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Perspective

Histamine prevents radiation-induced mesenchymal changes in breast cancer cells

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ABSTRACT

Radiotherapy is a prime option for treatment of solid tumors including breast cancer though side effects are usually present. Experimental evidence shows an increase in invasiveness of several neoplastic cell types through conventional tumor irradiation. The induction of epithelial to mesenchymal transition is proposed as an underlying cause of metastasis triggered by gamma irradiation. Experiments were conducted to investigate the role of histamine on the ionizing radiation-induced epithelial to mesenchymal transition events in breast cancer cells with different invasive phenotype. We also evaluated the potential involvement of Src phosphorylation in the migratory capability of irradiated cells upon histamine treatment.

MCF-7 and MDA-MB-231 mammary tumor cells were exposed to a single dose of 2 Gy of gamma radiation and five days after irradiation mesenchymal-like phenotypic changes were observed by optical microscope. The expression and subcellular localization of E-cadherin, β-catenin, vimentin and Slug were determined by immunoblot and indirect immunofluorescence. There was a decrease in the epithelial marker E-cadherin expression and an increase in the mesenchymal marker vimentin after irradiation. E-cadherin and β-catenin were mainly localized in cytoplasm. Slug positive nuclei, matrix metalloproteinase-2 activity and cell migration and invasion were significantly increased. In addition, a significant enhancement in Src phosphorylation/activation could be determined by immunoblot in irradiated cells. MCF-7 and MDA-MB-231 cells also received 1 or 20 μM histamine during 24 h previous to be irradiated. Notably, pre-treatment of breast cancer cells with 20 μM histamine prevented the mesenchymal changes induced by ionizing radiation and also reduced the migratory behavior of irradiated cells decreasing phospho-Src levels.

Collectively, our results suggest that histamine may block events related to epithelial to mesenchymal transition in irradiated mammary cancer cells and open a perspective for the potential use of histamine to improve radiotherapy efficacy.

1. Introduction

Breast cancer is a heterogeneous disease highly predominant among women worldwide. Histological and molecular classification of breast cancer describes four well-defined different phenotypes. The two major molecular classes of breast cancers are: luminal and basal-type tumors [1]. The luminal phenotype generally includes tumors that are estrogen receptor-positive (ER) and comprises about 70% of human invasive mammary tumors. The basal like carcinomas make about 15% of invasive mammary tumors. They are often defined as triple negative breast cancer (TNBC) since most of them are ER negative, progesterone receptor

Abbreviations: EMT, epithelial to mesenchymal transition; ER, estrogen receptor; FBS, fetal bovine serum; GPCR, G-protein coupled receptors; Gy, gray; HA, histamine; HER2, human epidermal growth factor receptor 2; MMPs, matrixmetalloproteinases; P-Src, phosho-Src; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PR, progesterone receptor; T-Src, total-Src; TBS, tris-buffered saline.

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(PR) negative and lack human epidermal growth factor receptor 2 (HER2) protein overexpression and gene amplification. In most instances, treatment of early and late stage breast cancers involves surgery followed by radiotherapy and/or chemotherapy. Conventional radiation therapy for breast cancer is delivered in fractionated doses to reduce deleterious effects on healthy tissues. The benefits of therapeutic strategies using ionizing radiation in reducing local-regional recurrence risk and increasing survival are recognized. However, ionizing radiation may paradoxically promote migration and invasion abilities of tumor surviving cells leading to local recurrences (primary tumor site) or metastases (distant organ) via epithelial to mesenchymal transition (EMT) induction [2–6].

EMT is a dynamic and reversible program. It is well documented that cancer cells undergo to EMT with loss or down-regulation of epithelial markers as E-cadherin and up-regulation of mesenchymal markers as vimentin, alpha smooth muscle actin and N-cadherin. The epithelial marker E-cadherin is a glycoprotein involved in cell-cell adhesion. β-catenin is the intracellular binding partner of E-cadherin. E-cadherin and β-catenin form a complex that is required for maintaining epithelial cell-cell contacts. One of the first steps of EMT is the disassembly of this complex with the re-localization and/or degradation of these proteins. Besides, EMT is associated to an enhancement of cell motility, an increase in the expression and activity of matrix metalloproteinases (MMPs) and in the invasiveness and metastatic ability. The Slug zinc-finger protein, a member of the Snail family, is a transcription factor involved in EMT regulation [7].

Histamine is a biogenic amine that exerts multiple physiopathological actions through the stimulation of four (H1–H4) histamine subtypes G-protein coupled receptors (GPCR) which are widely distributed in different tissues and organs. For the last 25 years histamine has displayed an important role in cell proliferation and tumor growth in a number of experimental models through the stimulation of different histamine receptors [8].

Increasing evidence has also been collected indicating that histamine might be a player in tumor progression modulating cell adhesion and MMPs activity in pancreatic and mammary tumor cells [9,10]. We have reported that ionizing radiation induced some EMT-related events in the TNBC cells MDA-MB-231 which were blocked upon histamine treatment before cell irradiation [9].

The proto-oncogene Src is a member of the Src family kinases linked to cancer progression and metastatic disease. Its interaction with different signaling proteins is involved in cell adhesion and migration. A growing body of research indicates that GPCR and Src family kinases are thoroughly implicated in multilayered forms of cross-talk that influence a host of cellular processes [11–15]. Recently, it has been reported that activated/phosphorylated Src is involved in the promotion of the radio-induced malignant phenotypes in breast cancer cells [16].

In this work we investigated whether histamine may modify EMT events induced by ionizing radiation in breast cancer cells with different invasive phenotype. The involvement of Src phosphorylation in histamine actions on migration of irradiated cells was also evaluated.

2. Materials and methods

2.1. Cell culture

Human breast carcinoma cells MCF-7 and MDA-MB-231 were from the American Type Cell Culture (ATCC). Cells were routinely maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS, Gibco, CA, USA), 0.3 g/l L-glutamine and 40 mg/l gentamicin at 37 °C in a humidified atmosphere with 5% CO2. Cells were subcultured according to the ATCC protocol within 2 months of resuscitation and used between passages 5 and 20. Cell cultures were mycoplasma tested monthly using DAPI as a direct DNA stain.

2.2. Irradiation

Tumor cells were seeded and treated or not with 1 μM or 20 μM histamine (Sigma-Aldrich, St Louis, MO, USA). The selective inhibitor of Src-family tyrosine kinases 4-amino-3-(4-chlorophenyl)-1-(*t*-butyl)-1H-pyrazolo[3,4-*d*]pyrimidine, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2, Sigma) was added to cell cultures 30 min before histamine in combined treatments. After 24 h cells were gamma-irradiated at a total dose of 2 Gy with an IBL 437C H type irradiator, ^{137}Cs source.

2.3. Morphological changes

Cell morphology and scattering were evaluated five days after irradiation. Cells were fixed with 4% formalin and stained with 0.1% toluidine blue dye solution. Cells were observed by an optical microscope Olympus Bx50 and photographed using an Olympus DP73 camera.

2.4. Immunoblotting

Cells were washed in ice-cold phosphate-buffered saline, taken up in 100 μl of lysis buffer, and heated to 95 °C for 5 min. 30–50 μg of protein were separated on a 12% SDS-PAGE gel and blotted onto a polyvinylidenedifluoride membrane. Membranes were blocked and probed with anti E-cadherin (1:400, Invitrogen, CA, USA), anti vimentin (1:500, Invitrogen), anti β-catenin (1:400, Invitrogen), anti Slug (1:100, Santa Cruz Biotechnology, TX, USA), anti phospho-Src and Src (1:250, Cell Signaling, MA, USA), anti β-actin or α-tubulin (1:2000, Sigma) antibody overnight and with anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibody (1:2000, Sigma) for 1 h at room temperature. The immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham Biosciences, Argentina). Densitometric analyses were performed using the software ImageJ 1.42q (NIH, USA).

2.5. Immunofluorescence studies

Cells grown on cover glasses were fixed, permeabilized, blocked in PBS with 1% bovine seroalbumin and incubated overnight at 4 °C with anti E-cadherin (1:100, Invitrogen), anti β-catenin (1:100, Invitrogen), anti Slug (1:50, Santa Cruz Biotechnologies), anti P-Src (1:100, Cell Signaling) or anti vimentin (1:100, Invitrogen) antibody and for 1 h at room temperature with Alexa Fluor 488 dye conjugated anti-mouse (1:400, Invitrogen) or FITC-conjugated anti-rabbit (1:100, Sigma) antibody. Nuclei were stained with 0.1 μg/ml DAPI or propidium iodide. Immunoreactivity was visualized by an immunofluorescence microscope (Olympus Fluo View FV1000).

2.6. Gelatin zymography

After 24 h in serum free RPMI, supernatants from cell cultures were collected, mixed with non-reducing buffer and electrophoresed on 7% sodium dodecyl sulfate-polyacrilamide gels with 0.1% gelatin (Sigma). The gels were washed with 0.5% Triton X-100 (v/v) in Tris-buffered saline (TBS), pH 7.4 for 30 min, rinsed briefly with TBS, pH 7.4, and incubated in TBS, pH 7.4 supplemented with 1 mM Ca^{2+} at 37° C for 24 h. Gelatinolytic activity was visualized by staining zymograms with Coomassie Brilliant Blue G250 (Sigma) and destaining in acetic acid-methanol-H_2O (1:3:6).

Densitometric analyses were performed using the software ImageJ 1.42q (NIH, USA).

2.7. Migration and invasion assay

Cell migration was investigated using a 24-well transwell unit with polyethylene terephthalate membranes showing a pore size of 8.0 μm (BD Falcon, Basel, Switzerland). Equal number of MCF-7 or MDA-MB-231 cells in serum-free RPMI was loaded into the upper compartment. The lower chamber contained RPMI medium plus 10% FBS. After 20 h at 37 °C non-migrated cells on the upper surface of membranes were gently scrubbed with a cotton swab. Cells migrated were fixed in 4% formalin and stained with 0.5% crystal violet. Total number of migrated cells was counted. For the invasion assay, transwell units were coated with Matrigel® (BD Biosciences, MA, USA)/RPMI (1:2). After 20 h at 37 °C, cells that invaded through Matrigel and reached to the reverse side were fixed, stained and counted under a microscope. Controls to verify that cell proliferation was not affecting migration/invasion results after 20 h incubation were performed (data not shown).

2.8. Statistical analysis

Data were analyzed by one-way ANOVA and Bonferroni post-test, using the GraphPad Prism Version 5.0 software (GraphPad Software Inc, Philadelphia, USA). *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of histamine and ionizing radiation on cell morphology

To clarify histamine and ionizing radiation actions on MCF-7 and MDA-MB-231 cell morphology we tested two doses of histamine (1 μM and 20 μM) on cells seeded at low densities to guarantee a clonogenic growth and 24 h later cell cultures were irradiated. After 5 days non-irradiated MCF-7 cells exhibited the characteristic epithelial phenotype with a cobblestone appearance and tight cell-cell junction. Irradiated cells treated or not with 1 μM histamine developed an irregular-shaped morphology with many protruding processes, loss of cell-to-cell contact and an increase in cell scattering. Additionally, MDA-MB-231 cells showed a more elongated appearance and cell spreading than usual when they were irradiated. However, pre-treatment of mammary tumor cells with 20 μM histamine prevented the phenotypic changes induced by ionizing radiation (Fig. 1).

3.2. Expression and subcellular localization of epithelial and mesenchymal markers in histamine-treated and irradiated cells

In order to investigate the effects of histamine and ionizing radiation on EMT molecular markers we performed immunoblot and immunofluorescence experiments five days post-irradiation.

A single dose of 2 Gy significantly reduced β-catenin expression in MCF-7 cells pre-treated with histamine. There was also a reduction in E-cadherin expression however it was not significant (Fig. 2A). Interestingly, immunofluorescence studies revealed different subcellular localization of these proteins. E-cadherin and β-catenin proteins were observed at cell membrane in non-irradiated cells while a cytoplasmic localization for E-cadherin and a cytoplasmic/perinuclear localization for β-catenin were mainly detected in irradiated MCF-7 cells, treated or not with 1 μM histamine. Notably, appreciable membrane-localized E-cadherin and β-catenin were visualized when cancer cells were irradiated in the presence of 20 μM histamine (Fig. 2B). Though the mesenchymal marker vimentin is not present in MCF-7 cells as reported elsewhere [17], emerging evidence from different radiation protocols supports that ionizing radiation may induce vimentin expression in these cells [5,16]. We could not detect vimentin by Western blot but our immunofluorescence data could confirm a low cytoplasmic expression of this protein when MCF-7 cells were irradiated in the presence or absence of 1 μM histamine (Fig. 2B). There was also an increase in the nuclear expression of the transcriptional factor Slug in MCF-7 cells (Fig. 2C). Remarkably, all these changes were not observed when cell cultures were treated with 20 μM histamine and then 2 Gy gamma-irradiated.

The TNBC cells MDA-MB-231 did not express the epithelial marker E-cadherin. However, a significant increase in the expression of the mesenchymal marker vimentin was determined in irradiated cells (Fig. 3A). In addition, our studies revealed a cytoplasmic, nuclear and perinuclear localization of β-catenin (Fig. 3B) and a significant augmentation in the number of Slug positive nuclei (Fig. 3C) when tumor cells were irradiated in the presence or not of 1 μM histamine. Interestingly, these findings were not observed when MDA-MB-231 cells received 20 μM histamine 24 h previous to be irradiated.

In addition, histamine actions in non-irradiated MDA-MB-231 and MCF-7 cells were also evaluated as a control. Immunofluorescence studies revealed that 1 μM and 20 μM histamine induced different subcellular localization of E-cadherin and β-catenin, and changes in EMT molecular markers expression (Supplementary Fig. S1).The lower dose of histamine produced similar results as those observed in 2 Gy-irradiated mammary tumor cells. On the contrary, cells receiving 20 μM histamine showed membrane-localized E-cadherin and β-catenin, and levels of vimentin and Slug like non irradiated control cells.

3.3. Enhancement of EMT functional markers in irradiated breast cancer cells

Since the expression of EMT molecular markers is tightly related to functional markers we next performed assays to investigate the gelatinolytic activity, cell migration and invasion. We have previously reported that histamine in high concentration could block the significant increase in the MMPs activity and the migratory/invasive capacity induced by a single dose of 2 Gy gamma-radiation in the TNBC cells MDA-MB-231 [9].

In the luminal breast cancer cells MCF-7 ionizing radiation induced a similar pattern expression of EMT functional markers. There was a significant increase in MMP2 activity and also in the number of migrated and invading cells when MCF-7 cells were 2 Gy-irradiated in the presence or absence of 1 μM histamine (Fig. 4A–C). However, these changes were not observed when cells received 20 μM histamine 24 h before irradiation. We performed these experiments five days after irradiation as in Section 3.2.

As a control we also monitored the effects of histamine on MCF-7 cells migration and invasion in the absence of irradiation. Different responses were noticed depending on histamine concentration. There was an increase in cell migration and invasion with 1 μM histamine. This increase was not observed when cancer cells received 20 μM histamine (Supplementary Fig. S2A). Remarkably, results were comparable with those previously reported in non irradiated MDA-MB-231 cells [9].

3.4. Histamine modulates phospho-Src levels in irradiated cells

In a recent report it was demonstrated that several breast cancer cells exposed to gamma-irradiation (2 Gy/day for 3 days) develop malignant phenotypes through Src activation promoting invasiveness [16].

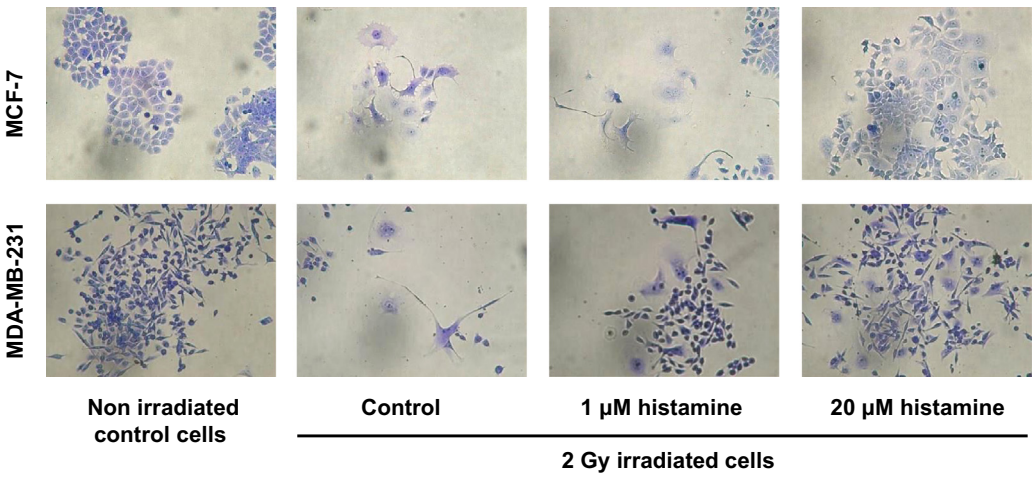

Fig. 1. Morphological changes in MCF-7 and MDA-MB-231 cells. Cells were seeded at low density, treated or not with histamine 24 h previously to irradiation and allowed to grow until well-defined individual colonies were formed. Representative optical micrographs (100x) show the difference in cell scattering and cellular morphology.

On the basis of our studies, a single dose of 2 Gy irradiation led to a pronounced and significant increase in cell migration and also in phospho-Src levels in MCF-7 and MDA-MB-231 cells. However, pre-treatment of both cell lines with 20 μM histamine significantly decreased cell migration as well as phospho-Src levels in irradiated cells (Fig. 5A and C). To further test whether phospho-Src levels are involved in the migratory ability of histamine-treated and irradiated cells we employed 2 μM PP2, a selective inhibitor of Src-family tyrosine kinases. Fig. 5B and D shows that Src phosphorylation and cell motility were almost undetectable in irradiated MCF-7 and MDA-MB-231 cells treated with 2 μM PP2 suggesting that modulation of phospho-Src level induced by 20 μM histamine might be responsible for the migratory behavior observed. In addition, our immunofluorescence studies revealed that MCF-7 treated with PP2 and then irradiated exhibited membrane-localized E-cadherin, a similar outcome to that observed in irradiated cells that received pre-treatment with 20 μM histamine (Fig. 5E). Thus, Src protein might be a potential key component of histamine effect on the radio-induced EMT.

We also evaluated the phosphorylation/activation of Src in non irradiated MDA-MB-231 and MCF-7 cells treated with histamine in order to check the effects of histamine alone. Immunofluorescence studies revealed a positive membrane staining for phospho-Src in control, and histamine treated MCF-7 cells. Interestingly, the number of positive cells for phospho-Src membrane staining was higher in cells receiving 1 μM histamine which also exhibited protrusions as irradiated cells did (Supplementary Fig. S2B). Western blot analysis showed different levels of phospho-Src dependent on histamine concentration in MDA-MB-231 cells (Supplementary Fig. S2C). In both cell lines there was only a stimulatory effect on Src phosphorylation when non irradiated cells were treated with 1 μM histamine.

4. Discussion

Breast cancer is the major malignancy diagnosed among women worldwide and an important cause of cancer mortality. Despite the improvements in outcomes due to enhanced combinations of surgery with chemo- and radiotherapy, research is going on within each treatment modality to increase therapeutic benefits.

Ionizing radiation may affect not only cancer cells but the cellular microenvironment and their reciprocal interactions in order to facilitate tumor progression and metastases [5,7,18]. Local recurrences or metastases that occur after radiotherapy are frequently associated to micrometastasis originated before tumor irradiation. However, increasing experimental evidence indicates that ionizing radiation may induce cellular changes leading to EMT and enhancement of invasiveness in surviving irradiated cells. For the treatment of breast cancer in women, 50 Gy in 25 daily fractions of 2 Gy over 5 weeks is recommended in terms of effects in normal breast. Diverse experimental conditions including many different irradiation schedules have been reported in literature to study the cells that remain alive after fractionated irradiation and the mechanisms involved in resultant malignant progression [4,16]. In the current study we chose 2 Gy as a clinically relevant subletal dose to evaluate whether histamine might modify the radiation induced mesenchymal changes in breast cancer cell lines since the better knowledge of the primary responses to initial radiation insults is the first step for a pharmacological intervention.

We could determine that luminal and TNBC cells irradiated with a single dose of 2 Gy exhibited mesenchymal changes supporting a more migratory and invasive phenotype five days after irradiation. Pre-treatment of breast cancer cells with 20 μM histamine prevented these changes opening a perspective for the use of histamine in prolonged experiments using multiple 2 Gy doses as an approach to clinical schedules.

Our experiments on cell morphology evidenced morphological features corresponding to a mesenchymal cellular appearance and cell scattering, probably due to the decrease in the intercellular adhesive forces, in agreement with other reports using different irradiation schedules [3,5,16]. Even if we detected a decrease in E-cadherin and β-catenin expression in irradiated MCF-7 cells, the distinctive finding was the disruption of E-cadherin-β-catenin complex leading to cytoplasmic localization of both proteins. The dissociation of the E-cadherin protein from β-catenin is considered an important feature of the EMT and sometimes it is defined as a cause and effect of the EMT [19,20]. As expected, the mesenchymal-like MDA-MB-231 cells did not express E-cadherin while a low β-catenin expression was detected in non irradiated control cells. In addition, cytoplasmic, perinuclear and nuclear localization of β-catenin were also observed in irradiated MCF-7 and MDA-MB-231 cells supporting its role as a transcriptional co-activator for genes linked to EMT and tumor invasion [21–23].

Evidence shows that histamine may regulate cell-cell adhesion and cadherin expression in normal and tumor cells of diverse origin. The reported effects are miscellaneous according to the tissue and the histamine receptor subtype involved [24–26]. The different results observed in this work using two histamine concentrations may be attributed to the activation of different receptor subtypes since MCF-7 and MDA-MB-231 cells express the four sub-

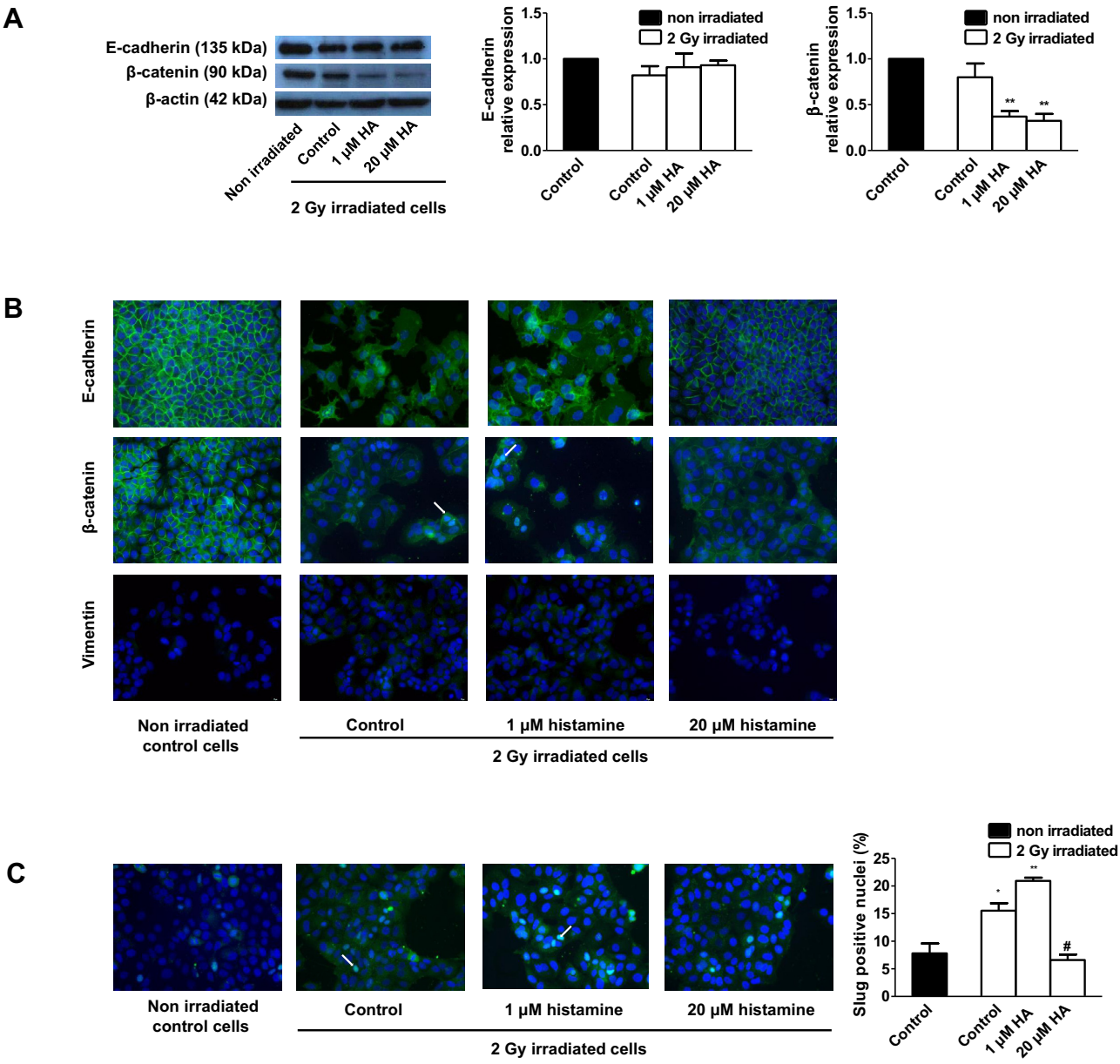

Fig. 2. Immunolocalization and protein levels of EMT molecular markers in MCF-7 cells. Cells were treated or not with histamine (HA) 24 h before irradiation. EMT molecular markers were evaluated 5 days post-irradiation. (A) Representative Western Blots showing the levels of E-cadherin and β-catenin in MCF-cells. β-actin was used for loading control. Densitometric analysis of Western Blots from three independent experiments are shown. Relative expression of E-cadherin and β-catenin was calculated as a ratio and normalized to non-irradiated control. Data represent as mean ± SD. $^{**}p < 0.01$ *vs* non irradiated control. (B) Cells were fixed and stained for immunofluorescence with anti-E-cadherin, anti β-catenin or anti-vimentin antibody and the appropiate secondary antibody. Nuclei were counterstained with DAPI. Arrows indicate nuclear localization of β-catenin. Representative photographs are shown. 400x. (C) Immunolocalization of Slug in MCF-7 cells. Cells were treated or not with histamine (HA) 24 h before irradiation. Subcellular localization of Slug was analyzed by immunofluorescence 5 days post-irradiation with anti-Slug antibody and a secondary antibody tagged with FITC. Nuclei were counterstained with DAPI. Representative photographs are shown, 400x. Arrows indicate nuclear localization of Slug. Bar chart shows the percentage of Slug positive nuclei from three independent experiments. At least five hundred cells were counted to determine the percentage of Slug positive nuclei. Data represent as mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs* non irradiated control, $^{\#}p < 0.05$ *vs* irradiated control.

types of histamine receptors [8]. Remarkably, our findings show that 20 μM histamine-treated and irradiated MCF-7 cells exhibited reduced expression of E-cadherin and β-catenin while preserved epithelial cell morphology with both proteins mainly observed at cell membrane. The most frequently described scenario for the decrease of E-cadherin and β-catenin expression is the finding of cytoplasmic localization associated to changes in cell morphology as described above for irradiated cells. However, there are some reports that account for a diminution in total protein levels coupled to membrane expression and preservation of epithelial morphology and cell-cell adhesion with dissimilar mechanisms ascribed, such as inhibition of proteosome degradation or different levels of p120-catenin expression [27,28]. Besides, we should consider that histamine concentration over 20 μM may simultaneously activate more than one receptor in MCF-7 cells and in that way affect different steps involved in E-cadherin and β-catenin trafficking leading to the atypical response observed [29].

Vimentin is an intermediate filament protein functionally involved in attachment, migration, and cell signaling, which is expressed in normal mesenchymal cells and a wide range of transformed cell types [30,31]. Vimentin is proposed as a positive regulator of EMT since its up-regulation appears to be a prereq-

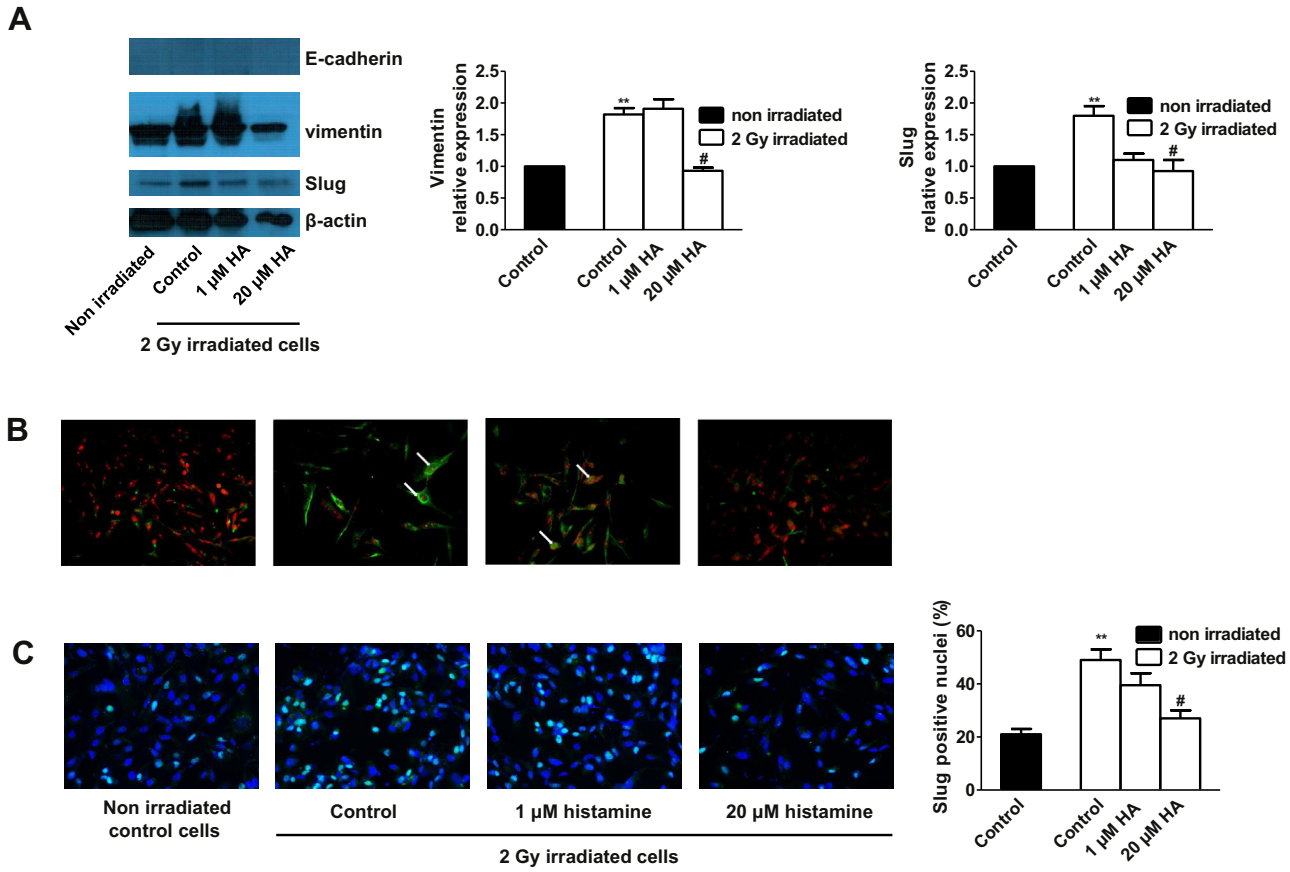

Fig. 3. Immunolocalization and protein levels of EMT molecular markers in MDA-MB-231 cells. Cells were treated or not with histamine (HA) 24 h before irradiation. EMT molecular markers were evaluated 5 days post-irradiation. (A) Representative Western Blots showing the levels of E-cadherin, vimentin and Slug in MDA-MB-231 cells. β-actin was used for loading control. Densitometric analysis of Western Blots from three independent experiments are shown. Relative expression of vimentin and Slug was calculated as a ratio and normalized to non-irradiated control. Data represent as mean ± SD. $^{**}p < 0.01$ *vs* non irradiated control and #p< 0.05 *vs* irradiated control. (B) Cells were fixed and stained for immunofluorescence with anti β-catenin antibody and the appropiate secondary antibody. Nuclei were counterstained with propidium iodide. Arrows indicate nuclear and perinuclear localization of β-catenin. Representative photographs are shown. 400x. (C) Immunolocalization of Slug in MDA-MB-231 cells. Cells were treated or not with histamine (HA) 24 h before irradiation. Subcellular localization of Slug was analyzed by immunofluorescence 5 days post-irradiation with anti-Slug antibody and a secondary antibody tagged with FITC. Nuclei were counterstained with DAPI. Representative photographs are shown, 400x. Bar chart shows the percentage of Slug positive nuclei from three independent experiments. At least five hundred cells were counted to determine the percentage of Slug positive nuclei. Data represent as mean ± SD. $^{**}p < 0.01$ *vs* non irradiated control and $^{\#}p < 0.05$ *vs* irradiated control.

uisite for EMT induction [32]. Further on, the *de novo* expression of vimentin is considered a mechanism associated with EMT [33]. In the present work, a single dose of 2 Gy significantly increased vimentin expression in MDA-MB-231 cells and induced the *de novo* expression of vimentin in MCF-7 cells in agreement with other reports [5,16] while the pre-treatment of irradiated cells with the higher dose of histamine prevented this effect in both cell lines. It is well documented that vimentin is necessary for Slug-induced EMT associated migration and required for induction of invasiveness-related genes in different breast cancer cell lines [34]. Recently, Tania et al. [35] have summarized the direct or indirect performance of EMT-inducing transcription factors, such as Twist, Snail, Slug, and Zeb, in cancer cell metastasis through different signaling pathways, with the final consequence of the up- or down-regulation of different mesenchymal and epithelial EMT-related proteins. Our findings strengthen this idea since the increase in cell migration and invasion was only verified in irradiated cells characterized by the cytoplasmic expression of vimentin and the higher number of Slug positive nuclei. Interestingly, the inverse relationship between membrane-localized E-cadherin and Slug nuclear expression was observed in irradiated MCF-7 cells when cells were pre-treated with 20 μM histamine.

It is known that loss of cell-cell contact is determinant for the migratory ability of cancer cells while the increase in MMPs lytic activities supports cell invasion [36]. In this regard, the enhancement of functional markers induced by ionizing radiation was not detected when mammary tumor cells were previously treated with 20 μM histamine. Thus, pre-treatment of MCF-7 and MDA-MB-231 cells with 20 μM histamine prevented the expression of vimentin, decreased the number of Slug positive nuclei and reduced cell migration and invasion in irradiated tumor cells.

Src is a non-receptor tyrosine kinase which participates in different signal transduction pathways regulating biological processes such as cell proliferation, differentiation, migration, angiogenesis, and survival [14]. Its level and activity are elevated in several human cancers including prostate, lung, breast, and colorectal [15]. The activation grade of Src is determined by the net phosphorylation status at its regulatory residues [37]. Src functions as a signal transducer from the cell surface receptors via phosphorylation of tyrosine residues on substrates in a sequential way being β-catenin one of these substrates. Tyrosine phosphorylation of β-catenin by activated Src alters the integrity of the cadherin-catenin complex [38]. Consequently a loss of cadherins-mediated cell-cell contacts and an increase in the level of cytoplasmic β-catenin are detected. The stabilization and accumulation of β-catenin in the cytoplasm activates β-catenin signaling in the nucleus increasing EMT related genes transcription like Slug [7]. Coluccia et al. [39] demonstrated that the employment of Src inhibitors in human col-

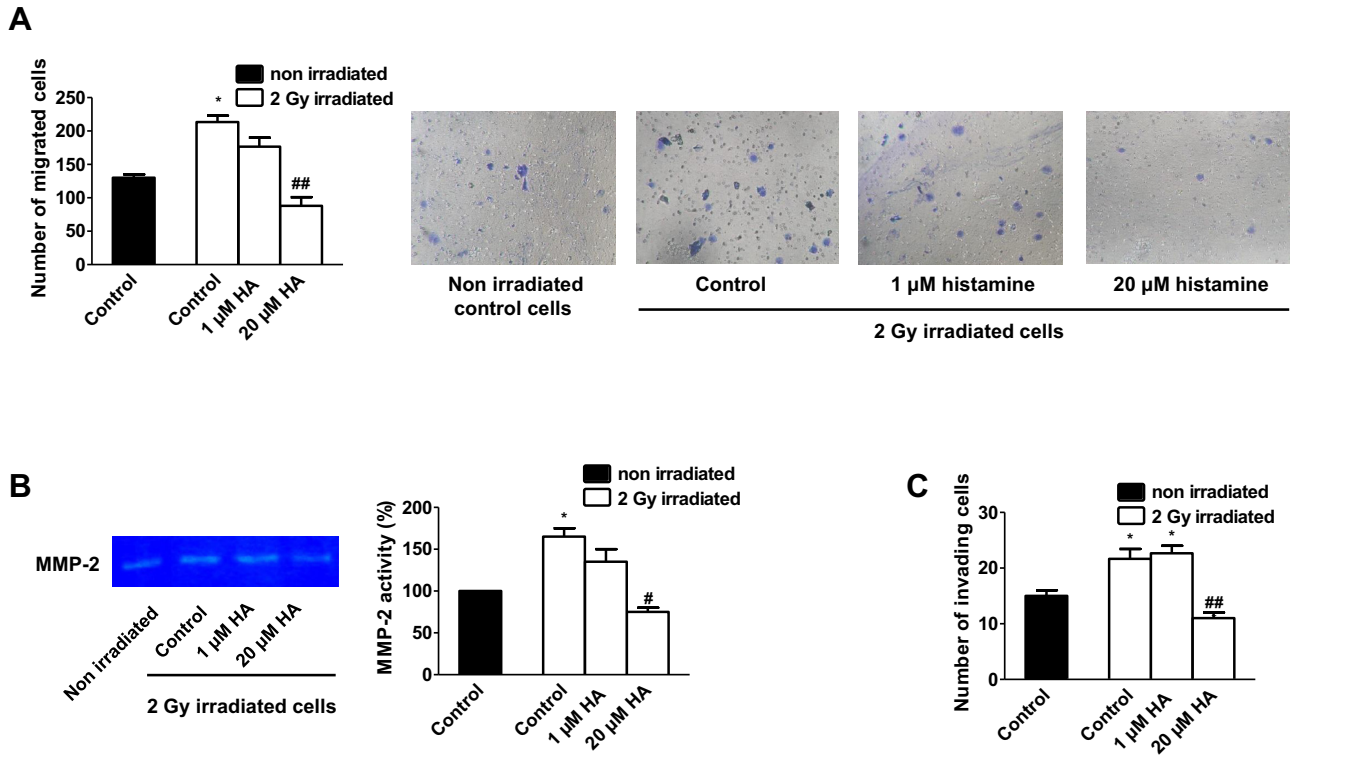

Fig. 4. EMT functional markers in MCF-7 cells. Cells were treated or not with histamine (HA) 24 h before irradiation and markers were analyzed 5 days post-irradiation. (A) Cell migration was evaluated using transwell units. MCF-7 cells in serum-free RPMI medium were seeded in the upper compartment. The lower chamber contained RPMI plus 10% FBS. After 20 h migrated cells were fixed and stained. Bar chart represents the total number of migrated cells (mean ± SD) from three independent experiments. $^{*}p < 0.05$ *vs* non irradiated control, $^{\#\#}p < 0.01$ *vs* irradiated control. Representative photographs are shown. 100x. (B) MMP2 activity was evaluated in 24 h serum-free cell culture medium. Bar chart represents gelatinolytic activity (%) for each experimental condition *vs* non irradiated control cells from three independent experiments. Data represent as mean ± SD. $^{*}p < 0.05$ *vs* non irradiated control, $^{\#}p < 0.05$ *vs* irradiated control. A representative zymogram is shown. (C) Cell invasion was evaluated as cell migration using inserts coated with Matrigel. Bar chart represents the total number of invading cells (mean ± SD) from three independent experiments. $^{*}p < 0.05$ *vs* non irradiated control, $^{\#\#}p < 0.01$ *vs* irradiated control.

orectal tumor cells reduced cell motility due to the prevention of β-catenin phosphorylation and its transcriptional activity. Though we did not determine the level of phospho-β-catenin in the present work we could observe that ionizing radiation induced a cytoplasmic localization of β-catenin and E-cadherin, an increase in nuclear Slug expression, vimentin expression, cell migration and phospho-Src level. Pre-treatment of irradiated breast cancer cells with 20 μM histamine prevented these events. Additionally, a positive association between vimentin expression and the invasive behavior of cancer cells has been reported. Wei et al. [40] demonstrated that vimentin may form aggregates with Src, E-cadherin and β-catenin supporting invasiveness of prostate tumor cells through the E-cadherin-β-catenin complex regulation by phospho-Src.

The migratory behavior of MCF-7 and MDA-MB-231 cells and the increase in phospho-Src levels observed with a single dose of 2 Gy in this work were similar to other reports with tumor cells exposed to 2 Gy/day for 3 days [16]. Besides, our present findings suggest that the inhibitory action of 20 μM histamine on irradiated breast cancer cells migration is mediated by the down-modulation of phospho-Src levels. Remarkably, the selective inhibition of Src is proposed as a potential clinical tool to be used in the reduction of tumor progression and in the prevention of cancer metastasis [20,37,41,42].

In summary, the present study provides evidence that the mesenchymal appearance and the EMT molecular and functional markers induced by ionizing radiation in breast cancer cells with different invasive phenotypes were reversed upon 20 μM histamine treatment. Data also identify Src as a key component of the signaling pathway involved in the migratory response attenuated by 20 μM histamine in irradiated breast cancer cells. Taken together these results demonstrate that histamine actions in irradiated mammary tumor cells are not cell line specific.

Clinical trials using histamine dihydrochloride as a subcutaneous formulation in conjunction with interleukin-2 have been carried out for the treatment of different cancers such as renal cell carcinoma, acute myelogenous leukemia and metastatic melanoma. When administered together, histamine enhances interleukin-2 antitumor activity by facilitating the activation of T cells and natural killer cells by IL-2. This therapy had an acceptable tolerability profile in patients in phase III trials [43,44]. In addition, histamine displayed antiproliferative effects in experimental cancer models *in vitro* and *in vivo* [8]. Recently, it has been reported that histamine over 10 μM exhibits a radiosensitizing action involving increased radiation-induced DNA damage, apoptosis and senescence in both mammary tumor cell lines, MCF-7 and MDA-MB-231 [45]. Moreover, *in vivo* radioprotective effects exerted by histamine on non-cancer tissues have also been described [46].

The research on the prospective use of histamine to enhance radiotherapy efficacy by improving cancer cells intrinsic radiosensitivity and also controlling metastatic behavior could lead to new therapeutic strategies in order to increase antitumor effectiveness and minimize undesirable side effects.

Competing interests

The authors declare that they have no competing interests.

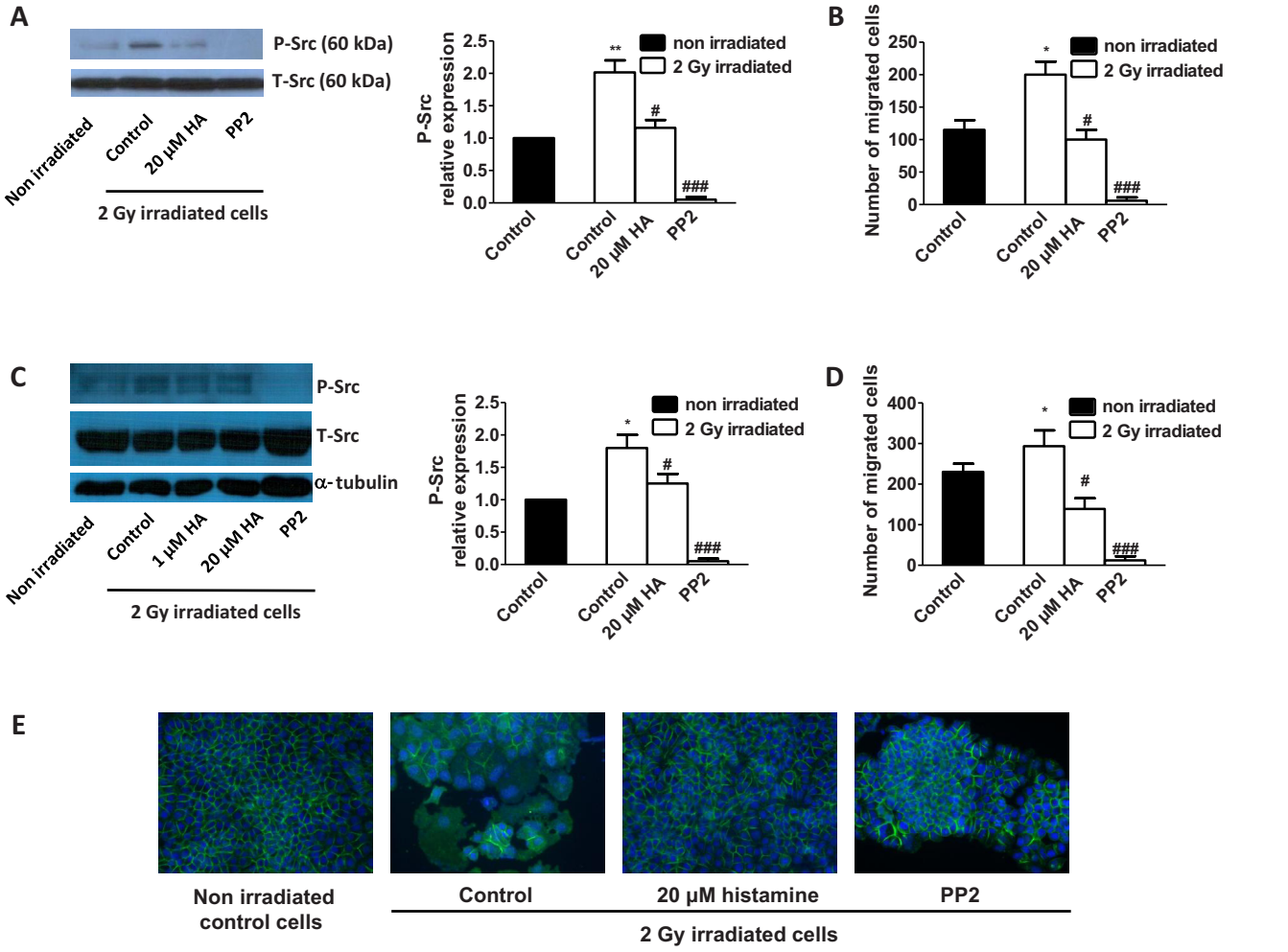

Fig. 5. Phospho-Src involvement in cell migration of histamine-treated and irradiated cells. Cells were treated with 20 μM HA or 2 μM PP2 Src inhibitor during 24 h before irradiation. (A) Effect of PP2 Src inhibitor on Src levels in irradiated MCF-7 cells. Phospho-Src (P-Src) and total Src (T-Src) level were evaluated by immunoblot 1 h post-irradiation. P-Src relative expression was calculated as the ratio P-Src/T-Src and normalized to non irradiated control. Representative Western Blots and densitometric analysis from 3 independent experiments (mean ± SD) are shown. $^{**}p < 0.01$ *vs* non irradiated control; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ *vs* irradiated control. (B) Effect of phospho-Src levels on the migratory behavior of irradiated MCF-7 cells. Cell migration was evaluated using the transwells units. After 20 h migrated cells were fixed and stained. Bar chart represents the total number of migrated cells from three independent experiments (mean ± SD). $^{**}p < 0.01$ *vs* non irradiated control; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ *vs* irradiated control. (C) Effect of PP2 Src inhibitor on Src levels in irradiated MDA-MB-231 cells. Phospho-Src (P-Src) and total Src (T-Src) level were evaluated by immunoblot 1 h post-irradiation. P-Src relative expression was calculated as the ratio P-Src/T-Src and normalized to non irradiated control. Representative Western Blots and densitometric analysis from 3 independent experiments (mean ± SD) are shown. $^{*}p < 0.05$ *vs* non irradiated control, $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ *vs* irradiated control. (D) Effect of phospho-Src levels on the migratory behavior of irradiated MDA-MB-231 cells. Cell migration was evaluated using the transwells units. After 20 h migrated cells were fixed and stained. Bar chart represents the total number of migrated cells from three independent experiments (mean ± SD). $^{*}p < 0.05$ *vs* non irradiated control; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ *vs* irradiated control. (E) Effect of PP2 Src inhibitor on E-cadherin localization in MCF-7cells. Cells were fixed and stained for immunofluorescence with anti E-cadherin antibody and the appropiate secondary antibody. Nuclei were counterstained with DAPI.400x.

Authors' contributions

GAM and GPC conceived the project and wrote the manuscript and contributed equally as senior authors. NAM performed the statistical analysis. TEG, MATD and GMV carried out most of the experiments including western blotting, cell migration and invasion, zymography, indirect immunofluorescence. EJC and RMB performed microscopic observations. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2016.07.039.

References

[1] S. Schnitt, Classification and prognosis of invasive breast cancer: from morphology to molecular taxonomy, Mod. Pathol. 23 (Suppl. 2) (2010) 60–64.
[2] J.H. Cho, W.G. Hong, Y.J. Jung, et al., γ-Ionizing radiation-induced activation of the EGFR-p38/ERK-STAT3/CREB-1-EMT pathway promotes the migration/invasion of non-small cell lung cancer cells and is inhibited by podophyllotoxin acetate, Tumour Biol. (December) (2015), Epub ahead of print.
[3] E. He, F. Pan, G. Li, J. Li, Fractionated ionizing radiation promotes epithelial-mesenchymal transition in human esophageal cancer cells through PTEN deficiency-mediated Akt activation, PLoS One 10 (5) (2015) e0126149.

[4] C. Moncharmont, A. Levy, J.B. Guy, A.T. Falk, M. Guilbert, J.C. Trone, G. Alphonse, M. Gilormini, D. Ardail, R.A. Toillon, C. Rodriguez-Lafrasse, N. Magné, Radiation-enhanced cell migration/invasion process: a review, Crit. Rev. Oncol. Hematol. 92 (2) (2014) 133–142.
[5] X. Zhang, X. Li, N. Zhang, Q. Yang, M.S. Moran, Low doses ionizing radiation enhances the invasiveness of breast cancer cells by inducing epithelial-mesenchymal transition, Biochem. Biophys. Res. Commun. 412 (1) (2011) 188–192.
[6] Y.C. Zhou, J.Y. Liu, J. Li, Y.Q. Xu, H.W. Zhang, L.B. Qiu, G.R. Ding, X.M. Su, G.Z. Mei-Shi Guo, Ionizing radiation promotes migration and invasion of cancer cells through transforming growth factor-beta-mediated epithelial-mesenchymal transition, Int. J. Radiat. Oncol. Biol. Phys. 81 (5) (2011) 1530–1537.
[7] S. Lamouille, J. Xu, R. Derynck, Molecular mechanisms of epithelial-mesenchymal transition, Nat. Rev. Mol. Cell Biol. 15 (3) (2014) 178–196.
[8] V.A. Medina, E.S. Rivera, Histamine receptors and cancer pharmacology, Br. J. Pharmacol. 61 (2010) 755–767.
[9] G. Cricco, N. Mohamad, M.S. Saez, E. Valli, E. Rivera, G. Martín, Histamine and breast cancer: a new role for a well known amine, in: M. Gunduz, E. Gunduz (Eds.), Breast Cancer-Carcinogenesis, Cell Growth and Signalling Pathways, Intech, Rijeka, Croacia, 2011, pp. 611–634 http://www.intechopen.com/articles/show/title/histamine-and-breast-cancer-a-new-role-for-a-well-known-amine.
[10] G. Cricco, N. Mohamad, G. Martín, Insights into histamine role in cell migration and matrix remodeling: special focus on tumor biology, in: L. Berhardt (Ed.), Advances in Medicine and Biology, Nova Science, Nueva York, 2012, pp. 29–49 https://www.novapublishers.com/catalog/product_info.php?products_id=37200.
[11] S. Gavi, E. Shumay, H.Y. Wang, C.C. Malbon, G-protein-coupled receptors and tyrosine kinases: crossroads in cell signaling and regulation, Trends Endocrinol. Metab. 17 (2) (2006) 48–54.
[12] D.K. Luttrell, L.M. Luttrell, Not so strange bedfellows: G-protein-coupled receptors and Src family kinases, Oncogene 23 (48) (2004) 7969–7978.
[13] D. McGarrigle, X.Y. Huang, GPCRs signaling directly through Src-family kinases, Sci. STKE (392) (2007) pe35.
[14] S.J. Parsons, J.T. Parsons, Src family kinases, key regulators of signal transduction, Oncogene 23 (48) (2004) 7906–7909.
[15] D.L. Wheeler, E.F. Mari Iida Dunn, The role of Src in solid tumors, Oncologist 14 (7) (2009) 667–678.
[16] R.K. Kim, Y.H. Cui, K.C. Yoo, I.G. Kim, M. Lee, Y.H. Choi, Y. Suh, S.J. Lee, Radiation promotes malignant phenotypes through SRC in breast cancer cells, Cancer Sci. 106 (1) (2015) 78–85.
[17] C. Gilles, M. Polette, M. Mestdagt, B. Nawrocki-Raby, P. Ruggeri, P. Birembaut, J.M. Foidart, Transactivation of vimentin by beta-catenin in human breast cancer cells, Cancer Res. 63 (10) (2003) 2658–2664.
[18] V. Bolós, H. Peinado, M.A. Pérez-Moreno, M.F. Fraga, M. Esteller, A. Cano, The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors, J. Cell Sci. 116 (Pt 3) (2003) 499–511.
[19] J.L. Andrews, A.C. Kim, J.R. Hens, The role and function of cadherins in the mammary gland, Breast Cancer Res. 14 (1) (2012) 203.
[20] J. Nam, Y. Ino, M. Sakamoto, S. Hirohash, Src family kinase inhibitor PP2 restores the E-cadherin/catenin cell adhesion system in human cancer cells and reduces cancer metastasis, Clin. Cancer Res. 8 (7) (2002) 2430–2436.
[21] D.M. Gonzalez, D. Medici, Signaling mechanisms of the epithelial-mesenchymal transition, Sci. Signal. 7 (344) (2014) re8.
[22] A.J. Karayiannakis, L. Nakopoulou, H. Gakiopoulou, A. Keramopoulos, P.S. Davaris, M. Pignatelli, Expression patterns of β-catenin in *in situ* and invasive breast cancer, Eur. J. Surg. Oncol. 27 (1) (2001) 32–36.
[23] E. López-Knowles, S.J. Zardawi, C.M. McNeil, E.K. Millar, P. Crea, E.A. Musgrove, R.L. Sutherland, S.A. O'Toole, Cytoplasmic localization of beta-catenin is a marker of poor outcome in breast cancer patients, Cancer Epidemiol. Biomarkers Prev. 19 (1) (2010) 301–309.
[24] Y. Komarova, A.B. Malik, Regulation of endothelial permeability via paracellular and transcellular transport pathways, Annu. Rev. Physiol. 72 (2010) 463–493.
[25] J.J. Lin, T.Z. Zhao, W.K. Cai, Y.X. Yang, C. Sun, Z. Zhang, Y.Q. Xu, T. Chang, Z.Y. Li, Inhibition of histamine receptor 3 suppresses glioblastoma tumor growth, invasion, and epithelial-to-mesenchymal transition, Oncotarget 6 (19) (2015) 17107–17120.
[26] W.H. Paik, J.K. Ryu, K.S. Jeong, J.M. Park, B.J. Song, S.H. Lee, Y.T. Kim, Y.B. Yoon, Clobenpropit enhances anti-tumor effect of gemcitabine in pancreatic cancer, World J. Gastroenterol. 20 (26) (2014) 8545–8557.
[27] M.A. Davis, R.C. Ireton, A.B. Reynolds, A core function for p120-catenin in cadherin turnover, J. Cell Biol. 163 (3) (2003) 525–534.
[28] L.A. Helguero, K. Lindberg, C. Gardmo, T. Schwend, J.A. Gustafsson, L.A. Haldosén, Different roles of estrogen receptors a and B in the regulation of E-cadherin protein levels in a mouse mammary epithelial cell line, Cancer Res. 68 (21) (2008) 8695–8704.
[29] D.M. Bryant, J.L. Stow, The ins and outs of E-cadherin trafficking, Trends Cell Biol. 14 (8) (2004) 427–434.
[30] J. Ivaska, H.M. Pallari, J. Nevo, J.E. Eriksson, Novel functions of vimentin in cell adhesion, migration, and signaling, Exp. Cell Res. 313 (10) (2007) 2050–2062.
[31] A. Satelli, S. Li, Vimentin in cancer and its potential as a molecular target for cancer therapy, Cell. Mol. Life Sci. 68 (18) (2011) 3033–3046.
[32] J. Ivaska, Vimentin central hub in EMT induction? Small GTPases 2 (1) (2011) 51–53.
[33] S. Bindels, M. Mestdagt, C. Vandewalle, N. Jacobs, L. Volders, A. Noël, F. van Roy, G. Berx, J.M. Foidart, C. Gilles, Regulation of vimentin by SIP1 in human epithelial breast tumor cells, Oncogene 25 (36) (2006) 4975–4985.
[34] K. Vuoriluoto, H. Haugen, S. Kiviluoto, J.P. Mpindi, J. Nevo, C. Gjerdrum, C. Tiron, J.B. Lorens, J. Ivaska, Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer, Oncogene 30 (12) (2011) 1436–1448.
[35] M. Tania, M.A. Khan, J. Fu, Epithelial to mesenchymal transition inducing transcription factors and metastatic cancer, Tumour Biol. 35 (8) (2014) 7335–7342.
[36] C. Gialeli, A.D. Theocharis, N.K. Karamanos, Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting, FEBS J. 278 (1) (2011) 16–27.
[37] B. Sen, F.M. Johnson, Regulation of SRC family kinases in human cancers, J. Signal Transduct. 2011 (2011) 865819.
[38] J. Lilien, J. Balsamo, The regulation of cadherin-mediated adhesion by tyrosine phosphorylation/dephosphorylation of beta-catenin, Curr. Opin. Cell Biol. 17 (5) (2005) 459–465.
[39] A.M. Coluccia, D. Benati, H. Dekhil, A. De Filippo, C. Lan, Gambacorti-Passerini C: SKI-606 decreases growth and motility of colorectal cancer cells by preventing pp60(c-Src)-dependent tyrosine phosphorylation of beta-catenin and its nuclear signaling, Cancer Res. 66 (4) (2006) 2279–2286.
[40] L.C. Kim, L. Song, E.B. Haura, Src kinases as therapeutic targets for cancer, Nat. Rev. Clin. Oncol. 6 (10) (2009) 587–595.
[41] D. Tryfonopoulos, S. Walsh, D.M. Collins, L. Flanagan, C. Quinn, B. Corkery, E.W. McDermott, D. Evoy, A. Pierce, N. O'Donovan, J. Crown, M.J. Duffy, Src: a potential target for the treatment of triple-negative breast cancer, Ann. Oncol. 22 (10) (2011) 2234–2240.
[42] J. Wei, G. Xu, M. Wu, Y. Zhang, Q. Li, P. Liu, T. Zhu, A. Song, L. Zhao, Z. Han, G. Chen, S. Wang, L. Meng, J. Zhou, Y. Lu, S. Wang, D. Ma, Overexpression of vimentin contributes to prostate cancer invasion and metastasis via src regulation, Anticancer Res. 28 (1A) (2008) 327–334.
[43] J.B. Perz, A.D. Ho, Histamine dihydrochloride for the treatment of acute myeloid leukemia, malignant melanoma and renal cell carcinoma, Future Oncol. 4 (2) (2008) 169–177.
[44] L.P. Yang, C.M. Perry, Histamine dihydrochloride: in the management of acute myeloid leukaemia, Drugs 71 (1) (2011) 109–122.
[45] D.J. Martinel Lamas, J.E. Cortina, C. Ventura, H.A. Sterle, E. Valli, K.B. Balestrasse, H. Blanco, G.A. Cremaschi, E.S. Rivera, V.A. Medina, Enhancement of ionizing radiation response by histamine in vitro and in vivo in human breast cancer, Cancer Biol. Ther. 16 (1) (2015) 137–148.
[46] V.A. Medina, M. Croci, N.A. Mohamad, N. Massari, G. Garbarino, G.P. Cricco, M.A. Núñez, G.A. Martín, E.J. Crescenti, R.M. Bergoc, E.S. Rivera, Mechanisms underlying the radioprotective effect of histamine on small intestine, Int. J. Radiat. Biol. 83 (10) (2007) 653–663.