in C33A cells (Figure 1a). Therefore, this study transfected the siRNA of

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Keywords: galectin-1; cervical cancer; radiosensitivity; radioresistance; H-Ras

Abbreviations: GFP, green fluorescent protein; IP, immunoprecipitation; ECs, endothelial cells; FBS, fetal bovine serum; PI, propidium iodide; RT-PCR, reverse transcriptase-polymerase chain reaction; PE, plating efficiency; PLA, *in situ* proximity ligation assay

Received 08.4.11; revised 30.8.11; accepted 12.9.11; Edited by P Salomoni

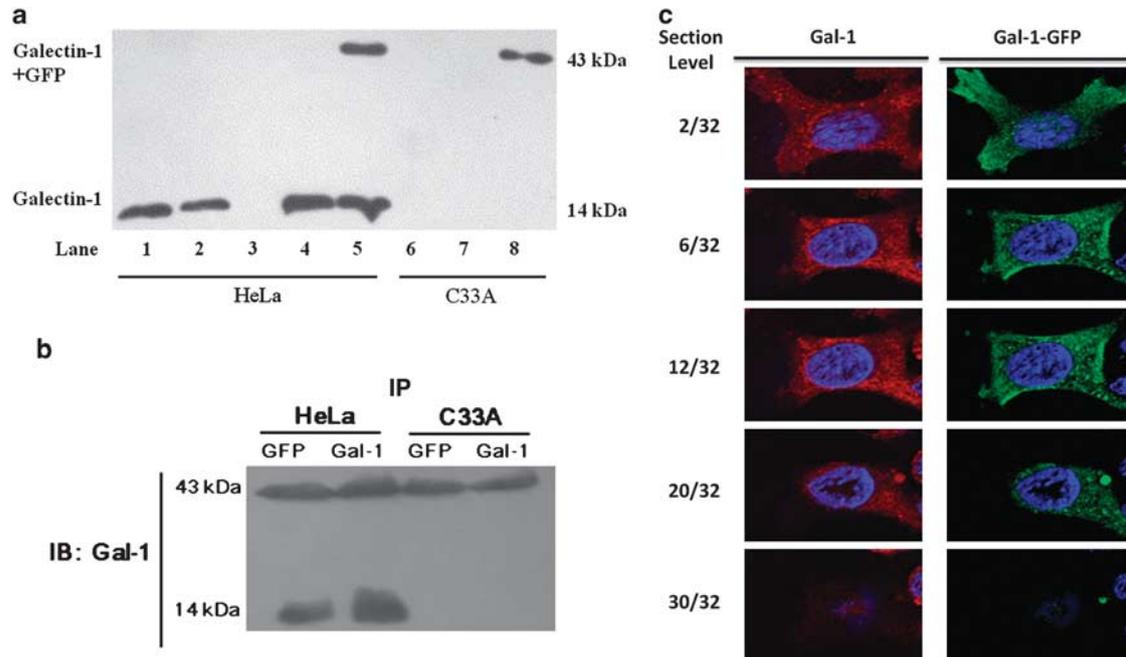


Figure 1 Expression of galectin-1 in HeLa and C33A cells. (a) Cells transfected with GFP only, GFP plus galectin-1, or shRNA were compared with cells without a vector. Lane 1: wild type; lane 2: empty vector; lane 3: shRNA; lane 4: GFP; lane 5: GFP + galectin-1; lane 6: wild type; lane 7: GFP; and lane 8: GFP + galectin-1. (b) Interaction between endogenous galectin-1 and GFP-galectin-1 fusion protein in C33A and HeLa cells. IP was performed for GFP or galectin-1, and then immunoblotting was performed for galectin-1 in both cells transfected with fusion protein. A band (43 kDa) on each lane indicates fusion protein expression formation. A band (14 kDa) on Lane 1 indicates heterodimer formation of endogenous galectin-1 and fusion protein. (c) Distribution of galectin-1 (red color) and GFP-galectin-1 fusion protein (green color) in HeLa cells transfected with GFP-galectin-1 fusion protein

galectin-1 in HeLa cells for knockdown and transfected the cDNA in C33A cells/HeLa cells for galectin-1 overexpression. Transfection of cDNA with green fluorescent protein (GFP) and galectin-1 increased the expression of galectin-1 in HeLa and C33A cells (Figure 1a). The siRNA of galectin-1 inhibited galectin-1 expression in RNA and protein levels (Supplementary Figure S1). In both cells transfected with GFP and galectin-1, immunoprecipitation (IP) for GFP and then immunoblotting for galectin-1 revealed positive galectin-1 (14 kDa) in HeLa cells, but not C33A cells (Figure 1b). The result demonstrated the interaction between endogenous galectin-1 and fusion protein in HeLa cells. In HeLa cells, similar cellular distributions of GFP-galectin-1 fusion protein (green) compared with those of galectin-1 (red) were observed using confocal microscopy (Figure 1c).

Galectin-1 involves the radioresistance of HeLa and C33A cells. The next step to examine the role of galectin-1 in the radiation response of cervical cancer cells is to perform a clonogenic assay using galectin-1 knockdown in HeLa cells or galectin-1 overexpression in HeLa and C33A cells. HeLa and C33A cells were transfected with scrambled RNA or galectin-1 siRNA 48 h before 0, 2, 4, 6, or 8 Gy of irradiation. HeLa or C33A cells were transfected with GFP vector or galectin-1 cDNA and then irradiated. Significantly lower surviving fractions were noted in HeLa cells (Figure 2a) with galectin-1 knockdown compared with that of scrambled RNA. The clonogenic survival of HeLa (Figure 2b) cells was enhanced by galectin-1 overexpression. Galectin-1 knockdown did not alter clonogenic survival in C33A cells

(Figure 2c). However, galectin-1 overexpression inhibited radiation-induced cell death in C33A cells (Figure 2d).

H-Ras, not K-Ras, has a significant role in galectin-1-mediated radioresistance. To assess whether the radioresistant effect of galectin-1 was dependent on H-Ras, this study knocked down H-Ras (Supplementary Figure S2a) to compare the survival curve in cancer cells with and without galectin-1 modulation. The galectin-1 knockdown did not further decrease clonogenic survival of HeLa cells with H-Ras knockdown (Figure 3a). Galectin-1 overexpression did not increase clonogenic survival of C33A cells with H-Ras knockdown (Figure 3b). To investigate the involvement of K-Ras in galectin-1-mediated radioresistance, this study performed the same experiment on HeLa cells and C33A cells with K-Ras knockdown (Supplementary Figure S2b). Galectin-1 knockdown significantly decreased clonogenic survival of HeLa cells, especially at high doses (Figure 3c). Galectin-1 overexpression significantly increased clonogenic survival, especially at high doses (Figure 3d). To assess whether the radioresistance effect of galectin-1 depended on activated H-Ras, we transfected constitutively active H-Ras into HeLa cells. Galectin-1 knockdown significantly decreased clonogenic survival of HeLa cells transfected with constitutively active H-Ras at high doses but not low doses (Figure 3e), defending against radiation-induced cell death in HeLa cells with gal-1 knockdown. To investigate the interaction between Ras and galectin-1, we performed immunocytochemistry using dual staining with two antibodies. Positive staining is considered protein-protein

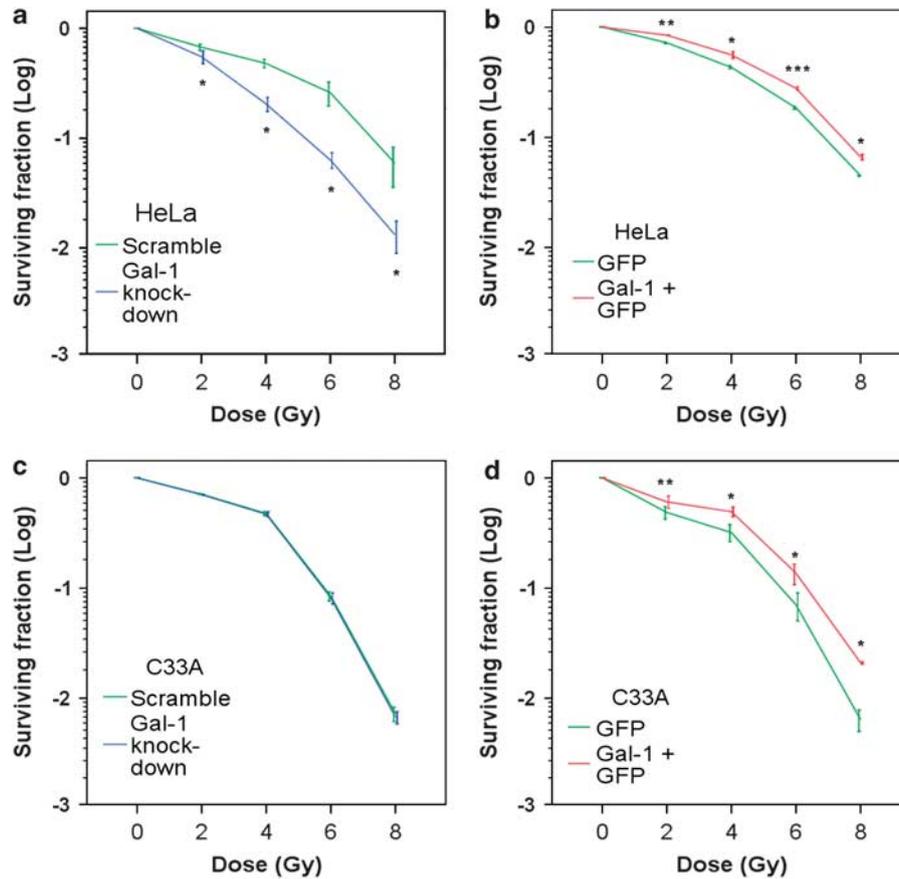


Figure 2 Galectin-1 inhibits radiation-induced cell death. Clonogenic survival curves were compared between (a) galectin-1 knockdown and scrambled RNA in HeLa cells 48 h after transfection (b) galectin-1 cDNA and GFP vectors in HeLa cells (c) galectin-1 knockdown and scrambled RNA in C33A cells 48 h after transfection (d) galectin-1 cDNA and GFP vectors in C33A cells. Cells were irradiated and clonogenic assay performed 12 to 14 days following irradiation. Clonogenic assays were performed during three independent experiments at each paired condition (with and without galectin-1 modulation). The error bar represents the standard error of mean: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

interaction. To study the interaction between H-Ras and galectin-1 in C33A cells with weak galectin-1 expression, we noted weak staining in GFP-transfected C33A cells. However, moderately enhanced dual staining was noted in galectin-1-transfected cells (Figure 4a). To study the interaction of K-Ras, we observed extremely weak staining in GFP-transfected C33A cells. However, mildly enhanced dual staining was noted in galectin-1-transfected cells (Figure 4b). Hence, the intensity of H-Ras and galectin-1 interaction was stronger than K-Ras and galectin-1 interaction in galectin-1-transfected C33A cells. In HeLa cells, the intensity of both H-Ras and galectin-1 was eliminated in shRNA-transfected cells compared with that of scrambled RNA cells (Figure 4c). No interaction between K-Ras and galectin-1 was noted in HeLa cells (Figure 4d). Anginex (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA), used for anti-angiogenesis and as a galectin-1 inhibitor, did not alter the interaction between H-Ras and galectin-1 (Figure 4e). To confirm interaction between GFP-galectin-1 and H-Ras, we performed IP on the galectin-1-overexpressing HeLa and C33A cells (Figure 4f). This study observed significant interaction between galectin-1 and H-Ras and positive immunoblotting of H-Ras during IP for

GFP in both cells. No interaction between K-Ras and galectin-1 was noted in HeLa cells, whereas weak interaction between K-Ras and galectin-1 was noted in C33A cells. These findings were consistent with similar profiles of radiosensitivity between wild-type and K-Ras-knockdown HeLa cells (Figures 2a and 3c) and C33A cells (Figures 2d and 3d). Therefore, the radioresistant effect of galectin-1 is H-Ras-dependent.

Galectin-1 enhanced H-Ras activation in cervical cancer cells following irradiation. Irradiation can activate Ras and its downstream signals, such as Raf-1 and ERK. To investigate whether the phosphorylation of Raf-1 and ERK is inhibited following irradiation by the galectin-1 knockdown, we used galectin-1 shRNA compared with scrambled RNA. An increased phosphorylation of both Raf-1 and ERK was observed in HeLa cells following irradiation. The effects of phosphorylated Raf-1 and ERK induction were inhibited by the galectin-1 knockdown (Figure 5). In C33A cells, the effects of phosphorylated Raf-1 and ERK induction were enhanced by the galectin-1 overexpression (Figure 5). Thus, the effects of galectin-1 on Ras activation are through H-Ras.

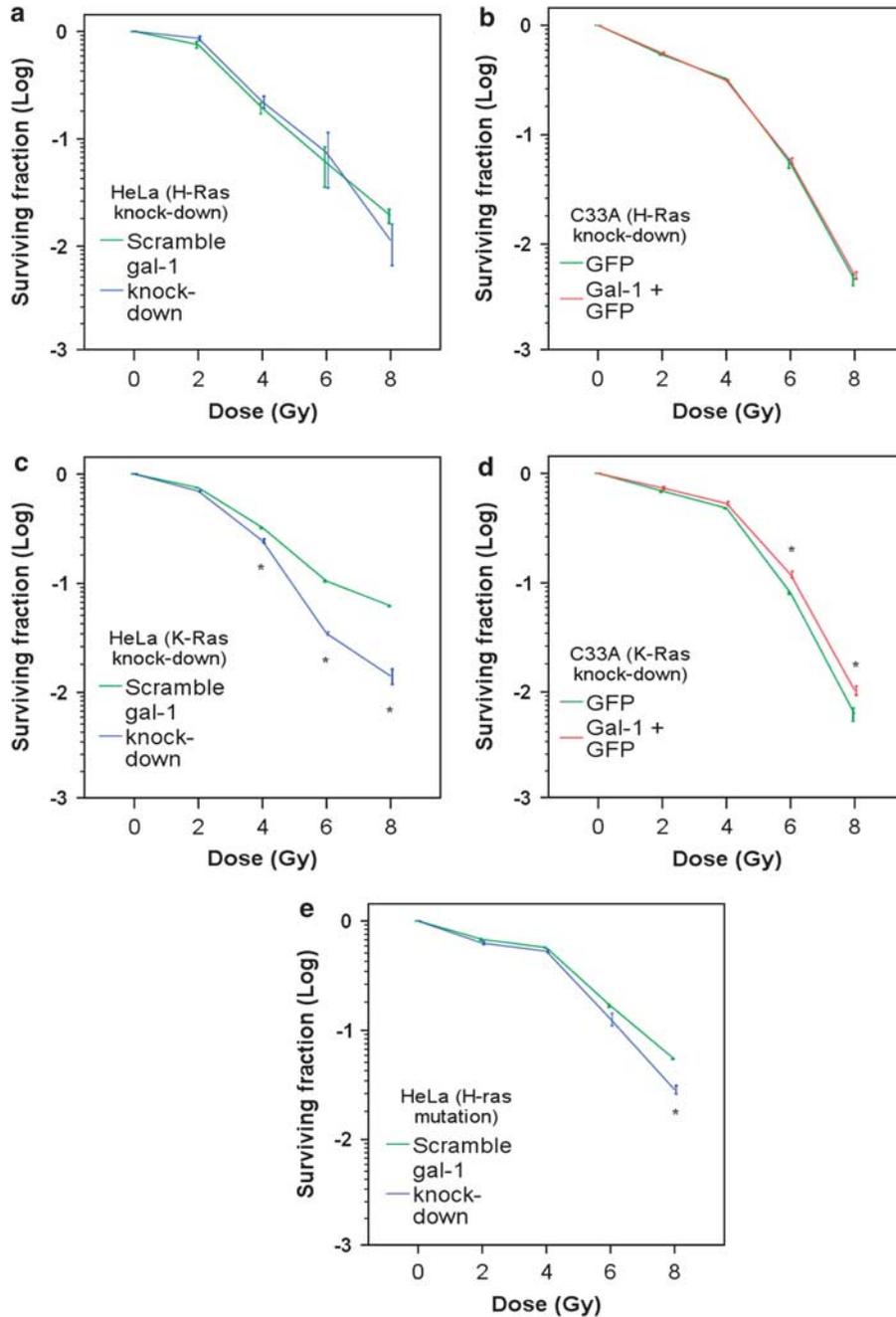


Figure 3 Galectin-1-mediated radioresistance was dependent on H-Ras but not on K-Ras. The knock down of HeLa cells by the siRNA of galectin-1 and H-Ras was confirmed by reverse transcription-polymerase chain reaction analysis and western-blot analysis in cells transfected with siRNA. Control cells were treated with scrambled RNA. The clonogenic survival curves were compared among scrambled RNA, H-Ras knockdown/scrambled RNA, and H-Ras knockdown/galectin-1 knockdown. After 48 h following transfection, cells were irradiated, and a clonogenic assay was performed 12 to 14 days after irradiation. Galectin-1 (a) knockdown or (b) overexpression did not affect the radiosensitivity of cervical cancer cells with H-Ras knockdown. However, galectin-1 (c) knockdown or (d) overexpression altered the radiosensitivity of cervical cancer cells with K-Ras knockdown. (e) Galectin-1 knockdown decreased clonogenic survival in HeLa cells transfected with mutated H-Ras at high doses but not at low doses. Clonogenic assays were performed during three independent experiments at each paired condition (with and without galectin-1 modulation). The error bar represents the standard error of mean: * $P < 0.05$

Galectin-1 enhanced DNA repair following irradiation. Because DNA repair is a significant factor of radioresistance, this study measured DNA damage in HeLa and C33A cells at various times following irradiation. Immediately following irradiation, DNA damage did not differ between

the scrambled RNA and galectin-1 siRNA of HeLa cells. Similarly, no difference existed between the GFP and galectin-1 with GFP of C33A cells. However, DNA damage was severer in the galectin-1-knockdown HeLa cells than in the control 4 and 24 h following irradiation (Figure 6a). DNA

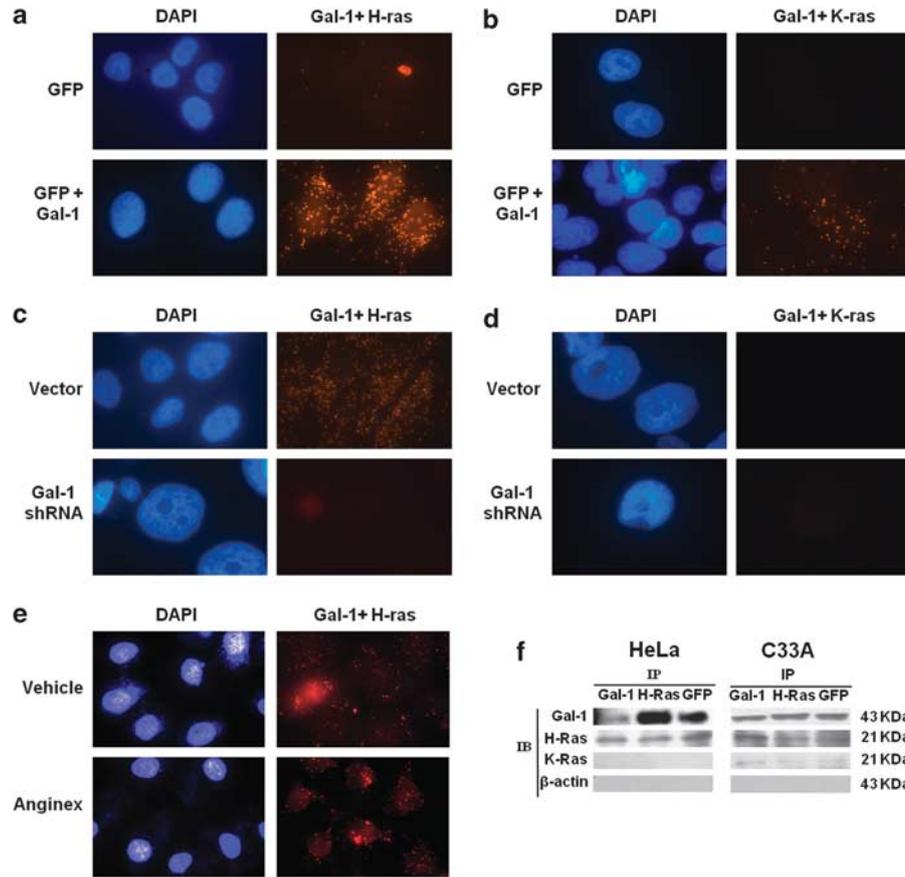


Figure 4 The interaction between H-Ras and galectin-1 is more intense than that between K-Ras and galectin-1 in C33A and HeLa cells. An *in situ* proximity ligation assay (PLA) and IP were performed for the interaction between galectin-1 and Ras. (a) PLA for galectin-1 and H-Ras in C33A cells with or without galectin-1 overexpression. (b) PLA for galectin-1 and K-Ras in C33A cells with or without galectin-1 overexpression. (c) PLA for galectin-1 and H-Ras in HeLa cells with or without galectin-1 knockdown. (d) PLA for galectin-1 and K-Ras in HeLa cells with or without galectin-1 knockdown. (e) PLA for galectin-1 and H-Ras in HeLa cells with or without the galectin-1 inhibitor anginex (10 μ M), which was administered for 24 h before cell fixation. (f) IP for galectin-1 and Ras in C33A and HeLa cells transfected with GFP plus galectin-1. IP was performed for GFP, H-Ras, and galectin-1 before immunoblotting for galectin-1, H-Ras, and K-Ras in both cells

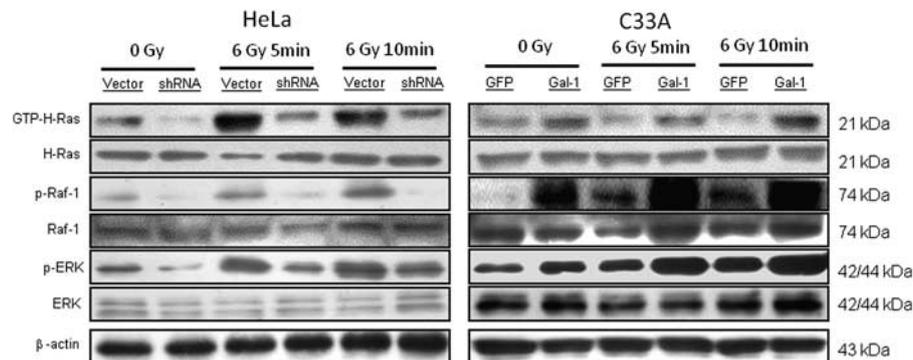


Figure 5 Effects of galectin-1 on the Ras-transmitted downstream signals. Scrambled RNA or galectin-1 shRNA were transfected in HeLa cells. GFP or galectin-1 cDNA were transfected in C33A cells. The cells were irradiated with 6 Gy and harvested 5 or 10 min later. Expressions of activated H-Ras, p-Raf-1, and p-ERK using western blots in HeLa and C33A cells were compared with or without galectin-1 modulation

damage was attenuated in the galectin-1-overexpression C33A cells than in the control 4 and 24 h following irradiation (Figure 6b). γ -H2AX is a marker of DNA damage. The expression of γ -H2AX did not differ between cancer cells with and

without galectin-1 modulation, immediately following irradiation. However, after 4 h of irradiation, enhanced expressions of γ -H2AX were observed in the galectin-1-silenced HeLa cells compared with the scrambled RNA-transfected

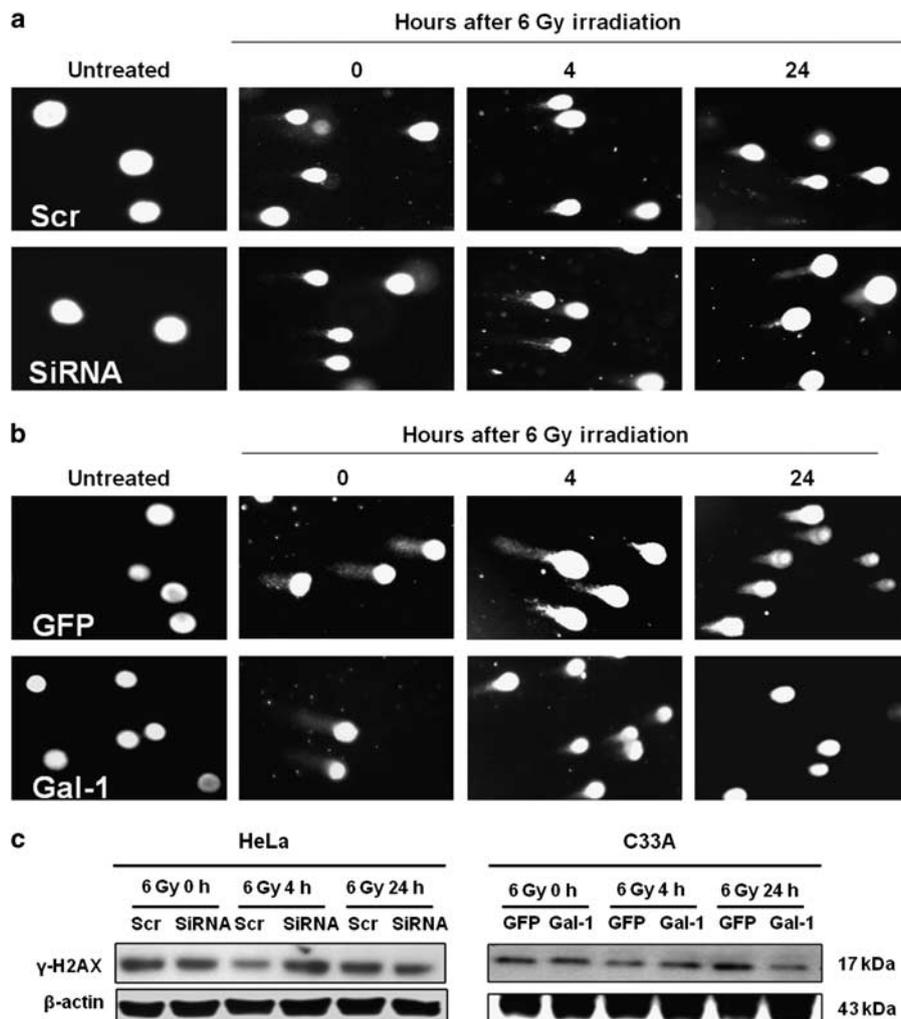


Figure 6 Galectin-1 enhanced the repair of DNA damage. GFP or galectin-1 plus GFP were transfected into C33A cells. Stable clones of scrambled RNA or galectin-1 shRNA were transfected into HeLa cells. Cells were harvested immediately, 4 or 24 h following 6-Gy irradiation. Comet assay was performed. (a) A longer tail was noted in galectin-1 shRNA compared with scrambled RNA HeLa cells 24 h following irradiation. (b) A shorter tail was noted in galectin-1-overexpressed cells compared with GFP-transfected C33A cells 24 h following irradiation. (c) Western blots were performed after harvesting the cells. Greater γ -H2AX expression was noted in galectin-1-knocked down HeLa cells and GFP-transfected C33A cells 4 h and 24 h following irradiation, respectively

cells (Figure 6c). The expression of γ -H2AX was attenuated in the galectin-1-overexpressing C33A cells compared with the GFP-transfected cells (Figure 6c).

Discussion

This study noted a significant difference in radiosensitivity between the scrambled RNA and galectin-1 siRNA-transfected HeLa cells. However, a small difference in radiosensitivity between GFP and galectin-1 plus GFP-transfected HeLa cells was observed. This is because HeLa is rich in galectin-1 protein. Hence, the galectin-1 knockdown significantly affects the clonogenic assay. By contrast, the overexpression of galectin-1 may have a ceiling effect on the clonogenic assay. To solve this problem, we selected galectin-1-deficient C33A cells (Figure 1a). As expected, the overexpression of galectin-1 in C33A cells significantly increased clonogenic survival. Thus, this study determined

that galectin-1 is involved in the radioresistance of cervical cancer cells. To the best of our knowledge, this study is the first investigation of the role of galectin-1 in radioresistance.

We are interested in the mechanism of galectin-1 involved in radioresistance. H-Ras is among the molecules associated with radioresistance^{20,21} and interacts with galectin-1.^{22,23} The common signaling pathways downstream of Ras are phosphoinositide 3-kinase (PI3K) and Raf-1. Elad-Sfadia *et al.*²⁸ found that galectin-1 involves Ras activation and diverts Ras signals to Raf-1 at the expense of PI3K.²³ Galectin-1 stabilizes GTP-bound H-Ras and enhances the activation of Raf-1 and ERK. Our results also demonstrated that galectin-1 was involved in radiation-induced H-Ras and Raf-1 and ERK activation. This study noted a weaker interaction between K-Ras and galectin-1 than between H-Ras and galectin-1, especially in HeLa cells. In addition, galectin-1-mediated radioresistance did not exist in H-Ras-silenced cells and was partially observed in K-Ras-silenced

cells. The transfection of constitutively active H-Ras could rescue the radiation-induced cell death of HeLa cells with galectin-1 knockdown (Figure 3e *versus* Figure 2a). Additionally, radiation-induced wild-type H-Ras activation was dependent on galectin-1 (Figure 5). The radioresistant effect of galectin-1 was also wild-type H-Ras dependent. Once H-Ras is constitutively activated, the downstream signals of radioresistance may be independent from galectin-1 following low irradiation doses (Figure 3e) because the level of GTP-form H-Ras may be sufficient to alleviate radiation damage. However, at high doses, endogenous H-Ras and galectin-1 may co-operate H-Ras mutant against radiation. Therefore, galectin-1 knockdown partially affects radiosensitivity at high doses, and wild-type H-Ras may have a significant role in galectin-1-mediated radioresistance. Though Paz *et al.*²² was the first to discover an interaction between galectin-1 and H-Ras, we reviewed literature regarding this interaction and noted that all the studies used transformed cells instead of cancer cells. In cell lines without transformations, the expression of GTP-H-Ras is low. However, the expression of GTP-H-Ras is high in cancer cells (Figure 5). Based on the study by Paz *et al.*,²² galectin-1 interacts with activated H-Ras. Our study using cancer cell lines reveals that galectin-1 interacts with H-Ras more than with K-Ras (Figure 4), and also correlates to the expression of GTP-form H-Ras (Figure 5). Furthermore, this phenomenon can be observed through clonogenic assays. The radiosensitive effect of a galectin-1 knockdown (Figure 2a) is similar to that of a H-Ras knockdown in HeLa cells (Figure 3a). The survival of C33A cells (Figure 2c) that are galectin-1 deficient is inferior compared with HeLa cells (Figure 2a) that are galectin-1 rich. Both cells possess abundant H-Ras expression. The knockdown of H-Ras (Figure 3a *versus* Figure 2a) alters the radiosensitivity of HeLa cells, whereas the knock down of K-Ras (Figure 3c *versus* Figure 2a) does not. However, the knock down of both H-Ras (Figure 3b *versus* Figure 2d) and K-Ras (Figure 3d *versus* Figure 2d) does not alter the radiosensitivity of C33A cells. These data prove that H-Ras, instead of K-Ras, has a significant role in radioresistance in the presence of galectin-1. Increased GTP-H-Ras can defend against radiation-induced cell death through either constitutive active H-Ras or galectin-1 and wild-type H-Ras cooperation.

Concerns exist regarding the function of GFP and galectin-1 fusion protein. This study noted a heterodimer formation based on the IP results (Figure 1b). Because almost no endogenous galectin-1 was expressed in C33A cells (Figure 1a), we propose that a monomer of fusion protein possesses the biological function of radioresistance (Figure 2d). Overexpression of the fusion protein enhanced radioresistance (Figure 2b) regardless of the abundant endogenous galectin-1 expression in HeLa cells (Figure 1a). In addition, both the fusion protein of C33A cells (Figure 4a) and the endogenous galectin-1 of HeLa cells (Figure 4c) interacted with H-Ras. Positive immunoblotting of H-Ras (Figure 4f) was noted in both cells transfected with GFP plus galectin-1 and immunoprecipitated with GFP. Thus, fusion protein possesses a similar biological function as endogenous galectin-1. However, differing amounts of endogenous galectin-1 expression was noted between C33A and

HeLa cells. Satelli *et al.*²⁹ noted that galectin-1 is silenced by promoter hypermethylation in human colorectal cancer cells. None of the cancer cell lines with endogenous galectin-1 expression possessed p53 mutation, whereas 66.7% of the cancer cell lines without endogenous galectin-1 expression exhibited p53 mutation. Because p53 mutation is noted in C33A cells but not in HeLa cells, the association between p53 mutation and galectin-1 promoter hypermethylation is worth investigating.

Raf-1 and ERK present an interesting issue regarding radioresistance because the shift of H-Ras transmits downstream signals to Raf-1 and ERK through galectin-1. Grana *et al.*³⁰ demonstrated that the mechanism of radioresistance is related to PI3K and Raf-1, but not to ERK. In addition, a number of studies support the involvement of Raf-1 in radioresistance.^{31,32} Hence, this study reasonably proposes that Raf-1 is associated with galectin-1-mediated radioresistance. Golding *et al.*³³ noted that Raf-1 enhanced radiation-induced DNA damage repair. Ras–Raf–MAPK signals enhance cell proliferation. However, the high-intensity Raf signal induces cell cycle arrest through p21.³⁴ The cell cycle is associated with radioresistance. A greater amount of cells in the G1 phase are expected to possess high radioresistance than cells with a lower G1 fraction.³⁵ Hence, we expected the involvement of galectin-1 in cell cycle regulation and distribution before and after irradiation based on a number of investigations. Fischer *et al.*³⁶ demonstrated that galectin-1 increased the G1 fraction of the cell cycle through the induction of p21 transcription. The comet assay and γ -H2AX expression revealed that galectin-1 enhanced the repair of DNA damage in both HeLa and C33A cells. Considered together, this study proposes (Figure 7) that galectin-1 enhances the activation of H-Ras/Raf-1. Raf-1 is involved in DNA damage repair and cell cycle arrest through p21. Radiation-induced cell death is inhibited following galectin-1-mediated H-Ras activation and the subsequent enhancement of DNA damage repair. Further studies are

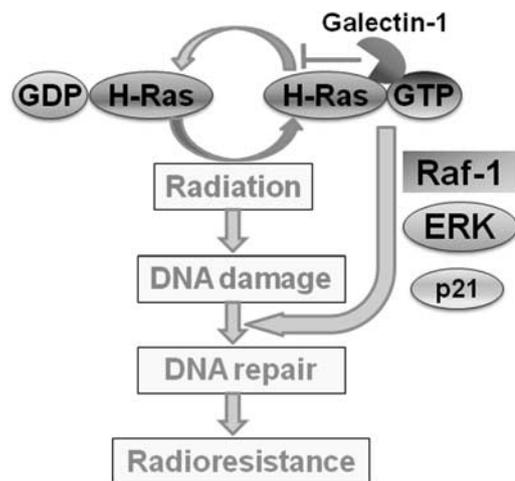


Figure 7 A proposed model of cooperation between H-Ras and galectin-1 for radioresistance. Galectin-1 enhances activation (GTP form) of H-Ras following irradiation. Galectin-1 potentiates downstream signals of H-Ras, such as Raf-1 and ERK, that may mediate DNA damage repair³³ and radioresistance.^{31,32} Galectin-1 may regulate p21 expression³⁶ through Raf-1.³⁴ Hence, Raf-1 may have a significant role in galectin-1-mediated radioresistance

suggested to verify the mechanisms of H-Ras downstream signaling regarding radioresistance.

This is the first study demonstrating galectin-1 to be a radioresistant marker of cervical cancer. Whether galectin-1 is also a radioresistant marker of other cancers still requires confirmation. Tumor-bearing mice with or without modulations of galectin-1 should be investigated in further study of radiosensitivity. Dings *et al.*³⁷ first noted that anginex induced radiosensitization in a murine mammary carcinoma model. However, during a clonogenic assay, anginex radiosensitized endothelial cells (ECs) but not cancer cells. In 2006, Thijssen *et al.*³⁸ discovered that anginex could inhibit various effects of galectin-1, such as angiogenesis, migration, and tumor progression. They also found that anginex could be detected at the membrane of intracellular vesicles in ECs. Pilch *et al.*³⁹ noted that anginex damaged the membrane of ECs but not red blood cells. This study demonstrates that anginex does not inhibit the interaction of H-Ras and galectin-1 (Figure 4e). Considered together, anginex may not translocate into cancer cells. The cell type-specific cytotoxicity of anginex may explain its anti-angiogenesis effect. Thus, different rationales of animal models for radiosensitivity using exist between the anti-angiogenesis of anginex and gene modulation of galectin-1. The mechanism of radioresistance based on the close interaction between galectin-1 and H-Ras is worth investigating. In conclusion, galectin-1 mediates the radioresistance of cervical cancer cells through H-Ras-dependent pathways. Targeting galectin-1 may be considered a radiosensitizing therapy for cancer.

Materials and Methods

Cell culture and antibodies. 293T cells (CRL-11268), HeLa cells (CCL-2), and C33A (HTB-31) were purchased from the American Type Culture Collection (ATCC). Cells were grown in DMEM medium (Gibco Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) (Gibco Life Technologies) and antibiotics (Gibco Life Technologies) at 37°C under an atmosphere of 10% CO₂. The medium was changed every 3 days. Cells were passaged by 0.25% trypsin-EDTA (Gibco Life Technologies). Subconfluent cancer cells were cultured in a 25-T flask for further experiments. Antibody to galectin-1 (H-45) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to phospho-histone H2AX (Ser139), phospho-Raf-1, Raf-1, phospho-p42/44 ERK, and p42/44 ERK were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies to α -actin, H-Ras, and K-Ras were purchased from Abcam Cambridge Science Park (Cambridge, UK). The secondary antibody (goat anti-rabbit IgG) of the detection system (Alexa Fluor 568) was purchased from Invitrogen (Carlsbad, CA, USA).

Short interfering RNA (siRNA). siRNAs (Invitrogen) were used to silence galectin-1, H-Ras, or K-Ras according to the protocol provided by the manufacturer. siRNA (7.5 μ l) and Opti-MEM (250 μ l) were mixed. Lipofectamine 2000 (7.5 μ l) was added to the other Opti-MEM (250 μ l) mixture and mixed for 5 min. The diluted siRNA and Lipofectamine were mixed for 20 min. The reagents were added into six-well plates, into which HeLa and C33A cells had been seeded (5×10^5 cells/well) for 4 h. Control cells were treated with Stealth RNAi Negative Control Duplex (Invitrogen).

Stable knockdown of galectin-1. All recombinant lentiviruses were produced by the transient transfection of 293T cells according to the manufacturer's instructions. Briefly, subconfluent 293T cells were cotransfected with 1 μ g of a hairpin-pLKO.1 vector shLGALS1 (Clone ID TRCN0000057423, National RNAi Core Facility, Taipei, Taiwan), 900 ng of psPAX2, and 100 ng of pMD2.G by Lipofectamine 2000 Transfection Reagent (Invitrogen). After 16 h, the medium was changed, and the recombinant lentivirus vectors were harvested 48 h later and then filtered through 0.45 μ m filters. The virus titers were calculated 72 h following virus infection by counting the number of GFP-expressing foci divided by

the dilution factor. For virus infection, culture cells were incubated with the medium-diluted culture virus supernatant supplemented with polybrene (8 μ g/ml) for 6 h. To achieve >95% infection efficiency, virus titers of 20 to 40 transduction unit/cells were used.

Stable transfected cell line. pCMV6-AC-GAL-1-GFP or pCMV6-AC-GFP was transfected by TrueORF destination vectors (OriGene, Rockville, MD, USA) with the Lipofectamine 2000 Transfection Reagent (Invitrogen). The C-terminal sequence encoding human Gal-1 was cloned into pCMV6-AC-GFP. To transfect HeLa or C33A cells with the plasmid vector, cells were plated into 24-well plates (2×10^5 cells per well) and allowed to adhere for 4 to 6 h. Lipofectamine 2000 (Invitrogen) was used for the transfection. After pCMV6-AC-GAL-1-GFP or pCMV6-AC-GFP transfection, the cells were cultured for 24 h. Then the medium was replaced with fresh medium supplemented with 10% FBS. G418 was used for selection; at the lowest drug concentration, G418 resulted in massive cell death after 3 days and killed all the cells within 2 weeks. Cells were diluted at a ratio of 1:10 into fresh growth medium 24 h after transduction; then, a selective medium with 600 to 1000 μ g/ml of G418 was added to stable cell lines the next day. The transduced cells were trypsinized, collected, and resuspended for flow cytometry to determine the percentage of green fluorescent cells at 48 h following transfection.

Measurement of GFP expression using flow cytometry. We placed approximately 10^5 cells into a 12 \times 15 mm test tube and washed them once with PBS by centrifugation for 5 min at $300 \times g$ and 2 to 8°C. Supernatant was removed through aspiration or rapid decanting, and 500 μ l of cold PBS was added to the cell pellet. Then, 500 μ l of cold, buffered 2% formaldehyde solution was added. The mixture was incubated at 2 to 8°C for 1 h. The cells were spun down by centrifugation for 5 min at $300 \times g$ and 2 to 8°C. Supernatant was removed by rapid decanting and the cells were washed once with cold PBS; then, 1 ml of 70% ethanol was added at -20°C to the cell pellet with the tube sitting on a vortex. The cell suspension was incubated overnight at 2 to 8°C; then, cells were spun down by centrifugation for 5 min at $300 \times g$ and 2 to 8°C. Supernatant was removed by aspiration or rapid decanting, and 1 ml of a solution containing 40 μ g/ml of propidium iodide (PI) and 100 μ g/ml of ribonuclease A was added. The cell suspension was incubated at 37°C in dark conditions for 30 min. The samples were filtered through a nylon mesh to remove clumps before acquisition on a flow cytometer.

Stable transfection of constitutively active H-Ras in HeLa cells. Retroviral packaging 293T cells (1×10^6) were seeded in a 10-cm dish, incubated for 24 h, and then transfected by Fugene 6 (Roche, Roche Applied Science, Indianapolis, IN, USA) with 1 μ g retroviral plasmids. The retroviral plasmids pBabe puro H-Ras G12V T35S and pBabe-puro control were obtained from Addgene (Cambridge, MA, USA). After 60 h, the retrovirus-containing medium was filtered (0.45- μ m filter; Acrodisc Syringe, Pall Corporation, Port Washington, NY, USA), concentrated 50-fold (from 25 ml to 500 μ l), and titrated. The HeLa cells were plated at 70 to 80% confluence in a 10-cm dish at night before infection. Each infection used 100 μ l of the concentrated virus (MOI 0.5) supplemented with 8 μ g/ml of polybrene (Sigma-Aldrich, St. Louis, MO, USA). The infected HeLa cells were selected in media containing 2 μ g/ml of puromycin for stable cell lines.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from the cells using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. The total RNA (1 μ g) was then reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen). The RNA content was measured using 260/280 UV spectrophotometry. The following primers were used: Gal-1: forward 5'-GCATGCATGGCTTGTGGTCTGGTCGC-3' and reverse 5'-AAGCTTTCAGTCAAAGGCCACACATTGA-3'. H-Ras: forward 5'-ATGACGGAATATAAGCTGGTGG-3' and reverse 5'-TCAGGAGAGCACACACTTGCAGC-3'. K-Ras: forward 5'-ACTGAATATAAACCTTGTGGTAG-3' and reverse 5'-TCAAAG AATGGTCTCGACC-3'. The internal control was β -actin.

Irradiation and clonogenic assay. Cells of different conditions (scrambled RNA, siRNA, overexpression, or GFP) were irradiated in 25 T flasks. For the clonogenic assay, 0, 2, 4, 6, and 8 Gy were delivered using a linear accelerator. Cells (100–10000/well, according to the radiation dose) were plated in six-well plates immediately following irradiation. In the 1 to 2 weeks following irradiation, glutaraldehyde (6.0% v/v) and crystal violet (0.5% w/v) were added to fix and stain colonies, respectively. Cells were counted using a stereomicroscope. A colony was

Table 1 Plating efficiency (%) of clonogenic assay

Cells and condition	Ras WT	H-Ras KD	K-Ras KD	H-Ras mutant
HeLa Scr	75.2 ± 2.1	33.7 ± 3.2	29.2 ± 1.2	72.4 ± 0.8
HeLa gal-1 KD	29.1 ± 0.8**	22.5 ± 7.6	23.5 ± 0.8*	59.3 ± 3.7*
HeLa GFP	71.2 ± 0.7			
HeLa gal-1+GFP	42.1 ± 13.0			
C33A Scr	73.1 ± 1.6			
C33A gal-1 KD	68.6 ± 0.9*			
C33A GFP	73.1 ± 1.6	34.2 ± 1.9	32.3 ± 0.2	
C33A gal-1+GFP	68.6 ± 0.9**	37.7 ± 2.9**	32.1 ± 0.8	

Abbreviations: gal-1, galectin-1; GFP, green fluorescent protein; KD, knock-down; Scr, scrambled RNA; WT, wild type. * $P < 0.05$; ** $P < 0.01$ versus control group

considered to be surviving when 50 or more cells were counted. Normalization to 0 Gy in each condition was according to the plating efficiency (PE) (Table 1). The surviving fraction equaled the number of colonies/(number of cells plated × PE). The survival curve was drawn according to the survival rate (log) and dose.

IP and western blotting. Cells (1×10^7) were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% NP40, 1 mM PMSF, $1 \times$ Roche complete mini protease-inhibitor cocktail, and $1 \times$ Pierce phosphatase-inhibitor cocktail). Co-IP was performed using the Catch and Release v2.0 Reversible Immunoprecipitation System (Millipore) according to the manufacturer's instructions. The immunoprecipitates or protein extracts (50 μ g) of cells were heated at 94°C for 3 min, resolved by 10% SDS-PAGE, and electrotransferred to PVDF membranes using semi-dry transfer. The membranes were blocked for 1 h at room temperature with PBS containing 5% milk powder, then incubated with the primary antibody (diluted concentrations of galectin-1 1 : 1000, Raf-1 1 : 500, pRaf-1 1 : 1000, ERK 1 : 2000, pERK 1 : 2000, and actin 1 : 10 000), and subsequently incubated with the horseradish peroxidase-conjugated secondary antibody (diluted concentration 1 : 3000; room temperature for 45 min). Following application of the peroxidase-conjugated secondary antibodies (Santa Cruz), the blots were developed using a chemiluminescent HRP substrate (Immobilon Western, Millipore, Billerica, MA, USA) for 1 min and then transferred onto a film (Amersham Hyperfilm MP, GE healthcare, Little Chalfont, UK).

H-Ras activation assays. HeLa or C33A cells were lysed in a RIPA buffer containing 10 mM of Tris-HCl (pH 7.6), 150 mM of NaCl, 10 mM of MgCl₂, 1% of Nonidet P-40, 1 mM of phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml of leupeptin, and 10 U/ml of aprotinin. Lysates were centrifuged at 14 000 g for 30 min at 4°C. The resulting supernatants were incubated for 1 h at 4°C with 20 μ g of Raf-1 RBD Agrose immobilized onto glutathione Sepharose beads (Cell Biolabs, San Diego, CA, USA). After incubation, the beads were collected by centrifugation for 10 s at 14 000 g and washed with $1 \times$ Assay buffer three times. The bound proteins were then analyzed by immunoblotting using the anti-H-Ras antibody.

Comet assay. The comet assay was performed using the CometAssay kit (Trevigen Inc., Gaithersburg, MD, USA), following the manufacturer's instructions. Briefly, an aliquot of 50 μ l of cells (1×10^5 cells/ml) was added to 500 μ l of molten LM agarose (0.5% low-melting agarose) stored at 37°C. Upon mixing the sample, a 75- μ l aliquot was pipetted onto an area of the comet slide. The slide was incubated at 4°C for 10 min to accelerate the gelling of the agarose disc and then transferred to a pre-chilled lysis solution for 10 min at 4°C. A denaturation step was performed in an alkaline solution (0.3 M NaOH, 1 mM EDTA) at room temperature (RT) and shielded from light for 5 min. The slide was then transferred to $1 \times$ TBE (10 mM Tris (pH 7.5), 1 mM EDTA) for 5 min to neutralize before undergoing electrophoresis in a horizontal chamber in fresh $1 \times$ TBE at 1 V/cm for 10 min. The slide was fixed in ice-cold, 70% methanol for 5 min and air dried. For observation, the samples were stained with SYBR Green (Molecular Probes, Eugene, OR, USA), diluted to 1 : 10 000 in 10 mM of Tris-HCl, pH 7.5, 1 mM EDTA, and observed using a fluorescence microscope with a 490-nm filter.

In situ proximity ligation assay (PLA). To investigate the protein-protein interaction, this study used the Duolink reagent kit (Olink Biosciences, Uppsala, Sweden). We seeded 1×10^3 cells in 200 μ l of medium into the chamber slide for culture. Cells were fixed in 1% paraformaldehyde for 15 min and washed by Wash

Buffer A for 5 min twice. Cells were incubated with a blocking solution at 37°C for 30 min, and then washed with Wash Buffer A for 5 min twice. The procedures for administrating the primary antibodies (galectin-1 versus H-Ras or galectin-1 versus K-Ras), PLA probes, hybridization, ligation, amplification, detection, and mounting followed the manufacturer's recommended protocol. The cells were observed using a fluorescence microscope (Axio Observer Z1, Carl Zeiss MicroImaging, Inc., Welwyn Garden City, UK), and photographed using an integrated camera with the appropriate filter for detection.

Confocal microscopy for the distribution of GFP-galectin-1 fusion protein. To compare the distribution of transfected GFP-galectin-1 fusion protein, we used a confocal microscope to observe galectin-1 staining and GFP expression. HeLa cells with GFP-galectin-1 fusion protein transfection were fixed in 1% paraformaldehyde for 15 min and washed using PBS for 5 min twice. The cells were incubated with a galectin-1 antibody at 37°C for 30 min, and then washed using PBS for 5 min twice. The cells were also incubated with a secondary antibody at 37°C for 30 min, and then washed using PBS for 5 min twice. Finally, the cells were incubated with a DAPI for 10 min and washed using PBS for 5 min twice. After mounting, the cells were observed using a confocal microscope (VivaTome, Carl Zeiss MicroImaging, Inc.). The procedure was performed according to the manufacturer's instructions. Red and green filters were used to observe galectin-1 and GFP, respectively.

Statistics. A comparison of the clonogenic assay of each pair was performed using a paired *t*-test. A *P*-value < 0.05 was considered statistically significant. Statistical analyses were performed using Statistical Package for Social Sciences version 17.0 (SPSS, Chicago, IL, USA).

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This study was partially supported by the grant for the Chang-Gung Medical Research Project (CMRPG880631-2).

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