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Silibinin attenuates ionizing radiation-induced pro-angiogenic response and EMT in prostate cancer cells

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ABSTRACT

Radiotherapy of is well established and frequently utilized in prostate cancer (PCa) patients. However, recurrence following therapy and distant metastases are commonly encountered problems. Previous studies underline that, in addition to its therapeutic effects, ionizing radiation (IR) increases the vascularity and invasiveness of surviving radioresistant cancer cells. This invasive phenotype of radioresistant cells is an upshot of IR-induced pro-survival and mitogenic signaling in cancer as well as endothelial cells. Here, we demonstrate that a plant flavonoid, silibinin can radiosensitize endothelial cells by inhibiting expression of pro-angiogenic factors. Combining silibinin with IR not only strongly down-regulated endothelial cell proliferation, clonogenicity and tube formation ability rather it strongly ($p < 0.001$) reduced migratory and invasive properties of PCa cells which were otherwise marginally affected by IR treatment alone. Most of the pro-angiogenic (VEGF, iNOS), migratory (MMP-2) and EMT promoting proteins (uPA, vimentin, N-cadherin) were up-regulated by IR in PCa cells. Interestingly, all of these invasive and EMT promoting actions of IR were markedly decreased by silibinin. Further, we found that potentiated effect was an end result of attenuation of IR-activated mitogenic and pro-survival signaling, including Akt, Erk1/2 and STAT-3, by silibinin.

1. Introduction

Radiotherapy is one of the leading therapeutic options for PCa patients. Successful use of radiotherapy is often limited by development of therapeutic resistance, local recurrence and distant metastases. Even though IR leverages cytotoxicity on cancer cells, recent studies have reported that IR paradoxically promotes the malignant cell phenotype in different cell types including lung, hepatocellular carcinomas and gliomas [1,2]. Moreover, a significant fraction of studies utilizing animal models have demonstrated that IR administered to primary tumours, accelerates their spread and formation of distant metastases *in vivo* [3]. One of the reasons attributed to this, is the induction of mitogenic signaling pathways by IR in these cells. IR is known to activate Erk1/2, Akt and STAT signaling pathways [1,2,4,5]. Through these molecules, IR can induce angiogenesis promoting and invasive phenotype in cancer cells. Other than accrediting aggressiveness to cancer cells, IR is also know to turn on the angiogenic switch. Low dose irradiation has been shown to enhance capillary tube formation by endothelial cells [6]. In addition, it revives VEGF (vascular endothelial growth factor) and bFGF (basic fibroblast growth factor) secretion by activation of HIF (hypoxia-inducing factor)-1α in tumor cells, which in turn promotes endothelial cell survival [7]. Another imperative connect between IR and angiogenesis was indicated by studies which showed enhanced clinical response when IR was able to affect tumor vasculature [8–10]. Hence, to improve the efficacy of radiotherapy, it could be combined with agents with an ability to target angiogenesis and epithelial to mesenchymal transition (EMT) [11]. Since the therapeutic efficacy of current anti-metastatic or anti-angiogenic chemical inhibitors are limited owing to resistance development and toxicity, combining radiotherapy with non-toxic plant phytochemicals with such activities could open newer avenues.

In the present study, we evaluated the role of silibinin, a plant flavonoid, in modulating the response of radiation on endothelial cell proliferation, clonogenicity and also its effect on PCa cell invasion, migration and IR-induced EMT. Studies conducted on various models *in vitro* and *in vivo* show that silibinin works by inhibition of various RTKs including epidermal growth factor receptor (EGFR),

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VEGFR and nuclear factor-kappa B (NF-κB) [12,13]. Therefore, by targeting IR activated pro-survival pathways, silibinin may prove to be effective in countering undesirable attributes of IR in these cells and hence improve radio-therapeutic response.

2. Materials and methods

2.1. Cell lines and reagents

HUVEC (human umbilical vein endothelial cells), DU145 and PC3 (human prostate cancer cells) were obtained from ATCC (Manassas, VA). HUVEC cells were cultured in EGM-2 medium supplemented with 5% FBS and growth supplements (EGM-2 MV bullet kit) (Walkersville, MD, USA). DU145 and PC3 cells were cultured in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum and 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate at 37 °C in a humidified 5% CO_2 incubator. All antibodies except, beta-actin, VEGF and MMP-2 were from Cell Signaling (Beverly, MA). Antibody against vimentin, VEGF and MMP-2 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Silibinin and antibody for beta-actin were from Sigma–Aldrich (St. Louis, MO, USA). ECL detection system was from Millipore.

2.2. γ- Irradiation using Co-60 source

Cells were irradiated at room temperature in gamma chamber (^{60}C, 240 TBq, Model 4000A) obtained from Bhabha Atomic Research Centre (BARC), Mumbai, India. The dose rate was estimated by Fricke's dosimetry and was found to be 4.6 Gy/min.

2.3. Cell growth and death assays

HUVEC (1×10^5) were seeded in complete EGM-2 medium. Next day, cells were treated with different doses of IR (2.5, 5 Gy) and/or 25 μM silibinin (SB) for 24 h. Cells were processed and counted after trypan blue staining as per earlier published protocol [14].

2.4. Clonogenic assay

HUVEC were seeded at 600 cells/well of a 6 well plate. After 24 h of seeding, cells were treated with 2.5, 5, 7.5 and 10 Gy of IR and/or 0.5 μM SB and the plate was incubated for 9 days in 37 °C incubator. Then colonies were fixed and processed as described earlier [15].

2.5. Tube formation assay

HUVEC (4×10^4) were cultured in EBM2 media on matrigel-coated plate as described earlier [16]. After 6 h of incubation, desired treatments were given and the cells were further incubated for 12 h. The tubular structure formation was imaged and quantified by counting the number of closed rings formed in each treatment group at 100× with inverted microscope with Zuiko Digital camera by Olympus Imaging Corp., Japan.

2.6. Wound healing assay

For *in vitro* migration assay, a fully confluent plate was wounded using a pipette tip and washed twice with media to remove detached cells, and photomicrographs of initial wounds were taken. Under direct irradiation protocol, treatment was done just after wounding. In the post-irradiation set-up, the cells were treated and wound was created 12 h after treatment. Wound closure was recorded by photography and quantified at 0, 12, 24 and 48 h at 100× magnification.

2.7. Cell migration and invasion assay using Boyden chamber

Cell migration and invasion under different treatment conditions were done using uncoated and matrigel-coated trans-well chambers from BD Biosciences, respectively (San Jose, CA). The cells were treated with SB, IR alone and in combination. After 24 h of treatment, the cells were trypsinised and replated onto the upper well of the trans-well chamber and incubated for 24 h. Then, cells on the bottom side of the membrane were fixed, stained and counted as described earlier [17].

2.8. Zymography assay for MMP-2 activity

After 48 h time period of treatment, conditioned-media was collected and was run onto a 10% polyacrylamide gel containing 0.1% gelatin under non-denaturing conditions for measuring the activity of MMP-2 as described earlier [17].

2.9. Reverse transcription-PCR

The cells were treated at 70% confluency with 25 μM SB and or IR (5 Gy) for 48 h. RNA was isolated and reverse transcription-PCR was done as per earlier published protocol [18]. The sequence of primers used are VEGF (Fwd: 5′GGAGTGTGTGCCCACTGAGGA3′; Rev: 5′TCACCGCCTCGGCTTGTCACA3′), MMP-2 (Fwd: 5′GCGGATG ATGCCTTTGCTC GTGCCT3′; Rev: 5′GCGGACAGAAGCCGTACTTG CCA3′), MMP-9 (Fwd: 5′CGGGA CAAGCTCTTCGGCTT3′; Rev: 5′GAGGTGCCGGATGCCATTCA3′), eNOS (Fwd: 5′GGTGTTCGGGG CTAAGCAGG3′; Rev: 5′CCAGCAGCATGTTGGACAC3′); E-cadherin (Fwd: 5′CGGAGGAGAGCGGTGGTCAAAG3′; Rev: 5′CTAGTCGTCCT CGCCGCCTCC3′), N-cadherin (Fwd: 5′GGAGGAGAAGAAGACCAGG-ACTA3′; Rev: 5′TCAGTCATCACCTCCACCATA3′), vimentin (Fwd: 5′GGAGTCCACTGAGTACCG GAGA3′; Rev: 5′TTATTCAAGGTCATC GTGATG-3′) and GAPDH (Fwd: 5′GGTCG GAGTCAACGGATTT GGTCG3′; Rev: 5′CCTCCGACGCCTGCTTCACCAC3′).

2.10. Western blotting

The cells were treated at 70% confluency with 25 μM SB and/or IR (5 Gy) for 48 h. Western blotting was performed as per earlier published protocol [19].

2.11. Confocal microscopy

Cells were grown on coverslips in 35-mm dish, washed with PBS, fixed in 4% formaldehyde (for 10 min, and permeabilized with 0.3% Triton X-100 for 15 min. Cells were blocked with [10% BSA in PBS] for 30 min, incubated with a primary rabbit anti-E-cadherin (1:250) or vimentin (1:500) overnight at 4 °C and then incubated with the secondary antibody Alexa Flour 594 (1:500; Invitrogen) for 1 h, and counterstained with 300 nM DAPI. Cells were examined using a confocal microscope (Nikon) at CIF, SLS.

2.12. Statistical analysis

Statistical analyses were performed using Sigma Stat software version 2.03 (Jandel Scientific, San Rafael, CA). The statistical significance of differences between control and treated-groups was determined by Student's *t*-test and $p < 0.05$ value was considered significant.

3. Results

3.1. Silibinin radiosensitizes HUVEC to suppress proliferation

Herein, we evaluated the effect of IR and silibinin on HUVEC growth and proliferation. Silibinin (25 μM) and IR (5 Gy) alone induced a moderate 34% and 32% inhibition of proliferation after 24 h, respectively, which was enhanced to 72% ($p < 0.001$) in the combined treatment. At a lower dose of 2.5 Gy IR, there was no anti-proliferative effect observed, in fact there was a slight but significant (8%; $p < 0.05$) increase in cell number, however, with silibinin addition, cell number was decreased by 45% ($p < 0.001$ vs. IR) (Fig. 1A). Silibinin and low dose IR (2.5 Gy) caused insignificant cell death, 5.6% and 4% respectively, compared to control (3.7%) (Fig. 1A). This was improved slightly in the combination, resulting in 8.5% cell death. However, at 5 Gy, there were 6.4% dead cells, which were profoundly enhanced by its combination with silibinin (17.8%; $p < 0.001$; Fig. 1A). Since radiation-induced inhibition of cell proliferation is more a result of reproductive death resulting from mitotic catastrophe, clonogenic assay gives a better measure of its efficacy. Different doses of IR with/without 0.5 μM silibinin were used to measure the effect on HUVEC clonogenicity. We found that combining IR with SB led to a striking attenuation of clonogenicity (20% at 2.5 Gy vs. 47%, 38% at 5 Gy vs. 70% at 5 Gy with SB; $p < 0.001$) compared to radiation alone (Fig. 1B). This difference was significant for the entire radiation range between 2.5 and 10 Gy.

3.2. Silibinin radiosensitizes HUVEC to inhibit capillary tube formation

Capillary tube formation by endothelial cells is one of the preliminary steps in angiogenesis and its inhibition is of great therapeutic value. Since there was a significant restraint on the growth of HUVEC on combining IR with silibinin, we examined whether it could also hinder capillary-tube formation of HUVECs on matrigel, under similar conditions. We found an almost complete inhibition in tube formation by HUVEC (96%, $p < 0.001$) compared to either 25 μM SB (48%) and 5 Gy (32%) at 24 h post-seeding (Fig. 1C). To further understand the cause of enhanced efficacy, we analyzed the effect of these treatments on angiogenic regulators. We found that IR moderately up-regulated VEGF and MMP-2 mRNA levels in these cells and it did not change the levels of eNOS and MMP-9. However, in combination treatment, there was a compelling down-regulation of IR induced MMP-2 and VEGF. Combination treatment also resulted in complete suppression of eNOS expression in these cells, which was otherwise unaffected (Fig. 1D).

3.3. Silibinin inhibits IR-induced migration of prostate cancer cells

Since our earlier studies suggest that silibinin can inhibit prostate tumor angiogenesis as well as growth and progression [12], next we assessed the effect of silibinin on migration of IR exposed DU145 and PC-3 cells (for 48 h), which are highly invasive in nature. IR and silibinin treatments were given immediately after the wound was made. IR paradoxically increased the wound closure

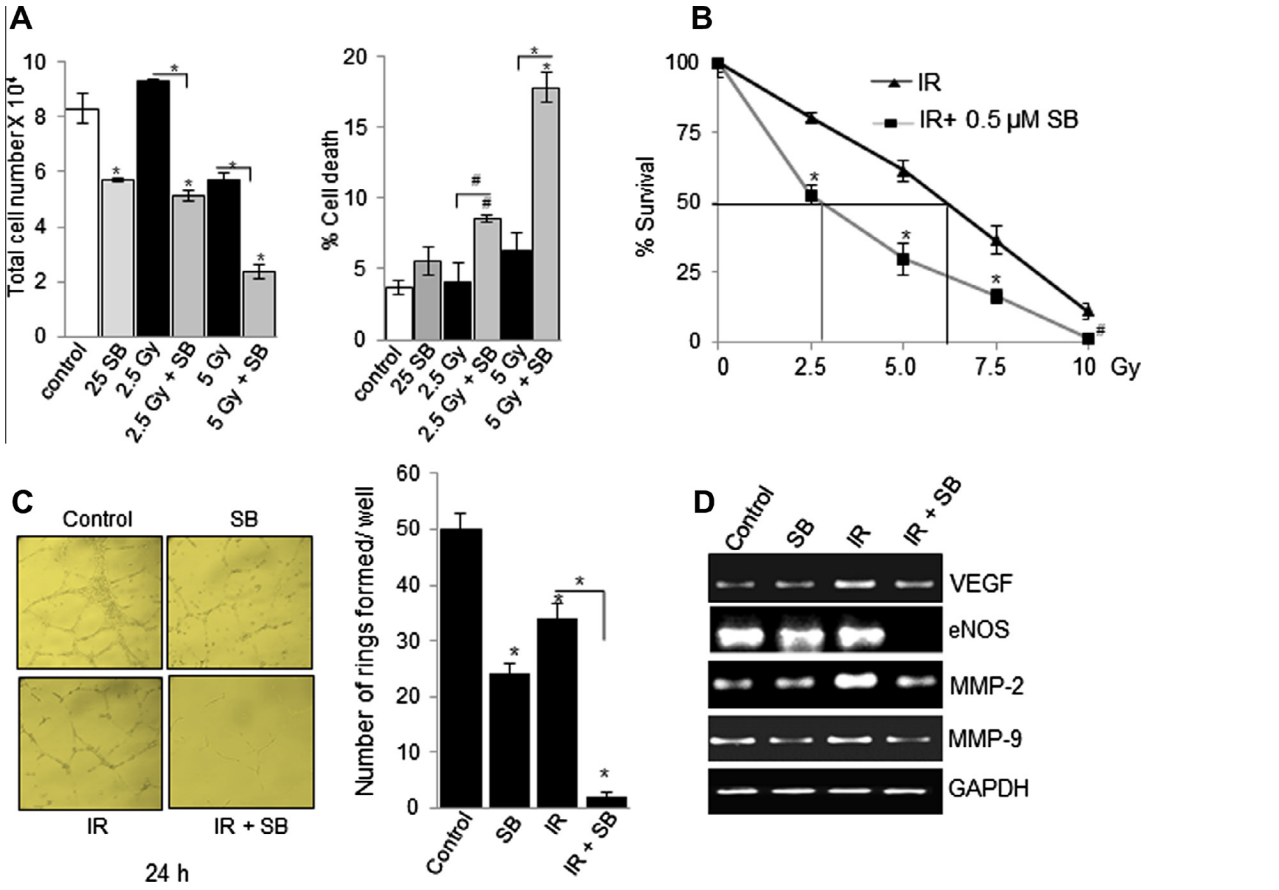

Fig. 1. Silibinin potentiates IR-induced inhibition of cell growth, survival and tube formation in HUVEC. (A) HUVEC proliferation and death after 24 h of silibinin and IR treatment. Cells were grown in complete EGM-2MV media with 5% FBS at the density of 1×10^5 cells/60 mm culture plates. After 24 h of seeding, cells were treated with 2.5 and 5 Gy with or without 25 μM of SB for 24 h in regular growth conditions. At the end of the treatment, total cell number and percent dead cells were quantified by trypan blue assay. (B) HUVEC cells at density of 600/well were seeded and treated with different doses of IR (2.5–10 Gy) with or without 0.5 μM SB after 24 h post seeding. The plate was incubated for 9 days and colonies formed were processed and counted as per the protocol. (C) Representative images and quantitative depiction of capillary tubes on matrigel by HUVEC after the treatments as mentioned in Section 2. (D). Modulation of mRNA expression of angiogenic factors by SB and IR after 24 h treatment, with GAPDH as the loading control. #, $p < 0.01$; *, $p < 0.001$.

rate, compared to control in DU145 cells. This is in line with reports suggesting the IR induces cell migration in many cancer cell types [20,21]. However, combining it with SB lead to 53% ($p < 0.001$) inhibition in wound closure compared with IR alone (Fig. 2A and C). We found a similar response in PC-3 as well, with 57% inhibition (data not shown). In another treatment condition where the wound was made 24 h post-treatment, we found IR had mild inhibitory effect (14%), however in the combination there was a substantial inhibition (55%; $p < 0.001$) in migration of DU145 cells as compared to IR alone (Fig. 2B and D). In PC-3, IR was more effective, with 60% inhibition, which was increased to 73% in combination with silibinin ($p < 0.001$) (Fig. 2D). This difference could be because DU145 cells are much more radioresistant as compared to PC-3 cells.

3.4. Combination of radiation with silibinin leads to potent inhibition of PCa cells migration and invasion through matrigel

Since we found inhibition of PCa cells migration in wound closure assay, to further verify the effects, Boyden chamber assay for studying invasion and migration was used. In the migration assay, we found 20% and 17% inhibition by silibinin and IR at the end of 20 h period, seeded post 48 h of treatment. In the combination, the inhibition was enhanced to 48% ($p < 0.001$) of the control and 28% ($p < 0.05$) from IR alone (Fig. 3A). In PC-3 cells, IR alone reduced migration by only 12%; however in combination there was 58% inhibition. In invasion assay, SB and IR caused 19% and 11% inhibition, however, their combination showed 32% inhibition in the invasive potential of DU145 cells. In PC-3 cells, IR showed 13% inhibition but when combined with silibinin, invasion was inhibited by 46% ($p < 0.001$) (Fig. 3B). To explain the effect at molecular levels, we did zymography analysis for MMP-2 activity in both these cells. We found that MMP-2 activity in DU145 cells did not change much with either SB or IR, however, in the combination the activity was noticeably reduced (Fig. 3C). In PC-3 cells, SB and IR alone could markedly reduce MMP-2 activity, which was further reduced in the combination treatment. DU145 colonies, which were formed by cells that survived irradiation treatment, showed more mesenchymal phenotype, which was reverted back to more epithelial like in combination with silibinin (Fig. 3D). Hence, it could be one of the mechanisms by which SB could inhibit invasion and migration processes in radiation-exposed PCa cells which are pre-requisite for angiogenesis and metastasis.

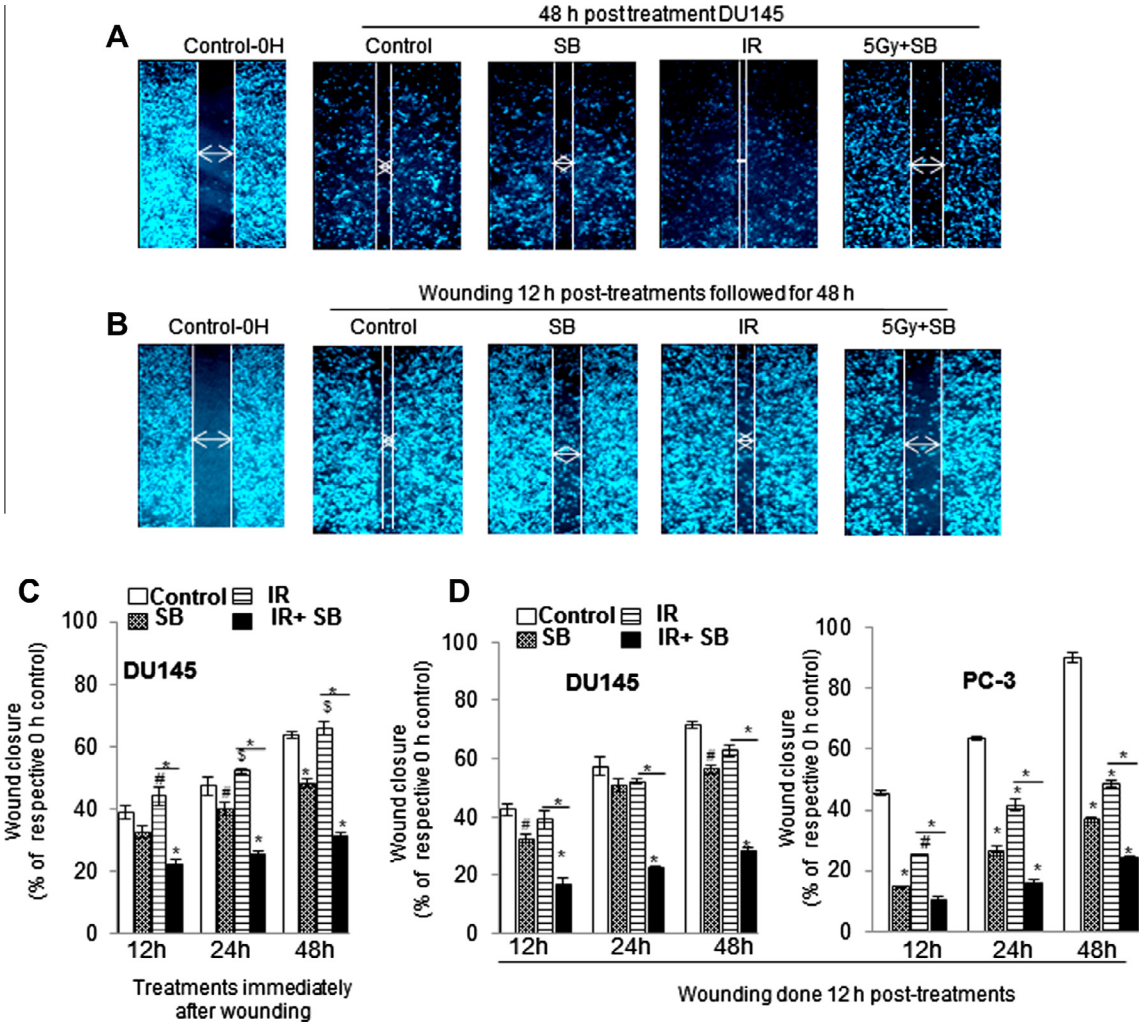

Fig. 2. Silibinin potentiates the inhibition of migration of IR-exposed PCa cells. Representative images depicting wound closure in DU145 cells. (A) Wound was created on confluent monolayer, followed with treatment with IR (5 Gy) and SB (25 μM) and the wound closure was followed for the next 48 h. (B) Cells were treated with IR and/or SB and after 12 h a wound was created and followed for closure till next 48 h. (C) Quantitative data for wound closure with treatment done immediately after wounding. (D) Quantitative data for wound closure post treatment in DU145 and PC-3 cells. Data are shown as percent cell migration compared to 0 h control the time of wounding. $, $p < 0.05$; #, $p < 0.01$; ∗, $p < 0.001$.

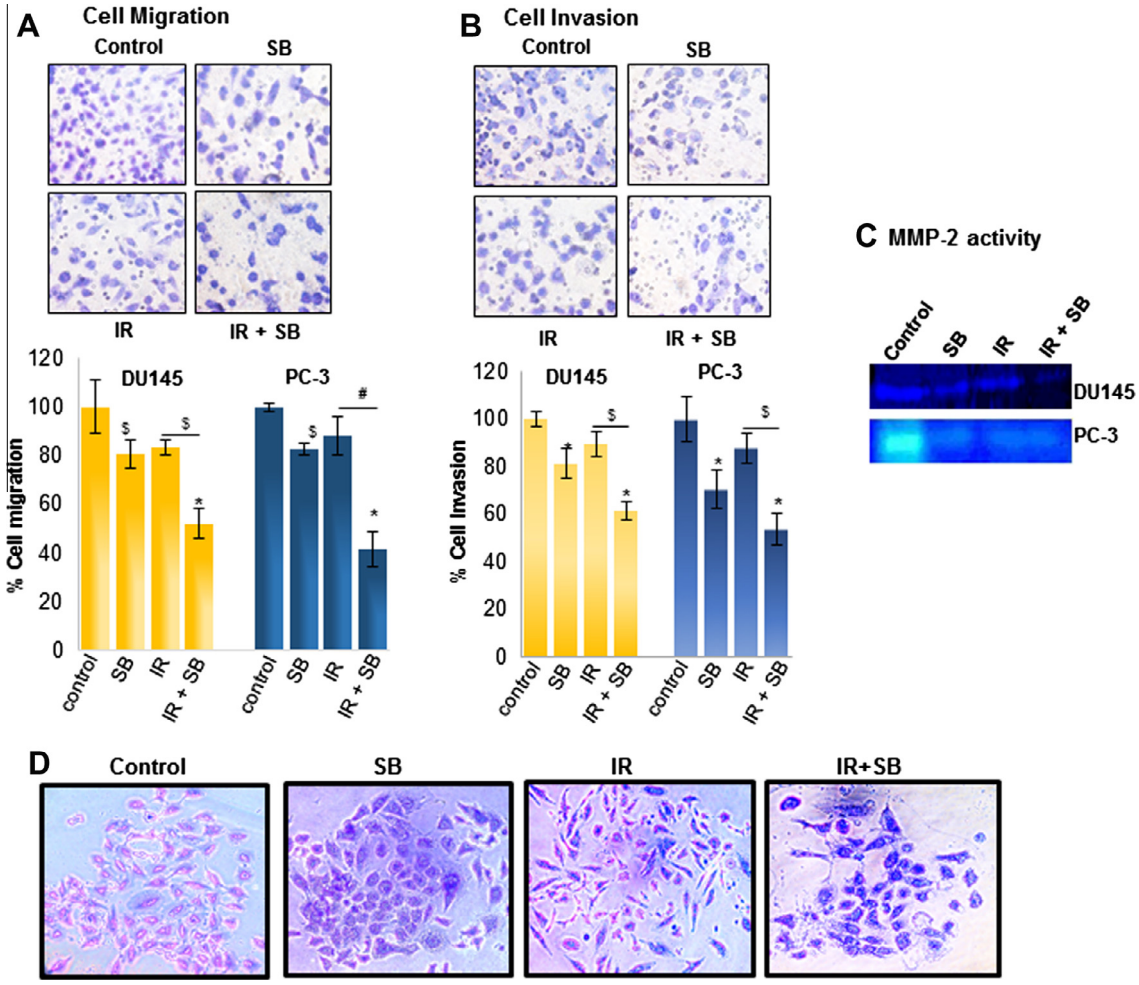

Fig. 3. IR with silibinin leads to enhanced inhibition of prostate cancer cell migration and invasion. (A) Representative images of migrated DU145 cells and quantitation of DU145 and PC-3 migration. (B) Representative images of invaded DU145 cells and quantitation of DU145 and PC-3 invasion after 24 h of treatment, followed for migration and invasion for 20 h on either uncoated/matrigel-coated (invasion) Boyden chambers. Five independent areas were scored in each sample and data are shown as percent cell migration/invasion compared to control. (C) MMP-2 activity in DU145 and PC-3 was measured after 48 h of treatment as described [18]. (D) Representative pictures of crystal violet-stained DU145 colonies formed after 10th day of treatments. The quantitative data shown are mean ± SE of two samples for each treatment. Experiments were repeated with similar results. $, $p < 0.05$; #, $p < 0.01$; ∗, $p < 0.001$.

3.5. Silibinin inhibits IR-induced mitogenic signaling and modulates molecules regulating angiogenesis, migration and invasion in PCa cells

One of the reasons for radio-therapeutic resistance and reduction in the efficacy of IR in cancer therapy is the induction of the pro-survival signaling by cells in order to counter radiation induced stress. Hence, we studied whether silibinin could modulate this effect of IR. We found that IR (5 Gy) induces Akt (Ser 473) and Erk1/2 and STAT-3(Tyr 705) phosphorylation in DU145 cells. Addition of silibinin with IR down-regulated these pathways, especially Akt and Erk1/2 (Fig. 4A). Further, we examined whether silibinin could modulate IR-induced activation of their downstream targets. The acquisition of EMT phenotype upon irradiation and its reversal by silibinin was studied using Western blotting and confocal microscopy. We found a prominent decrease in E-cadherin by IR, which was reversed by silibinin treatment. Concomitantly, silibinin also strongly reversed IR-induced vimentin expression in these cells (Fig. 4B and C). Like vimentin, similar results were observed for N-cadherin protein expression. A strong induction of iNOS and uPA by IR was observed which were down-regulated by silibinin (Fig. 4D). We also found reduced expression of VEGF, eNOS and MMP2 which were otherwise moderately affected by IR (Fig. 4D). The data hence suggest that silibinin mediated potentiation of response of IR to endothelial cell proliferation and cancer cell migration and invasion are mediated through down-regulation of IR activated signaling pathways which contribute to the therapeutic resistance and reduced efficacy of IR.

4. Discussion

Failure of conventional treatment regimens including chemo- and radio-therapy in curbing the rising cancer mortality, has led to approaches which advocate multiple targeting of the disease to avoid development of resistance and recurrence against the single agent used. Radiotherapy has been successfully used in more than 50% of patients with both curative and palliative intent for treatment of solid cancers [11]. However, it suffers from issues of normal tissue toxicity and development of resistance. Combinatorial approaches combining different classes of chemotherapeutic agents along with IR has shown to be effective [9,22–24]. In this study, we analyzed the role of a plant flavonoid silibinin, with anti-cancer efficacy with IR. Use of IR though effective in initially reducing the cell number, is belittled by activation of pro-survival signaling within the cancer cells. The activation of mitogenic pathways lead to angiogenesis and EMT induction [25,26]. IR is also

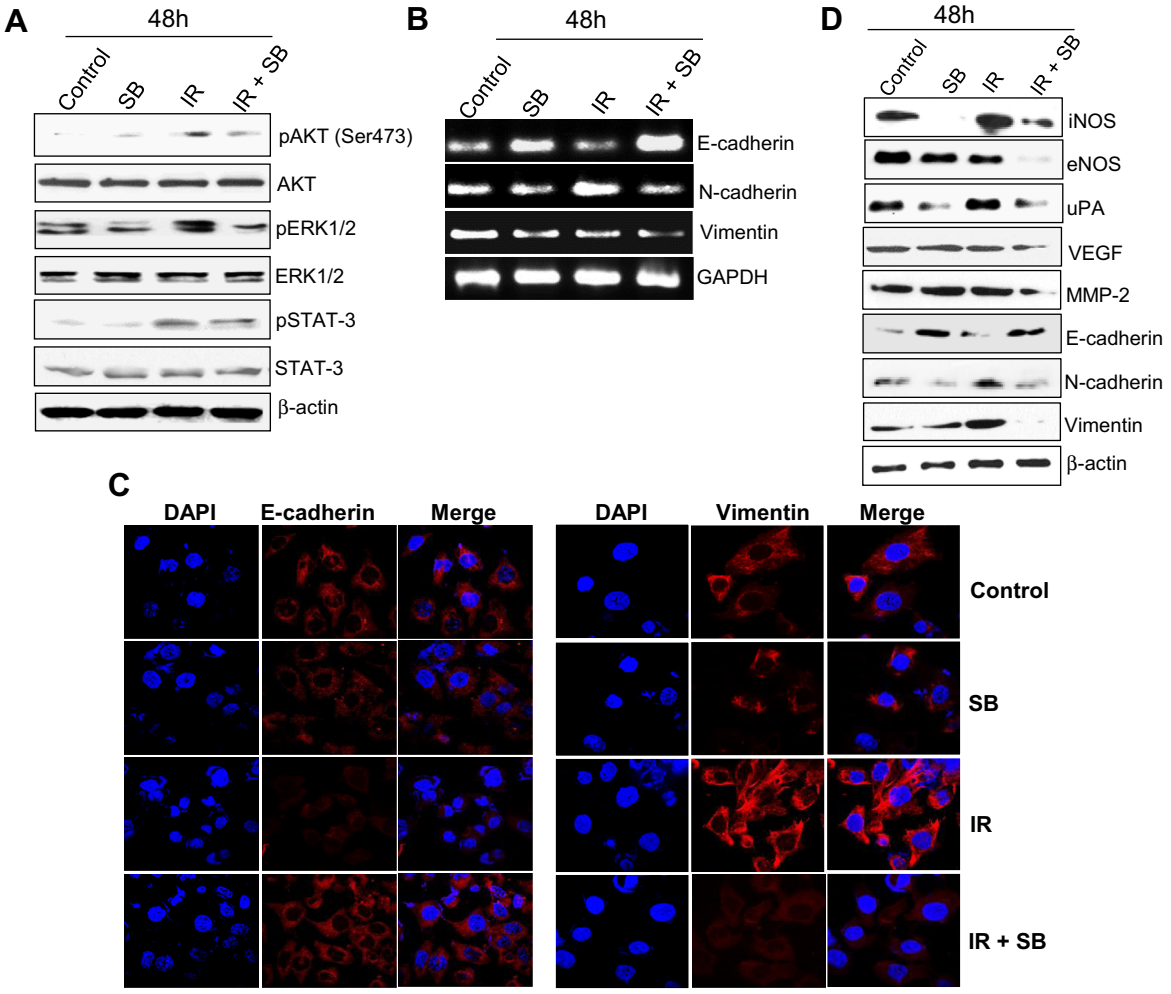

Fig. 4. Silibinin inhibits IR-induced mitogenic signaling and modulates molecules governing angiogenesis, migration and invasion. DU145 cells were treated with the indicated doses of IR and SB for 48 h. (A) At the end of the treatments, lysates were analyzed by immunoblotting using specific primary antibodies for pAKT(Ser473); AKT; pERK1/2; ERK1/2; pSTAT-3(Tyr705); STAT-3. (B) RNA was isolated and RT-PCR was done for expression of EMT markers, E-cadherin, N-cadherin and vimentin. (C) Immunofluorescence analysis of E-cadherin and vimentin (red, Alexa Fluor 594) expression in DU145 cells counterstained with DAPI (blue) after 48 h of respective treatments. (D) Immunoblotting was done using specific antibodies for iNOS, eNOS, uPA, VEGF, MMP-2, E-cadherin and N-cadherin, vimentin followed by detection with HRP-labeled appropriate secondary antibodies as mentioned in Methods. β-Actin was probed after stripping the membrane as protein loading control. Experiments were repeated with similar results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

known to stimulate the production of pro-angiogenic molecules, such as VEGF, NOS and MMPs in diverse cell types [7,27]. These changes equip cells with a more invasive and migratory phenotype.

We found that silibinin could not only radiosensitize endothelial cells by reducing the levels of VEGF, eNOS and MMPs but also showed potent inhibition of PCa cell migration and invasion. Studies have shown that IR induces decrease in E-cadherin with a concomitant increase in vimentin after radiation, suggesting that the irradiated epithelial cells acquire a more mesenchymal-like morphology [26]. We found a similar response in DU145 cells with reduced E-cadherin levels and up-regulation of N-cadherin and vimentin after IR treatment. Silibinin was able to reverse this effect of IR. This is highly significant considering that many studies report that after radiotherapy to primary tumor, the radioresistant cells acquire more mesenchymal nature and hence result in accelerated metastasis [3]. Other than stimulating a pro-angiogenic response in endothelial cells, IR is also known to activate these factors in tumor cells. Importantly, earlier studies have shown that exposure to a single dose or fractionated IR significantly induced the expression of iNOS, which was linked to stemness and resistance to therapies including IR in glioma cells [28]. The uPA–uPAR system has also been implicated in several processes governing tumorigenesis including adhesion, migration and angiogenesis [29]. IR potentiates uPA activity as well as the invasive and angiogenic properties. These effects of IR were also evident in PCa cells. By paracrine mode of actions, these factors further enhance the growth and proliferation of endothelial cells and aid the process of angiogenesis. Hence IR-induced signaling strengthens the positive feedback loop between the tumor cells and endothelial vessels, helping them sustain and negate the inhibitory effects of radiotherapy. Our study showed that silibinin could strongly reduce the IR-induced activation of these molecules in both endothelial cells and PCa cells, thereby acting as a double-edged sword.

In summary, we have demonstrated that IR induces activation of mitogenic signaling which in turn leads to activation of iNOS, VEGF and EMT modulating genes. Silibinin inhibits these factors and thus restrains IR-induced migratory and invasive phenotype in cancer cells. The study also points to the fact that silibinin could also be effective in inhibiting tumor-stromal interactions, which are key to metastatic progression especially in radioresistant tumors and may substantially improve clinical outcome.

Acknowledgments

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References

[1] J.N. Ho, G.Y. Kang, S.S. Lee, et al., Bcl-XL and STAT3 mediate malignant actions of gamma-irradiation in lung cancer cells, Cancer Sci. 101 (2010) 1417–1423.
[2] J.C.H. Cheng, C.H. Chou, M.L. Kuo, et al., Radiation-enhanced hepatocellular carcinoma cell invasion with MMP-9 expression through PI3K/Akt/NF-kappaB signal transduction pathway, Oncogene 25 (2006) 7009–7018.
[3] K. Camphausen, M.A. Moses, W. Beecken, et al., Radiation therapy to a primary tumor accelerates metastatic growth in mice, Cancer Res. 1 (2001) 2207–2211.
[4] M. Martinou, E. Giannopoulou, G. Malatara, et al., Ionizing radiation affects epidermal growth factor receptor signalling and metalloproteinase secretion in glioma cells, Cancer Genomics Proteomics 8 (2011) 33–38.
[5] J.A. Bonner, H.Q. Trummell, C.D. Willey, et al., Inhibition of STAT-3 results in radiosensitization of human squamous cell carcinoma, Radiother. Oncol. 92 (2009) 339–344.
[6] D.R. Maddirela, D. Kesanakurti, M. Gujrati, et al., MMP-2 suppression abrogates irradiation-induced microtubule formation in endothelial cells by inhibiting αvβ3-mediated SDF-1/CXCR4 signaling, Int. J. Oncol. 42 (2013) 1279–1288.
[7] B.J. Moeller, Y. Cao, C.Y. Li, M.W. Dewhirst, Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules, Cancer Cell 5 (2004) 429–441.
[8] C. Timke, H. Zieher, A. Roth, et al., Combination of vascular endothelial growth factor receptor/platelet-derived growth factor receptor inhibition markedly improves radiation tumor therapy, Clin. Cancer Res. 14 (2008) 2210–2219.
[9] C. Clémenson, C. Chargari, E. Deutsch, Combination of vascular disrupting agents and ionizing radiation, Crit. Rev. Oncol. Hematol. 86 (2013) 143–160.
[10] M. Garcia-Barros, F. Paris, C. Cordon-Cardo, et al., Tumor response to radiotherapy regulated by endothelial cell apoptosis, Science 300 (2003) 1155–1159.
[11] A.C. Begg, F.A. Stewart, C. Vens, Strategies to improve radiotherapy with targeted drugs, Nat. Rev. Cancer 11 (2011) 239–253.
[12] R.P. Singh, K. Raina, G. Sharma, et al., Silibinin inhibits established prostate tumor growth, progression, invasion, and metastasis and suppresses tumor angiogenesis and epithelial-mesenchymal transition in transgenic adenocarcinoma of the mouse prostate model mice, Clin. Cancer Res. 14 (2008) 7773–7780.
[13] R.P. Singh, R. Agarwal, A cancer chemopreventive agent silibinin, targets mitogenic and survival signaling in prostate cancer, Mutat. Res. 555 (2004) 21–32.
[14] T.A. Bhat, D. Nambiar, A. Pal, et al., Fisetin inhibits various attributes of angiogenesis in vitro and in vivo–implications for angioprevention, Carcinogenesis 33 (2012) 385–393.
[15] G. Deep, N.H. Oberlies, D.J. Kroll, et al., Identifying the differential effects of silymarin constituents on cell growth and cell cycle regulatory molecules in human prostate cancer cells, Int. J. Cancer 50 (2008) 41–50.
[16] T.A. Bhat, D. Nambiar, D. Tailor, et al., Acacetin inhibits *in vitro* and *in vivo* angiogenesis and downregulates STAT signaling and VEGF expression, Cancer Prev. Res. 6 (2013) 1128–1139.
[17] R.P. Singh, S. Dhanalakshmi, C. Agarwal, et al., Silibinin strongly inhibits growth and survival of human endothelial cells via cell cycle arrest and downregulation of survivin, Akt and NF-kappaB: implications for angioprevention and antiangiogenic therapy, Oncogene 24 (2005) 1188–1202.
[18] D. Tailor, E.R. Hahm, R.K. Kale, et al., Sodium butyrate induces DRP1-mediated mitochondrial fusion and apoptosis in human colorectal cancer cells, Mitochondrion 16 (2014) 55–64.
[19] C.V. Kavitha, C. Agarwal, R. Agarwal, et al., Asiatic acid inhibits pro-angiogenic effects of VEGF and human gliomas in endothelial cell culture models, PLoS One 6 (2011) e22745.
[20] A.C. Pickhard, J. Margraf, A. Knopf, et al., Inhibition of radiation induced migration of human head and neck squamous cell carcinoma cells by blocking of EGF receptor pathways, BMC Cancer 11 (2011) 388–400.
[21] C. Moncharmont, A. Levy, J.-B. Guy, et al., Radiation-enhanced cell migration/invasion process: a review, Crit. Rev. Oncol. Hematol. (2014) (Epub ahead of print).
[22] C. Belka, V. Jendrossek, M. Pruschy, et al., Apoptosis-modulating agents in combination with radiotherapy-current status and outlook, Int. J. Radiat. Oncol. Biol. Phys. 58 (2004) 542–554.
[23] C.E. Leonard, D.C. Chan, T.C. Chou, et al., Paclitaxel enhances in vitro radiosensitivity of squamous carcinoma cell lines of the head and neck, Cancer Res. 56 (1996) 5198–5204.
[24] S. Burdak-rothkamm, C.E. Rübe, T.P. Nguyen, et al., Radiosensitivity of tumor cell lines after pretreatment with the EGFR Tyrosine Kinase Inhibitor ZD (Iressa ®), Strahlenther. Onkol. 2005 (1839) 197–204.
[25] S. Asuthkar, K.K. Velpula, A.K. Nalla, et al., Irradiation-induced angiogenesis is associated with an MMP-9-miR-494-syndecan-1 regulatory loop in medulloblastoma cells, Oncogene 33 (2014) 1922–1933.
[26] H. Tsukamoto, K. Shibata, H. Kajiyama, et al., Irradiation-induced epithelial-mesenchymal transition (EMT) related to invasive potential in endometrial carcinoma cells, Gynecol. Oncol. 107 (2007) 500–504.
[27] E.T. Oh, M.T. Park, M.J. Song, et al., Radiation-induced angiogenic signaling pathway in endothelial cells obtained from normal and cancer tissue of human breast, Oncogene 33 (2014) 1229–1238.
[28] R.-K. Kim, Y. Suh, Y.-H. Cui, et al., Fractionated radiation-induced nitric oxide promotes expansion of glioma stem-like cells, Cancer Sci. 104 (2013) 1172–1177.
[29] K. Dass, A. Ahmad, A.S. Azmi, et al., Evolving role of uPA/uPAR system in human cancers, Cancer Treat. Rev. 34 (2008) 122–136.