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Original Article

Sensitizing endometrial cancer to ionizing radiation by multi-tyrosine kinase inhibition

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ABSTRACT

Objective: Endometrial carcinoma is the most frequent gynecological cancer. About 15% of these cancers are of high risk and radiotherapy still remains the most suitable treatment. In this context, agents able to promote radiosensitization are of great interest. Here, we describe for the first time the radiosensitization ability of sunitinib in endometrial carcinoma.
Methods: Four endometrial carcinoma cell lines were used for the study. The activation of apoptosis signalling pathways and tyrosine kinase receptors were analysed by Western blot, luciferase assays and Immunoprecipitation. Radiosensitization effects were assessed using clonogenic assays. p65 and phosphatase and tensin homolog (PTEN) were upregulated by lentiviral transduction.
Results: We discovered that ionizing radiation activates the pro-oncogenic proteins and signalling pathways KIT, protein kinase B (AKT), and nuclear factor kappa B (NF-κB) and these activations were abrogated by sunitinib, resulting in a radiosensitization effect. We found out that AKT pathway is greatly involved in this process as PTEN restoration in the PTEN-deficient cell line RL95-2 is sufficient to inhibit AKT, rendering these cells more susceptible to ionizing radiation and sunitinib-induced radiosensitization. In Ishikawa 3-H-12 cells, radiosensitization effects and inhibition of AKT were achieved by PTEN restoration plus treatment with the phosphoinositide-3-kinase inhibitor LY294002. This suggests that endometrial tumors could have different sensitivity degree to radiotherapy and susceptibility to sunitinib-induced radiosensitization depending on their AKT activation levels.
Conclusions: Our results provide the rationale of using sunitinib as neoadjuvant treatment prior radiotherapy which could be a starting point for the implementation of sunitinib and radiotherapy in the clinic for the treatment of recalcitrant endometrial cancers.

Keywords: Endometrial Carcinoma; Radiotherapy; Tyrosine Kinase Receptors; Sunitinib

INTRODUCTION

Endometrial cancer is the most common gynecological malignancy and the sixth most occurring cancer in women worldwide [1]. In 2018, endometrial cancer was responsible for almost one third of the deaths related to cancers of the feminine tract [2]. According to current international guidelines, systemic therapy is the standard of care for advanced endometrial

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carcinoma. In addition, radiotherapy either alone or combined with chemotherapy is a frequent treatment choice for these cancers [3,4]. Different strategies have been applied to sensitize these tumors to ionizing radiation and in turn optimize tumor response and decrease patients' side-effects such as employing cisplatin [5] or 5-fluorouracil [6].

Ionizing radiation has been shown to activate cell survival and metastatic processes through the activation of tyrosine kinase receptors (TKRs) such as epidermal growth factor receptor (EGFR) [7,8], epithelial-mesenchymal transition factors such as hypoxia-inducible factor 1 (HIF1) [9] and zinc finger E-box-binding homeobox 1 (ZEB1) [10] and signalling pathways such as phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT) [11], mitogen-activated protein kinase (MAPK) [8] and nuclear factor kappa B (NF-κB) [12], leading to adaptative radioresistance. This has inspired the utilization of new therapeutic approaches able to counteract oncogenic signalling induced by radiotherapy [13]. The PI3K/AKT pathway is particularly attractive for therapeutic intervention in endometrial carcinoma given its frequent hyperactivation, which is due to PTEN loss, mutations in *PI3KCA* and *PI3KR1/2* [14] and, its role in radioresistance. PI3K/AKT inhibitors have been shown to sensitize several cancers to ionizing radiation such as LY294002 in pancreatic cancer cells [15] or the dual PI3K/mammalian target of rapamycin (mTOR) inhibitor NVP-BEZ235 in endometrial carcinoma [16]. Sunitinib is also able to inhibit AKT [17,18] and has demonstrated successful radiosensitization activity in clinical trials for several cancers [19]. However, such radiosensitizing properties have not yet been studied in endometrial carcinoma. Sunitinib (SU011248; Sutent®, Pfizer, New York, NY, USA) is a multi-TKR inhibitor approved by the US FDA in 2006 for the treatment of renal cell carcinoma and gastrointestinal tumors [20]. Sunitinib exhibits its highest inhibitory potency for KIT, platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) receptors [21] and has also been shown to inhibit MAPK [22] and NF-κB [23].

Herein, we explore the possibility of sensitizing endometrial cancer to ionizing radiation by using a multi-TKR inhibition approach. We discovered that radiotherapy triggers apoptotic processes but also activates KIT phosphorylation, NF-κB and AKT signalling pathways in endometrial carcinoma. Interestingly, these activations are abrogated by sunitinib, which results in a decrease in clonogenic survival. Furthermore, PTEN restoration together with the treatment of sunitinib and LY294002, conditions where AKT activity is suppressed, renders endometrial carcinoma cells more sensitive to ionizing radiation and to sunitinib-mediated radiosensitization, suggesting the involvement of AKT pathway in this process. This study unveils the utility of a multi-TKR inhibitor approach to sensitize endometrial carcinoma cells to ionizing radiation which mechanistically depends on AKT. Future studies can be performed to implement this promising combined therapy in the clinic to overcome radioresistance mechanisms occurring in endometrial carcinoma.

MATERIALS AND METHODS

1. Cell lines and drugs

Ishikawa 3-H-12 (IK), HEC-1-A, RL95-2 and KLE cells were purchased from the ATCC (Manassas, VA, US). The four cell lines were cultured in DMEM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FBS (Invitrogen, Barcelona, Spain), 20 mmol/L of L-Glutamine and penicillin/streptomycin. Sunitinib was kindly provided by Pfizer and LY294002 was purchased from Calbiochem (La Jolla, CA, USA). All treatments were carried out in complete media, performed in triplicate and repeated at least 3 times.

2. Ionizing radiations regimens

Cell irradiation (X-rays) was performed using a 6MV Varian 2100 linear accelerator, at a dose rate of 300 cG/min. Medium was changed 24 hours after irradiation.

3. Clonogenic assays

To perform the clonogenic assays, 1×10^3 IK cells, 3×10^3 RL95-2 cells, 3×10^3 HEC-1-A cells and 5×10^3 KLE cells were seeded in 6-wells plates in complete media. The next day, cells were irradiated at the indicated Gy doses or pre-treated with sunitinib for 1 hour or 24 hours at 10 µmol/L or LY294002 for 24 hours at 20 µmol/L and then irradiated. Clonogenic assays were performed in wild type (empty vector; EV) cells and cells lentivirally engineered to overexpress p65 or PTEN. 14 days after the seeding, colonies were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide during one hour at the final concentration of 0.5 mg/mL. Then, colonies were fixed with 10% formalin for 10 minutes, washed thrice with phosphate-buffered saline (PBS) and automatically counted using the Quantity One 1-D Analysis software (Bio-Rad, Hercules, CA, USA).

4. Dose enhancement factor (DEF) determination

The DEF was calculated to determines whether sunitinib acted as a radiosensitizer, radioprotector or whether it was radioneutral. DEF was calculated by the following formula:

$$\text{DEF} = \frac{\text{Dose with radiation alone}}{\text{Dose with radiation+drug}} \text{ for the same biological effect}$$

Our biological effect was 50% of clonogenic survival inhibition or IC50. IC50 (in doses of Gy) were determined by using the software GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA) from the clonogenic survival curves of the four endometrial carcinoma cell lines treated with the doses 0, 1, 1.5, 2, and 4 Gy of ionizing radiation in the absence or presence of sunitinib pretreatment at 10 µM for 24 hours.

5. Western blot and immunoprecipitation

Western blot and immunoprecipitation protocols have been described before [17]. Antibodies anti-cleaved caspase-3, γ-H2AX, platelet-derived growth factor receptor (PDGFR) α, PDGFRβ, vascular endothelial growth factor receptor (VEGFR) 2, phospho (P)-AKT ser 473, P-p65 ser 536, P-IκBα ser 32, P-p42/44 MAPK Thr202/Tyr204 and PTEN were purchased from Cell Signaling (Beverly, MA, USA). The antibodies anti-KIT and anti-tubulin were from Dako (Glostrub, Denmark) and Sigma-Aldrich (St Louis, MO, USA) respectively.

6. Immunofluorescence

IK cells were seeded in complete media on top of 13 mm^2 glass coverslips inserted into 24-well plates. Cells were irradiated at 2 Gy, treated with sunitinib at 10 µM or treated with the combination of ionizing radiation and sunitinib 10 µM. 24 hours after the treatments, cells were fixed with 4% paraformaldehyde for 15 minutes and then washed thrice with PBS. Cells were permeabilized with 0.1% triton X-100 for 20 minutes, washed and non-specific antibody binding was blocked for 1 hour with 1% bovine serum albumin (BSA) diluted in PBS. After that, primary antibody anti-γ-H2AX was added overnight in the wells diluted in 1% BSA in PBS in a 1:100 dilution. Later, cells were washed thrice with PBS and a secondary goat anti-mouse conjugated with Alexa Fluor 488 (Jackson ImmunoResearch, West Grove, PA, USA) was added for one hour at room temperature (dilution 1:500). After that, bis-benzimide fluorescent dye (Hoechst 33258) was added at the final concentration of 5 µg/mL to stain the nuclei and cells were washed thrice. Finally, coverslips were mounted on slides and visualized

using the Nikon Ti-E inverted confocal microscope. Images from different fields were taken and at least 100 cells were counted for each condition.

7. NF-κB transcriptional activity assays

Endometrial carcinoma cell lines were plated in 24-wells plates and transfected using Lipofectamine 2000 following the manufacturer's instructions, with the NF-κB-LUC plasmid construct together with a plasmid encoding the β-galactosidase gene used for normalization as previously reported [23]. The luciferase construct containing five NF-κB sites (NF-κB-LUC) (Stratagene, AF 053315; La Jolla, CA, USA) was a gift from Dr. Giles Hardingham. 24 hours after the finalization of treatments and/or irradiations, cells were lysed with 60 µL of lysis buffer (25 mmol/L glycylglycine, pH 7.8, 15 mmol/L Mg2SO4, 1% Triton X-100, 5 mmol/L EGTA) and rocketed on ice for 15 min. 25 µL of lysates were transferred to 96-wells plates and 25 µL of luciferase assay buffer was added to a final concentration of 25 mmol/L glycylglycine, 15 mmol/L KHPO4, pH 7.8, 15 mmol/L Mg2SO4, 1% Triton X-100, 5 mmol/L EGTA, 1 mmol/L dithiothreitol containing 2 mmol/L ATPm 100 mmol/L acetyl-coenzyme A, and 100 mmol/L luciferine). Luciferase was measured using a microplate luminometer. Subsequently, 60 µL of β-galactosidase buffer (200 mmol/L NaPO4, 20 mmol/L KCl, 2 mmol/L Mg2SO4, 4 mg/mL o-Nitrophenyl β-D-galactopyranoside) were added to each well and β-galactosidase activity was measured on a microplate reader at 415 nm. Treatment of tumor necrosis factor, a potent NF-κB inductor, was used as a control of the assay.

8. Gene upregulation experiments

p65 and PTEN were upregulated in the PTEN-deficient cell lines IK and RL95-2 by lentiviral transduction. Lentiviruses carrying PTEN cDNA were produced in HEK293T cells by transfection using polyethylenimine of VSV-G, D8.9, and p65 or PTEN cDNA cloned into a pDSL vector. Three days post-transfection, infective lentiviral particles were collected by centrifugation for one hour at 4,000 rpm using a filter column of 100kDa (VWR International LLC, West Chester, PE, USA). Host endometrial cancer cells were incubated overnight in medium containing lentiviral particles. Then, cell media was freshly replaced, and cells were further incubated for 72 hours to allow p65 or PTEN cDNA expression.

9. Statistical analysis

Student's t-test was performed between the indicated groups.

RESULTS

1. Ionizing radiation induces γ-H2AX and apoptosis in endometrial carcinoma cells

The phosphorylated form of the variant histone H2AX (γ-H2AX) is a biomarker of DNA damage being formed at the site of DNA double-strand break, which is the most immediate consequence of ionizing radiation. Also, it is widely known that ionizing radiation causes apoptosis in cancer [24]. Here, we wanted to validate the functionality of ionizing radiation in endometrial cancer by performing Western blot in RL95-2 cells to detect γ-H2AX and cleaved caspase-3, a well-known executor of the apoptotic cascade, at 0, 3 and 24 hours after radiation at the doses of 2, 4, and 6 Gy. As seen in **Fig. 1**, γ-H2AX presented a characteristic dynamic pattern of phosphorylation with a phosphorylation peak at 3 hours post-irradiation. Alternatively, we performed an immunofluorescence for the detection of γ-H2AX in IK 24 hours after the ionizing radiation treatment at 2 Gy, sunitinib at 10 µM and the combination

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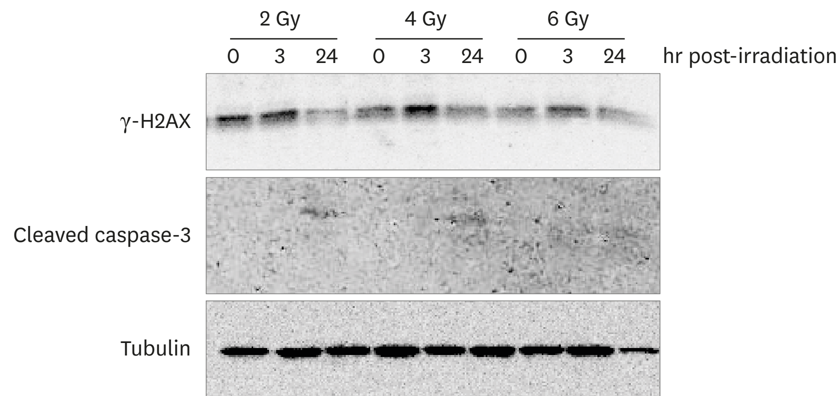

Fig. 1. Effects of ionizing radiation effects in endometrial carcinoma.
Western blot of RL95-2 cell lysates extracted at 0, 3 and 24 hours after irradiation at the doses of 2, 4, and 6 Gy. Tubulin was used as loading control.

of radiation and sunitinib (**Supplementary Fig. 1**). Interestingly, we observed that ionizing radiation induced an average of 30.5 γ-H2AX foci per cell ($p<0.001$) and sunitinib originated 13.8 γ-H2AX foci per cell ($p<0.001$). Interestingly, cells treated with the combination of radiotherapy and sunitinib presented an average of 63.6 γ-H2AX foci per cell ($p<0.001$). This result suggests that the treatment with sunitinib resulted in a high persistence of radiation-induced DNA double-strand breaks. Additionally, we observed that RL95-2 cells underwent apoptosis 24 hours after the radiotherapeutic insult (**Fig. 1**). The quantification of cleaved caspase-3 in the Western blot can be found in **Supplementary Fig. 2**.

2. Sunitinib sensitizes endometrial carcinoma cell lines to ionizing radiation

Next, we wanted to investigate the ability of sunitinib in sensitizing endometrial cancer to radiotherapy. For that, IK, HEC-1-A, KLE, and RL95-2 cells were treated with sunitinib at 10 μM 24 hours before irradiation. The administration of sunitinib was carried out before radiotherapy for its superior activity compared to the concurrent treatment [19]. Cells were irradiated at 1, 1.5, and 2 Gy and clonogenic assays were performed, which are reference cell-based assays for the assessment of growth inhibition induced by radiotherapy [25]. Our clonogenic assays show that pre-treatment of sunitinib sensitized IK, RL95-2, HEC-1-A, and KLE cells to radiotherapy (**Fig. 2**). In particular, sunitinib significantly sensitized IK at low doses of ionizing radiation ($p=0.022$) whereas HEC-1-A, RL95-2, and KLE were sensitized at higher doses ($p=0.011$, $p=0.018$, and $p=0.029$, respectively). Additionally, cell lines were treated with 4 Gy of ionizing radiation (**Supplementary Fig. 3**). This dose of ionizing radiation greatly compromised cell clonogenicity and sunitinib also showed radiosensitizing ability at this dose of 4 Gy. The different dynamics of sensitization to ionizing radiation is in accordance with the sensitivity of the endometrial carcinoma cell lines to sunitinib observed in a previous work [23]. To further demonstrate the radiosensitization ability of sunitinib, we determined the DEF in all 4 endometrial carcinoma cell lines (**Supplementary Fig. 4**). The DEF for IK cells was 1.389, for RL95-2 was 3.177, for HEC-1-A was >4 and for KLE was 1.061. These results demonstrate that sunitinib acts as a radiosensitizer (DEF >1) in the endometrial carcinoma cell lines tested.

3. Ionizing radiation activates KIT, NF-κB, and AKT in endometrial carcinoma and these activations are inhibited by TKR inhibition

Ionizing radiation activates multitude of transcription factors and signalling pathways that promote cancer cell survival [26]. However, little is known about the radiotherapy-induced

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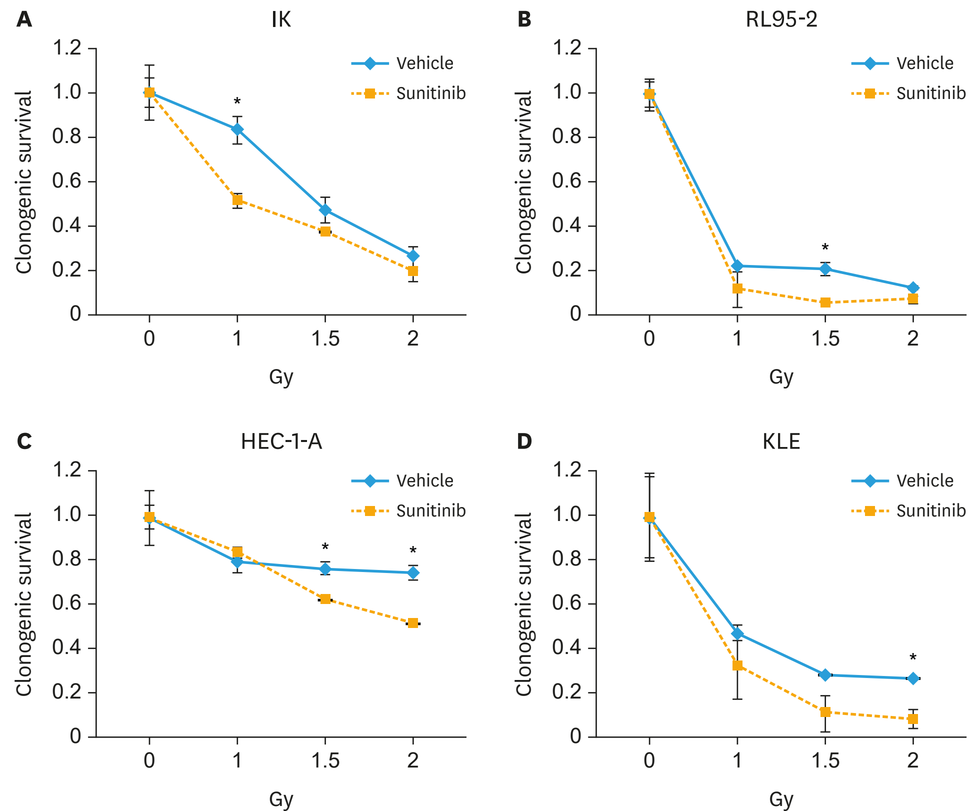

Fig. 2. Sunitinib sensitized endometrial carcinoma cells to ionizing radiation.
Representative images of clonogenic assays and corresponding clonogenic survival graphs of (A) IK, (B) RL95-2, (C) HEC-1-A, and (D) KLE cells exposed to 10 μM sunitinib for 24 hours followed by irradiation at 0, 1, 1.5 and 2 Gy.
IK, Ishikawa 3-H-12.
*Means $p<0.05$.

oncogenic pathways in endometrial carcinoma. Here, we wanted to investigate whether ionizing radiation activates TKR and pro-tumorigenic signalling pathways. As IK cells expressed high levels of KIT, we first performed immunoprecipitation of KIT in IK cell lysates irradiated at 1, 3, 6, and 10 Gy for 30 minutes followed by Western blot for the detection of tyrosine phosphorylation (**Fig. 3A**). Intriguingly, ionizing radiation activated tyrosine phosphorylation in KIT in absence of the natural KIT ligand, the stem cell factor (SCF). This KIT activation was particularly evident at 6 and 10 Gy (**Fig. 3A**). Alternatively, cells were pre-treated with sunitinib at 10 μM for 1 hour to investigate whether this TKR inhibitor was able to inhibit KIT phosphorylation. In this condition, sunitinib totally abrogated ionizing radiation-induced KIT phosphorylation (**Fig. 3A**). It is worth mentioning that sunitinib treatment alone at 10 μM for one hour did not change neither the phosphorylation status of KIT nor the expression levels of KIT.

Next, we wanted to investigate the effect of radiotherapy and sunitinib on the activation of survival signalling pathways in endometrial carcinoma cells. To address this point, we performed Western blot for the detection of AKT activation (P-AKT ser 473), NF-κB (P-p65 ser 536 and P-IκBα ser 32) and MAPK (P-p44/42 MAPK Thr202/Tyr204) in IK cells at short timepoints (15 and 30 minutes) and at the long timepoint of 24 hours after cell irradiation. Prior radiotherapy, cells were pre-treated with vehicle or sunitinib at 10 μM for 1 hour. As shown in **Fig. 3B**, shortly after the administration of ionizing radiation, we detected

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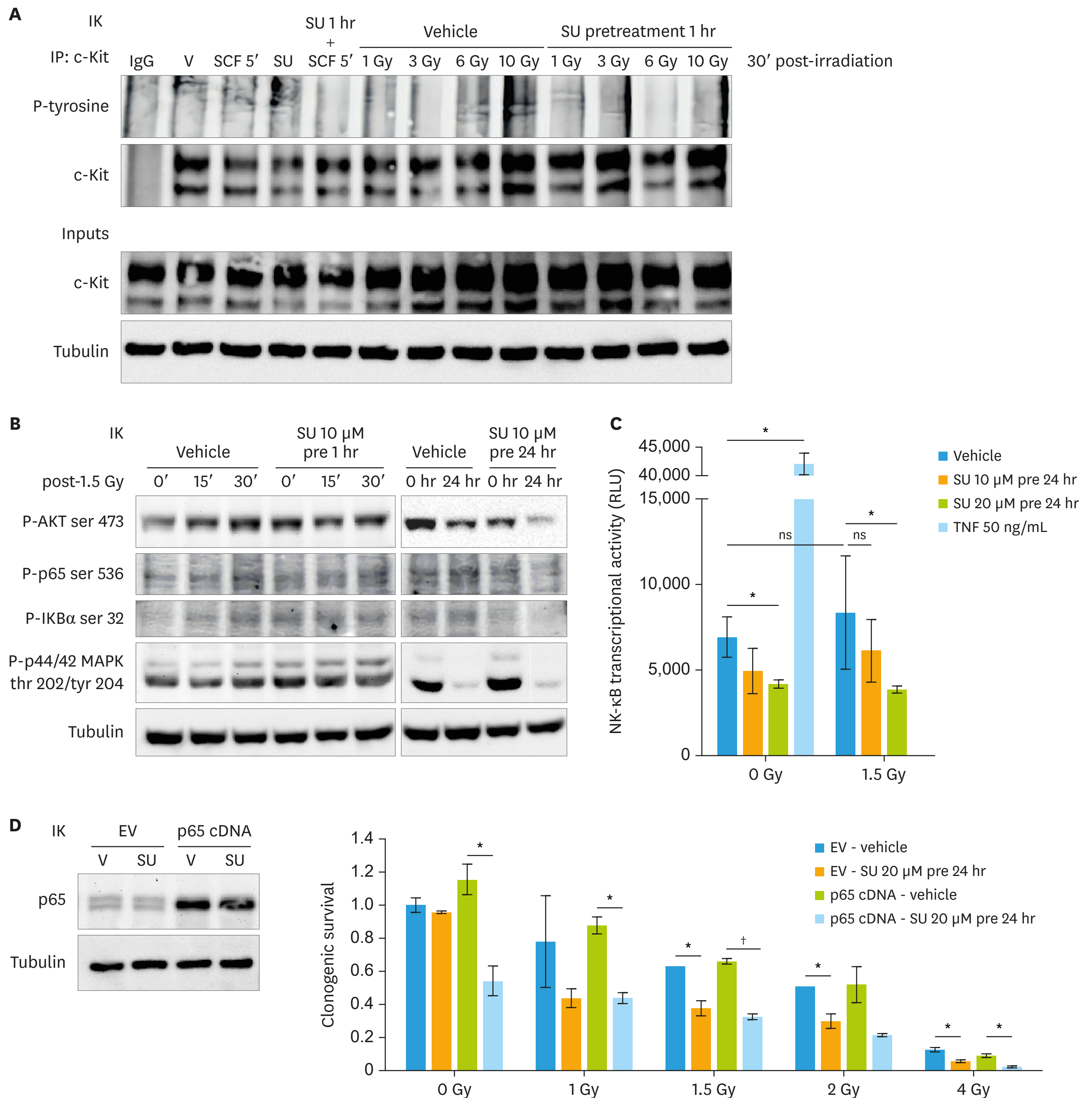

Fig. 3. Ionizing radiation activated KIT, AKT, and NF-κB in endometrial carcinoma and they were inhibited by sunitinib. (A) Immunoprecipitation of KIT in IK cells. Rabbit IgG was used as immunoprecipitation control (B) Western blot of IK cells. (C) NF-κB-luciferase assay graph in IK cells. RLU values were normalized to β-galactosidase activity. (D) Western blot (left) and clonogenic survival graphs of IK cells (right). Tubulin was used as loading control of the Western blots.
AKT, protein kinase B; EV, empty vector; IgG, immunoglobulin G; IK, Ishikawa 3-H-12; NF-κB, nuclear factor kappa B; ns, not significant; RLU, relative light units; SU, sunitinib; TNF, tumor necrosis factor; V, vehicle.
*Means $p<0.05$, †means $p<0.005$.

P-AKT ser 473, P-p65 ser 536 and P-IκBα ser 32. In contrast, the phosphorylation status of p44/42 MAPK Thr202/Tyr204 remained unaffected after 15 or 30 minutes of irradiation. Interestingly, AKT and NF-κB activation by ionizing radiation at short timepoints was

prevented by 1 hour of sunitinib pre-treatment. At 24 hours, ionizing radiation activated NF-κB at different levels, at p65 and IκBα, and these phosphorylated forms disappeared with 24 hours of sunitinib pre-treatment. Ionizing radiation inhibited AKT and MAPK 24 hours after the radiotherapeutic insult. In these conditions, sunitinib further inhibited AKT and also the activation of p65 and IκBα induced by radiotherapy. In contrast, sunitinib pre-treatment did not alter the phosphorylated levels of MAPK. The quantification of the expression levels P-p65 ser 536 and P-IκBα ser 32 in the Western blots can be found in **Supplementary Fig. 2**.

We also performed luciferase assays using a NF-κB gene construct responsive to p65 [23] to determine whether ionizing radiation and sunitinib could affect p65-dependent transcription. Radiotherapy increased NF-κB transcriptional activity in a 17% and sunitinib at 10 and 20 μM almost reversed this activation (**Fig. 3C**). Next, we pursued rescue experiments by overexpressing p65 cDNA to determine up to which extend NF-κB is involved in the radiosensitization effect exerted by sunitinib (**Fig. 3D**). For that, we overexpressed p65 cDNA in IK cells and studied their clonogenic ability in the absence or presence of radiation and sunitinib. As expected, p65 overexpression slightly enhanced clonogenic survival after irradiation and sunitinib sensitized IK cells to radiotherapy. In the presence of sunitinib and ionizing radiation, p65-overexpressing cells remained more resistant to radiation compared to non-irradiated cells. However, this resistance phenomenon did not result in a total rescue of clonogenic survival.

4. Total abrogation of AKT is needed to promote sunitinib-induced radiosensitization in endometrial carcinoma

Given the observed involvement of AKT in the radiosensitization effect of sunitinib, we wanted to investigate the extend of this AKT dependency. To do so, we intended to inhibit the AKT signalling pathway in IK and RL95-2 cells through 2 different approaches: By restoring physiological PTEN expression and by using the PI3K inhibitor LY294002. IK and RL95-2 cells were lentivirally transduced with PTEN cDNA or the EV and then treated with sunitinib at 10 μM for 24 hours. As seen in **Fig. 4A**, PTEN overexpression was able to suppress AKT phosphorylation in RL95-2 cells but not in IK cells. Also, sunitinib on its own was able to inhibit P-AKT in both PTEN-overexpressing IK and RL95-2 cells. When performing clonogenic assays (**Fig. 4B**), we observed that PTEN upregulation affected clonogenic survival after irradiation at 3 Gy, decreasing the clonogenic ability in RL95-2 cells, but not in IK cells. Moreover, sunitinib significantly sensitized RL95-2^{PTEN+} cells to irradiation by almost reaching 0% of survival whereas this did not happen in IK^{PTEN+} cells. This finding correlates with the different level of inhibition of AKT observed. AKT phoshorylation was more inhibited in RL95-2 cells than in IK cells (**Fig. 4A**). This result indicates that AKT pathway plays a crucial role in sunitinib-induced radiosensitization in endometrial carcinoma. To further prove this point, we pharmacologically treated IK cells with LY294002 in order to totally abrogate AKT pathway and then performed clonogenic assays. IK^{EV} and IK^{PTEN+} were pre-treated with vehicle or LY294002 at 20 μM for 24 hours in the presence or absence of 1.5 Gy. As shown in **Fig. 4C**, LY294002 was able to totally suppress the activation of AKT pathway in IK^{PTEN+} cells at both 0 and 1.5 Gy. Importantly, this reduction in AKT activation resulted in a significant decrease in clonogenic survival after irradiation (p=0.004; **Fig. 4D**). This result suggests that when AKT pathway is totally inhibited by sunitinib, sunitinib treatment results in a radiosensitization effect in endometrial carcinoma. This discovery, however, do not exclude the possibility of engagement of other signalling pathways that could participate in the radiosensitization effect exerted by sunitinib. In **Fig. 5**, we showed the mechanism by which Sunitinib induces radiosensitization in endometrial carcinoma cells.

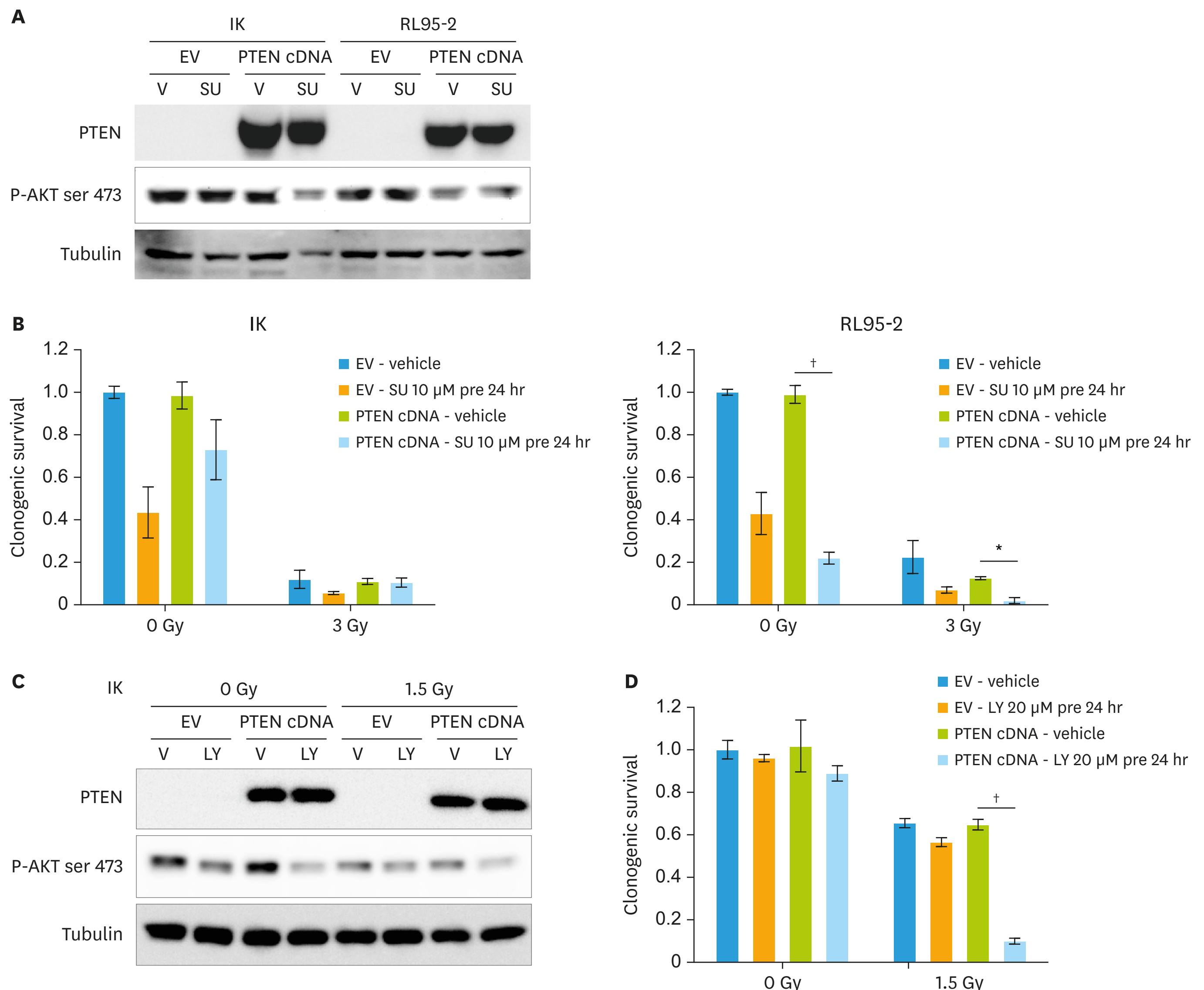

Fig. 4. PTEN upregulation and treatment with LY294002 enhance the radiosensitization effect of sunitinib. (A) Western blot in IK cells. (B) Graph showing clonogenic survival of EV and PTEN-overexpressing IK and RL95-2 cells. (C) Western blot of EV and PTEN-overexpressing IK cells. (D) Clonogenic survival graph of EV and PTEN-overexpressing IK cells. Tubulin was used as loading control in the Western blots.
EV, empty vector; IK, Ishikawa 3-H-12; LY, LY294002; PTEN, phosphatase and tensin homolog; SU, sunitinib; V, vehicle.
*Means $p<0.05$ and †means $p<0.005$.

DISCUSSION

Ionizing radiation has been for decades the most prescribed treatment after surgery to prevent tumor relapse in high-risk endometrial carcinomas. Recent findings pointed out an improved 5-year free survival with the use of chemoradiotherapy plus radiotherapy compared to radiotherapy alone in these cancers [4], suggesting that current trends go towards chemoradiation treatments. In this context, compounds that could sensitize to radiotherapy and thus maximize tumor response and reduce patient´s secondary effects are of high interest.

The PI3K/AKT/mTOR pathway is downstream of TKR and promotes resistance to ionizing radiation apart from cancer cell growth and proliferation [11,15]. This signalling pathway

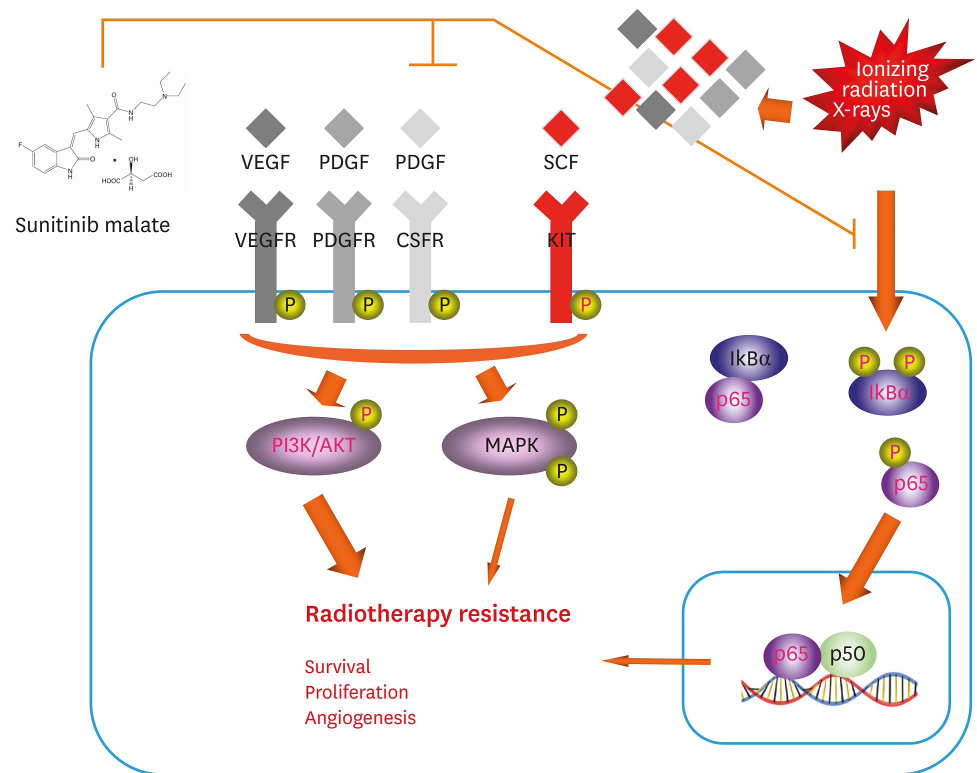

Fig. 5. Schematic representation of the radiosensitization mechanism of sunitinib in endometrial carcinoma. AKT, protein kinase B; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PI3K, phosphoinositide-3-kinase; SCF, stem cell factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

plays an important role on the pathogenesis of the disease [27] and is frequently activated in endometrial carcinoma, mostly due to loss-of-function *PTEN* mutations which account for up to 70%–80% of endometrioid endometrial tumors and 90% of high grade tumors [28], together with *PI3K* mutations [14]. Therefore, targeting PI3K/AKT seems to be an excellent candidate to promote radiosensitization.

In this work, we have explored the radiosensitization ability of sunitinib in endometrial carcinoma. Sunitinib showed some discrete response in patients with metastatic and recurrent endometrial carcinoma in a phase II clinical trial [29]. Another phase II clinical trial is currently ongoing (NCT00478426) for sunitinib in patients with metastatic and recurrent endometrial carcinoma with results yet to report. However, its radiosensitization power in endometrial carcinoma has not been described in the bibliography as yet. Sunitinib is a multi-TKR inhibitor able to inhibit ligand-dependent TKR phosphorylation and consequently the PI3K/AKT signalling pathway [17]. Our results demonstrate that sunitinib sensitized the endometrial carcinoma cell lines IK, HEC-1-A, RL95-2, and KLE to ionizing radiation. This is particularly interesting given the radioresistance characteristics of IK and HEC-1-A cells observed here and by others [30]. Similarly to our study, sunitinib has shown to radiosensitize head and neck cancer cells [31], oesophageal carcinoma cells [32], prostate cancer cells [33], and in vivo Lewis lung carcinoma or glioblastoma tumors [34]. Upon radiation treatment, we observed that the maximum activation of γ-H2AX occurs at three hours post-irradiation and then declines at 24 hours. At this timepoint, γ-H2AX phosphorylation is lower than at 0 hours post-irradiation. We would like to indicate that the time “0 hours” refers to the time

when the cell lysates were collected after the radiation insult. Technically, this time is not possible to be 0 but represents a very few minutes delay. According to the kinetic studies of Mariotti et al., the authors described a very quick activation of H2AX that starts a few minutes after irradiation, reaching a maximum count of γ-H2AX foci after 30 minutes of irradiation. After this peak, the number of γ-H2AX foci decreases following an exponential trend while the cell machinery is repairing the DNA breaks. At 24 hours post radiation, γ-H2AX is almost nil. The results of γ-H2AX expression in our western blot is consistent with the observations of the radiation kinetics studies in the literature. Regarding TKR, sunitinib has been reported to inhibit KIT with an IC_{50} of up to 0.001–0.01 μM and, PDGFR and VEGFR with an IC_{50} of up to 0.01 μM in NCI-H526 and NIH-3T3 cells expressing these receptors respectively [21]. The expression levels of the main targets of sunitinib in endometrial carcinoma have been previously published [23]. KIT was found to be strongly expressed in IK cells and VEGFR2 was present in both IK and KLE cells. As for PDGFRα and PDGFRβ, there was no detectable expression of these receptors (**Supplementary Fig. 5**) by Western blot. This expression analysis suggests that KIT is the main target of sunitinib in endometrial carcinoma.

In an attempt to decipher the mechanism of sensitization of sunitinib to ionizing radiation, we first analysed the effects of ionizing radiation in the activation of KIT and associated pro-tumorigenic signalling pathways (AKT, NF-κB, and MAPK) and then, we investigated how this activation was affected with the presence of sunitinib. Interestingly, we found that ionizing radiation activated KIT, AKT, and NF-κB and that the administration of sunitinib prior radiation inhibited their activation, suggesting the involvement of AKT and NF-κB in sunitinib-induced radiosensitization. In agreement to this, it has also been shown that ultraviolet B enhances KIT expression in epidermal melanocytes [35]. Also, radiotherapy activates AKT [11] and NF-κB [36]. Moreover, sunitinib has been shown to inhibit both basal and proteasome inhibitor-induced NF-κB activation in endometrial carcinoma [23] and to abrogate AKT in melanoma [17]. Futhermore, Gorski et al. [37] demonstrated that ionizing radiation increased the production of the TKR ligand VEGF and that treatment with an anti-VEGF-165 antibody resulted in radiosensitization in vivo in different tumor models. Additionally, Abdollahi et al. [38] proved that radiotherapy upregulated VEGF and FGF in prostate cancer cells and VEGFR2 in endothelial cells when co-cultured and that inhibition of this paracrine loop by the TKR inhibitors SU5416 and SU6668 enhanced the radiotherapeutic effect. The same TKR inhibitors improved the anti-tumoral effects of radiotherapy in in vivo models of prostate cancer and glioblastoma [39]. The maximum doses of ionizing radiation used in our clonogenic assays and signaling pathway analyses are 4 and 3 Gy respectively. This is in agreement with previous published works deploying low doses of ionizing radiation in endometrial carcinoma cell lines [16]. These doses of ionizing radiation are opposed to the high doses of radiation used clinically for postoperative endometrial carcinoma patient (45–50 Gy). The difference in the radiation usage between the in vitro and in vivo setting is expected as targeting and eliminating the cancer cells in the patient's tumor needs higher doses of radiation than the one needed to kill cells cultured in monolayer due to the existence of natural body barriers and the higher degree of complexity and heterogeneity of the tumors.

According to our results, p65 overexpression does not completely rescue the decrease in clonogenicity induced by the combination of radiotherapy and sunitinib. In contrast, PTEN restoration in RL95-2^{PTEN-} cells clearly potentiates the radiosensitization effect of sunitinib. Moreover, the addition of LY294002 together with PTEN overexpression strongly improves the radiosensitization ability of sunitinib in IK^{PTEN-} cells. In these conditions, AKT is totally inhibited. Interestingly, we observed an important decrease in cell clonogenicity in non-

irradiated IK cells lentivirally transduced with the empty vector and pre-treated with sunitinib for 24 hours. We believe that the infection with lentiviruses made the cells more vulnerable to the effects of sunitinib. The insertional mutagenesis at the site of the lentiviral vector integration as a consequence of the lentiviral infection could have disturbed the expression of genes responsible of the regulation of sunitinib sensitivity. Changes in sensitivity to rituximab were also observed by Ranjbar et al. in B cells transduced with lentiviruses. Clearly, due to the complexity of gene regulation and the stochastic integration of the lentiviral vectors within the genome, many hypotheses may be valid to explain the enhanced sunitinib sensitivity.

Altogether these results suggest that AKT pathway is more involved than NF-κB in the radiosensitization properties of sunitinib in endometrial carcinoma. Similarly, the involvement of the AKT pathway in radiosensitization events has been demonstrated in prostate cancer for LY294002 [15] and in endometrial cancer for the PI3K/mTOR inhibitor NVP-BEZ235, which went through the suppression of VEGF [16]. Finally, the different response to radiotherapy observed in IK and RL95-2 could be due to the higher P-AKT ser 473/total AKT ratio observed in IK compared to RL95-2 cells [40], which makes the phosphorylation inhibition of AKT more difficult for sunitinib.

In this work, we demonstrate the radiosensitization properties for sunitinib in endometrial carcinoma. We discovered that this radiosensitization effect is AKT-dependent. This finding has important implications for therapy as the treatment success will depend on the level of AKT activation in each tumor. To sum up, our results provide valuable information for the use of sunitinib as a radiosensitization agent in endometrial carcinoma, which could inspire future pre-clinical and clinical trials. We present a study in vitro with the inherent limitations of this kind of work such as the pending the validation of the results in the in vivo setting to draw conclusions that can be used clinically. Hence, future in vivo studies utilizing progression models of endometrial carcinoma such as the transgenic mouse PTEN$^{+/-}$, which spontaneously develops endometrial cancer, can be performed. Such studies can be based on the useful information that we provide in the present work.

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SUPPLEMENTARY MATERIALS

Supplementary Fig. 1

γ-H2AX expression induced by the treatments with sunitinib, ionizing radiation and the combination of ionizing radiation with sunitinib. (A) Representative images of confocal microscopy images of an immunofluorescence for the detection of γ-H2AX in IK cells after 24 hours of treatment with 2 Gy of ionizing radiation, sunitinib 10 µM and the combination of ionizing radiation with Sunitinib. Cell nuclei are stained in blue (Hoechst) and γ-H2AX is stained in green. (B) Bar graph showing the average number of the double strand breaks originated from the treatments.

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Supplementary Fig. 2

Western blot quantification. Tables showing the band quantification of the Western blots corresponding to cleaved-caspase 3 (**Fig. 1**), P-p65 ser 536 and P-IKBa ser 32 (**Fig. 3B**). The table includes the lane number (from left to right), the condition of the Western blot, the total lane volume and the relative lane volume which is relative to the respective control condition.

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Supplementary Fig. 3

Clonogenic survival at higher doses of ionizing radiation in endometrial carcinoma cell lines. IK, RL95-2, HEC-1-A, and KLE cells were treated with a dose of 4 Gy of radiation alone (vehicle) or with sunitinib pretreatment at 10 μM for 24 hours.

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Supplementary Fig. 4

Half maximal inhibitory dose of radiation and DEF in all 4 carcinoma cell lines after irradiation. (A) Table indicating the IC50 (in Gy) in IK, RL95-2, HEC-1-A and KLE cells treated with ionizing radiation (0, 1. 1.5, 2, and 4 Gy) alone (vehicle) or in the presence of sunitinib pretreatment at 10 μM for 24 hours. (B) values of DEF in IK, RL95-2, HEC-1-A, and KLE cells corresponding to the biological effect of 50% inhibition of clonogenic survival.

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Supplementary Fig. 5

Expression levels of sunitinib targets in endometrial carcinoma cell lines. Western blot of whole cell lysates of IK, HEC-1-A, RL95-2, and KLE cells for the detection of KIT, PDGFRα, PDGFRβ, and VEGFR2. The melanoma cell line M16 was used as positive control for KIT, PEGFRα and PDGFRβ, and the melanoma cell line M28 was used as positive control for VEGFR2. Tubulin was used as loading control.

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