

Research Paper

VEGFR-2 Inhibitor Apatinib Hinders Endothelial Cells Progression Triggered by Irradiated Gastric Cancer Cells-derived Exosomes

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Abstract

Background: Radiotherapy is a standard treatment for a significant fraction of cancer patients. Nonetheless, to this day radiation resistance is a key impediment in gastric cancer (GC) treatment. Moreover, GC is characterized by its substantial neo-angiogenesis, driven by high levels of vascular endothelial growth factor (VEGF) correlated with the presence of stomach cancer. The aim of our study was to address if VEGFR inhibitors treatments impact the negative effect of radiotherapy regimens of gastric cancer.

Materials and methods: Isolation of exosomes released by SGC-7901 and BGC-823 lines after irradiation at 0 Gy or 6 Gy was performed by differential ultra-centrifugation. Incubation of Human Umbilical Vein Endothelial Cells (HUVEC) was carried out with different concentrations of exosomes from non- or irradiated GC cells to address their proliferation and survival fraction (SF) by MTS. 6 Gy irradiated cells exosomes at concentration of 20 µg/ml were compared to EC incubated with the same exosome concentration from non-irradiated human GC cells over 72-hour time course. Wound-healing and Transwell assays were performed in a migration buffer consisting of exosomes released by non- or irradiated SGC-7901 and BGC-823 cells over 24-hour time course. HUVEC cells stained with DAPI that have passed through a gluten gel were counted in order to monitor their invasion capacity. Employing IC50, 60 µg/ml was determined as the optimal Apatinib (YN968D1) concentration for the half-life of HUVEC, and incubated with exosomes from irradiated GC cells. The aforementioned assays were performed in the background of the same conditions in order to analyse the effect of Apatinib on HUVEC progression.

Results: We show that proliferation, motility and invasive capacity of HUVEC are enhanced upon incubation with exosomes released by irradiated GC cell lines. Importantly, the latter is counteracted by the VEGFR-2 inhibitor Apatinib which hinders ECs progression.

Conclusion / Significance: Combining radiotherapy and VEGFR inhibitors treatment can provide potentially a substantial impact in decreasing cancer death rates by averting the negative effect of radiotherapy regimens and provide better standard for cancer patients.

Key words: gastric cancer cells, SGC-7901, BGC-823, exosome, radiotherapy, angiogenesis, Human Umbilical Vein Endothelial Cells (HUVEC), Apatinib

Introduction

Radiotherapy is widely used therapeutic medical treatment for up to 50% of all cancer patients worldwide [1]. Its successful outcome relies on the

property of cancer cells to divide faster than the surrounding normal tissue, limiting their ability to cope with DNA repair, contrary to normal cells

allowed more time to recover and withstand radiation [2]. Nevertheless, certain cancer type is resistant and even shows enhanced local invasion and metastasis capacities upon radiotherapy. It has been demonstrated pancreatic, glioma and rectal cancer cells display enhanced invasion potential on an irradiation dose-dependent manner [3-7]. For decades, studies to enhance radiotherapy have been focused on the cancer itself, however the significance of the interaction between the cancer cells and the underlying microenvironment has been underrated. Essentially, for metastasis to occur tumor cells require the acquisition of motility to escape the primary region and invasiveness, to penetrate the surrounding tissues and disseminate. Irradiation has complex effects on the tumor microenvironment contributing to metastasis, such as hypoxia, development of fibrosis and immunity system responses [8].

Exosomes are small 30-120 nm membrane-derived vesicles released by many normal and cancer cell types, *in vivo* and *in vitro* [9]. They play crucial role in tumour proliferation and metastasis as mediators of intracellular communication in localized and remote microenvironments by means of conditioning the development of pre-metastatic niche and metastasis [10]. It has been shown that exosomes transport various proteins, lipids, miRNAs and mRNAs, and importantly their composition alters upon irradiation [11, 12]. Nevertheless, to this day little is known on how recipient cells recognise specific exosomal cargo involved in cancer progression.

Angiogenesis is a biological process in which signals, such as the vascular endothelial growth factor (VEGF), coordinate the development of ECs, resulting in the generation of new blood vessels. In its course, the activated ECs produce and secrete proteases that enable them to proliferate, migrate and form primary vascular system [13]. Angiogenesis occurs normally during development or wound healing, however vascularisation contributes to tumour progression by supplying oxygen and nutrients to cancer cells [14]. Previous studies show that the VEGF transcription positive regulator Hypoxia-Inducible Factor (HIF-1) is upregulated in irradiated tumour cells [15]. VEGF pharmacological inhibitors anti-VEGF antibody (bevacizumab), VEGF-trap (aflibercept), VEGF-receptor (VEGFR)-inhibiting antibody (DC101) and receptor tyrosine kinase inhibitors (TKIs, sunitinib, sorafenib, pazopanib) show low efficiency in tumour treatment but increase its sensitivity to radiotherapy due to reduced hypoxia [1]. Similar inhibitors are subject to extensive studies and new compounds that target VEGF or other angiogenesis pathways are currently being tested in clinical trials

[16]. Apatinib is a tyrosine kinase inhibitor that selectively inhibits the VEGFR-2 and is approved in China, Europe, South Korea, and expects similar faith in the USA [17].

Radiation can have deleterious effect in cancer treatment and is particularly controversial as means of digestive tract cancer regiment. We hypothesize that Apatinib may have a negative effect on angiogenesis-dependent GC cell progression. Therefore, in this study we assess whether exosomes isolated from irradiated GC cells affect ECs proliferation, migration and invasion capacity. We show that exosomes released by irradiated human GC cell lines SGC-7901 and BGC-823 promote HUVEC cells proliferation and promote their motility and invasiveness. Significantly, we demonstrate that the enhanced progression of HUVEC incubated with exosomes derived from irradiated GC cell lines is counteracted by Apatinib.

Materials and Methods

Cell culture and irradiation

The human gastric cancer cell lines, SGC-7901 and BGC-823, and human umbilical vein endothelial cell (HUVEC) which was a similar normal control cell line were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. All cells were grown in a humidified incubator at 37°C with 5% CO₂. For irradiation, SGC-7901 and BGC-823 cells grown at 80% confluence were washed using phosphate-buffered saline (PBS), and changed to serum-free medium, then cells were treated by electron radiation with a dose of 0 or 6 Gy, and incubated for another 48 h before exosome extraction.

Exosomes isolation

Exosomes extraction was performed essentially as described before [18]. Briefly, 48h after irradiation, the medium was decanted, centrifuged at 2000 g for 10 min to sediment cells, then at 10,000g for 30 min to deplete cell debris. Exosomes were further isolated by ultracentrifugation at 120,000 g for 60 min and the pellets were re-suspended using PBS. After another two repeats, the exosomes were finally re-suspended in PBS and stored at -70 °C for use. Concentration of exosomes was determined using BCA method as recommended by the manufacturer (Thermo Scientific, USA).

Morphological Characterization of exosomes

Isolated exosomes were evaluated for morphology by transmission electron microscopy (TEM). Briefly, exosomes were firstly diluted using PBS, then put on copper grids. After 1 min, the grids were stained with 1% (v/v) uranyl acetate in ddH₂O,

and the exosome samples were examined immediately using a transmission electron microscope (Hitachi, Japan).

Cell proliferation assay

MTS method was used for cell proliferation assay. Briefly, the HUVEC cells were seeded into 96-well plates at density of 3000 cells/well. After incubating for 12 hours, the medium was replaced by serum-free DMEM, then exosomes of indicated concentration, Apatinib (Selleck, USA) or both were added to the cells. The MTS reagents were added at the indicated time points and the optical density was measured at 520 nm after incubating for 2 h.

Cell Migration Assay (Transwell)

Cell migration assay was performed using Transwells (6.5 mm diameter; 8 μm pore size polycarbonate membrane) obtained from Corning. 10^5 Cells in 0.5 ml serum-free medium were placed in the upper chamber, then exosomes, Apatinib, or both were added. After incubating for 36 h, the lower chamber was loaded with 0.8 ml medium containing 10% FBS. The migrated cells were stained with DAPI and then imaged under a confocal microscope (Olympus IX70, Japan) and counted after 36 h of incubation at 37°C with 5% CO₂.

Wound-Healing Assay

HUVEC cells were plated in triplicates on uncoated 6-well culture dishes at density of 7×10^5 cells/well. In order to allow the cells to adhere and grow, they were incubated at 37 °C for about 24 h. The samples were then scratched manually using a pipette tip. Cell debris were then removed by washing the samples 3 times with phosphate-buffered saline (PBS) and the wounded cell samples were covered with serum-free culture medium. Then exosomes, Apatinib, or both were added, and after 0 h and 24 h of incubation pictures were taken under a microscope after and the rate of migration was calculated by measuring the distance moved toward the central of the wound.

Western blot analysis

Total proteins were extracted from exosomes released by non- or irradiated SGC-7901 and BGC-823 cells, and whole cell lysates from SGC-7901 and BGC-823 cells were used as control. The protein concentration was measured using the BCA assay (Thermo Scientific, USA). Equal amounts of proteins (20 μg/well) were separated on 10% SDS polyacrylamide gels and transferred to NC membranes. After the membranes were blocked with 5% fat-free milk in TBST buffer (0.1% Tween-20) for 30 minutes at room temperature, they were incubated

with antibodies against CD9 (1:1000, Abcam, USA) and GAPDH (1:1000, Abcam, USA) at 4°C overnight. The membranes were then incubated with HRP-conjugated secondary antibody (Promega, USA) at RT for 45 minutes. The protein bands were detected using chemiluminescence (Millipore, USA) and exposed to X-ray films.

Statistical analysis

Data were expressed as mean \pm SD, and student's t test was used to determine the significance of differences between two groups. All tests carried out were two-sided. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 13.0 software.

Results

Irradiation alters exosomes released by human gastric cancer cells thereby promoting endothelial cell proliferation

To address whether exosomes released by non- or irradiated GC cell lines have distinctive effect on HUVEC cells progression, we carried out isolation by differential ultra-centrifugation of exosomes released by SGC-7901 and BGC-823 cells after irradiation at 0 Gy or 6 Gy. The isolation was next confirmed employing transmission electron microscopy which revealed round, alveolate-shaped structures with diameter of 20-50 nm (Figure 1A). To further verify the exosome identity, we probed the exosomal marker CD9, with GAPDH as loading control, on western blots. As shown in Figure 1B, CD9 was detected in exosome isolates, but much less in lysates of SGC-7901 and BGC-823 cells. Together, these data indicate successful isolation of exosomes.

Next, we addressed the influence of exosomes on HUVEC cells proliferation capacity. Using a 96-well plate, we carried out incubation of 3000 HUVEC cells per well with different concentrations of exosomes from irradiated and non-irradiated GC cells. Subsequently, proliferation and determination of SF of endothelial HUVEC cells was determined by MTS which generates soluble formazan products in cell culture medium and is termed as MTS assay [18]. In accordance with previously observed proliferation-enhancement of head and neck cancer cells [19], HUVEC cell proliferation was significantly boosted upon their incubation with 6Gy irradiated cell exosomes at concentration of 20 μg/ml or more, compared to ECs incubated with the same exosome concentration from non-irradiated human GC cells (Figure 2A). In addition, proliferation of HUVEC increased over 72-hour time course incubation with exosomes and was significantly enhanced upon

incubation with exosomes from irradiated, compared to non-irradiated SGC-7901 and BGC-823 cells (Figure 2B). Taken together, these results suggest that irradiation alters the cargo quality of exosomes

released by GC cells. Importantly, the latter results in enhanced proliferation of HUVEC cells on a concentration- and time-dependant manner.

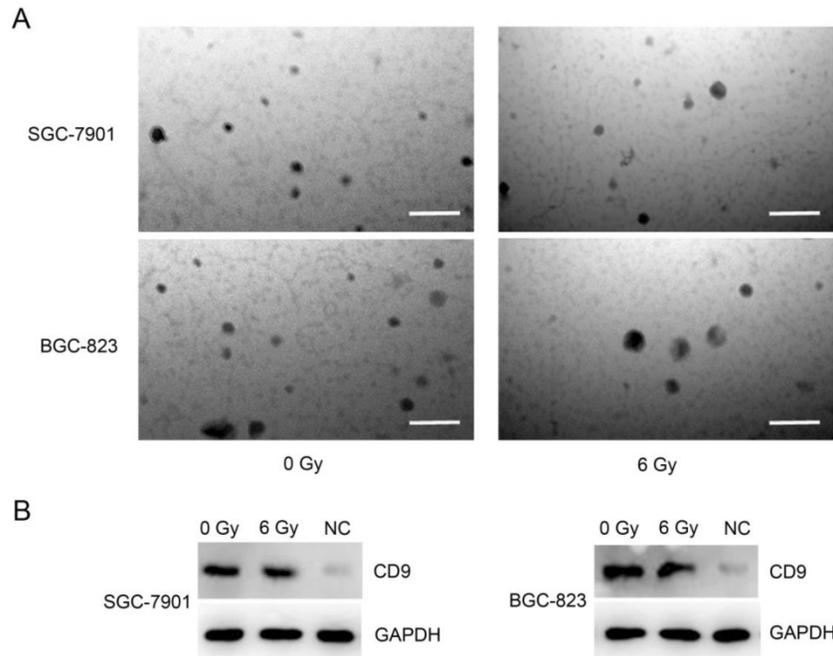


Figure 1. Isolation of exosomes secreted from gastric cancer cells SGC-7901 and BGC-823 treated by irradiation. **A:** Electron microscopic images of exosomes released from SGC-7901 and BGC-823 cell lines. Exosomes were isolated 24 h after irradiation at either 0 Gy or 6 Gy. Bars = 0.2µm. **B:** Western blot analysis of CD9 protein in cell lysates prepared from exosomes of SGC-7901 or BGC-823 cell line. Whole cell extract from SGC-7901 or BGC823 cells was used as negative control (NC) and GAPDH was detected as loading control.

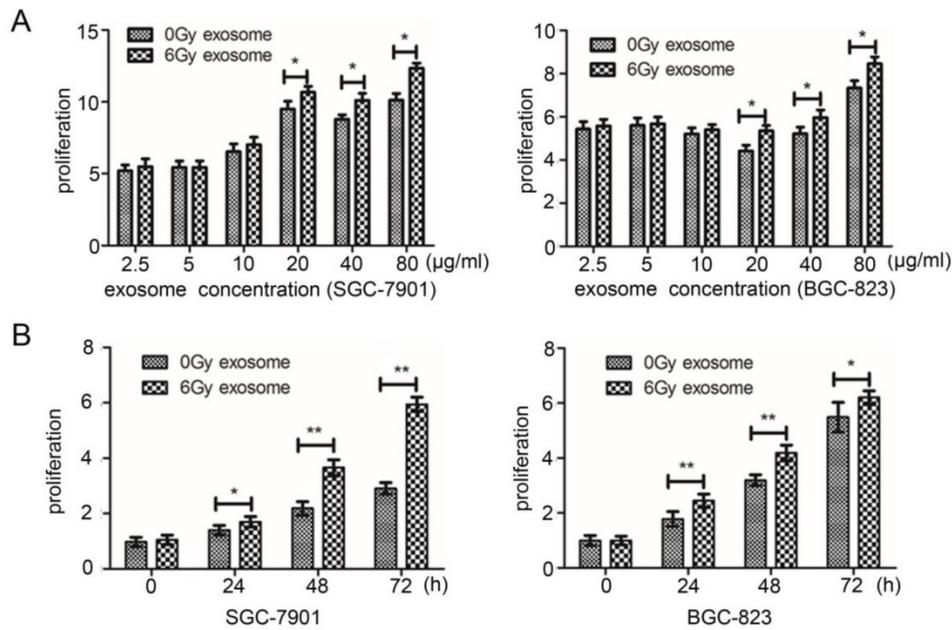


Figure 2. Characterizing effect of irradiated and non-irradiated SGC-7901 or BGC-823 exosomes on proliferation of HUVEC cells. **A:** Effect of indicated concentrations of SGC-7901 or BGC-823 exosomes on proliferation of HUVEC cells. For each experiment, 3×10^3 HUVEC cells and MTS method were used to determine the cell viability. Data indicate means + SD of three biological replicates. Student's t test; * $P < 0.05$, ** $P < 0.01$. **B:** Cell viability assay of HUVEC cells after incubation with 20 µg/ml either SGC-7901 or BGC-823 exosomes for the indicated time. The experiment was performed similarly to that in A. Data indicate means + SD, Student's t test; * $P < 0.05$, ** $P < 0.01$.

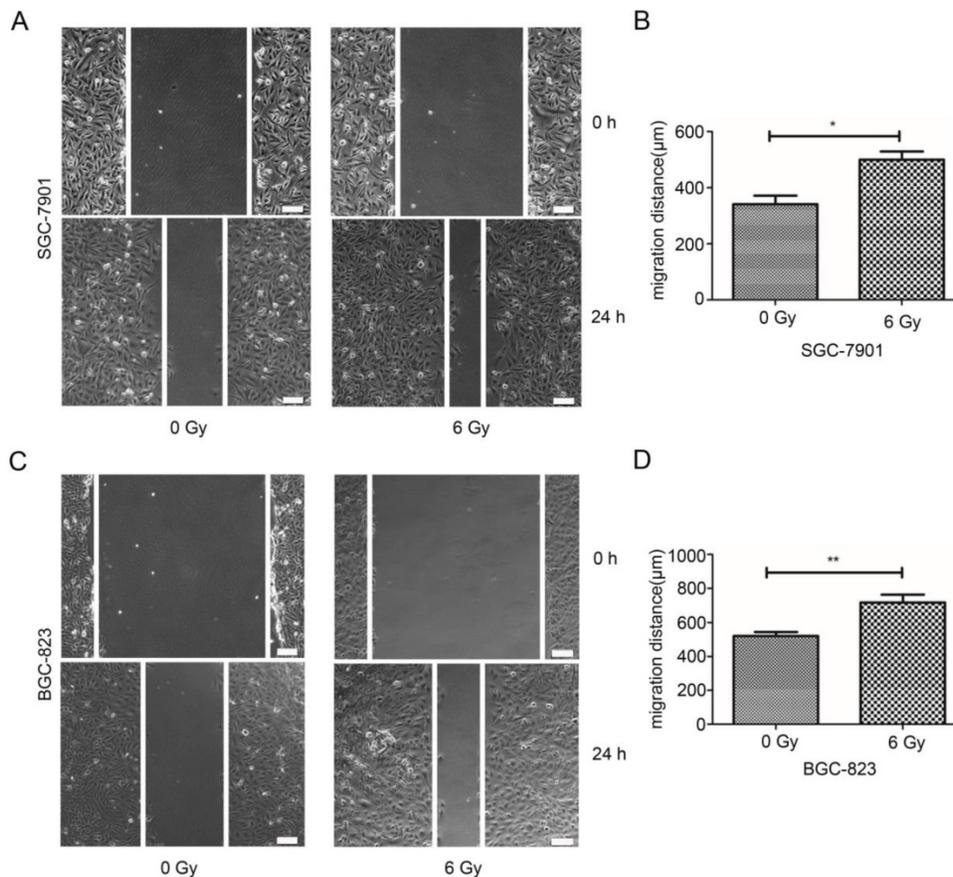


Figure 3. Effect of irradiated and non-irradiated SGC-7901 or BGC-823 exosomes on migration of HUVEC cells. **A,C:** representative results of cell motility assessed by scratch wound-healing assay. 20 $\mu\text{g/ml}$ non-irradiated or irradiated exosomes were incubated with 7×10^5 HUVEC cells for indicated time before pictures were taken. Vertical white lines define the area lacking cells. Each experiment was performed in triplicate. Bars = 100 μm . **B,D:** Migration distance calculated for non-irradiated or irradiated exosome treatment as shown in A and C. Data indicate means + SD, Student's t test; * $P < 0.05$, ** $P < 0.01$.

Exosomes secreted by irradiated human gastric cancer cells stimulates endothelial cell migration

In order to investigate the effect of exosomes from irradiated or non-irradiated SGC-7901 and BGC-823 cells on the cell motility HUVEC cells, wound-healing and Transwell migration assays were conducted. On a 6-well plate, 7×10^5 HUVEC cells per well were re-suspended in the migration buffer containing 20 $\mu\text{g/ml}$ exosomes released by non-irradiated or irradiated SGC-7901 and BGC-823 cells for 24 hrs. Pictures were taken at 0 and 24 hours post incubation using a time-lapse Olympus IX51 microscope. Representative pictures at 0 and 24 hours are shown and scale bars (100 μm) are added for motility width measurement. As demonstrated on the micrographs (Figure 3A, 3C), the results of the wound-healing assay demonstrated that 24 hrs after incubation of HUVEC cells with 20 $\mu\text{g/ml}$ of exosomes released by SGC-7901 and BGC-823 cells irradiated at 6 Gy significantly promoted the motility of the ECs by 42.99% ($P = 0.032$) and 38.09% ($P = 0.009$) as compared to HUVEC incubated with exosomes

originating from non-irradiated SGC-7901 and BGC-823 lines (Figure 3B, 3D).

Similarly, results obtained by Transwell cell migration assay represented on the Zeiss, AXIO Scan.Z1 micrograph with DAPI stained HUVEC on a 24-well plate with 1×10^5 cells per well, confirmed that the invasion capacity of HUVEC cells, 24 hours post incubation with 20 $\mu\text{g/ml}$ of exosomes released by SGC-7901 and BGC-823 cells irradiated at 6 Gy, was obviously increased (Figure 4A), compared to HUVEC cells incubated with exosomes originating from non-irradiated GC cells. Quantitatively, HUVEC cells incubated with 20 $\mu\text{g/ml}$ of exosomes from SGC-7901 and BGC-823 cells irradiated at 6 Gy, were migrating through the gluten gel by 30.01% ($P = 0.0159$) and 28.25% ($P = 0.0135$), respectively, compared to ECs incubated with the same concentration of exosomes originating from non-irradiated SGC-7901 and BGC-823 cell lines (Figure 4B). Taken together, the results obtained from the wound-healing and Transwell migration assays suggest that the exosomes secreted by irradiated GC cells exhibit the capacity to enhance EC motility and migration capacity.

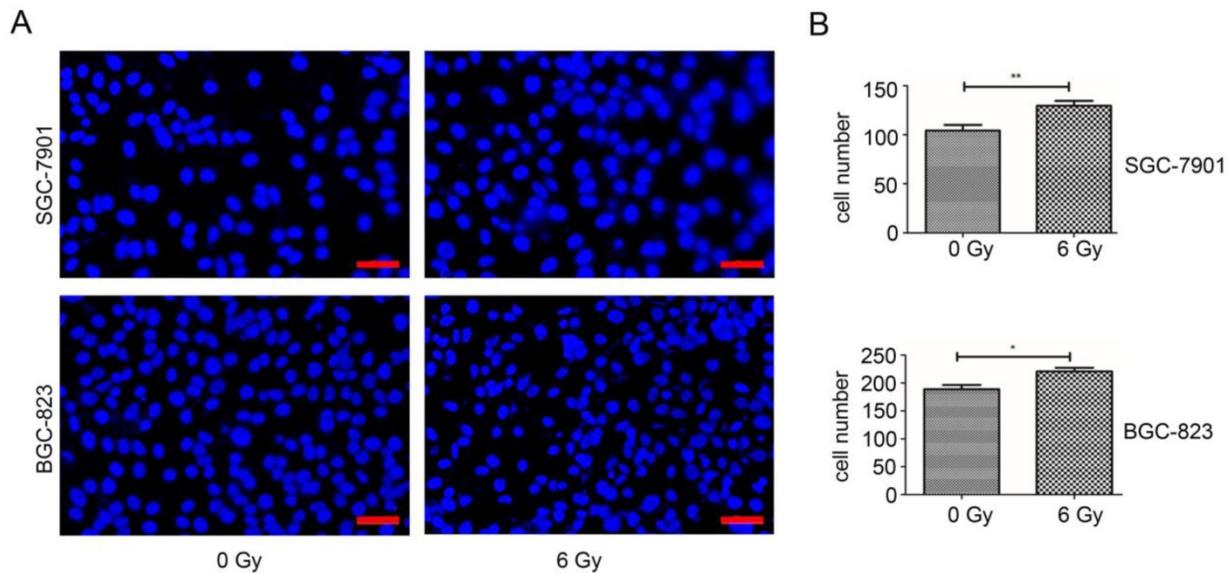


Figure 4. Effect of irradiated and non-irradiated SGC-7901 or BGC-823 exosomes on motility of HUVEC cell. **A:** Transwell migration assay of HUVEC cells treated by either non-irradiated or irradiated exosomes. 1×10^5 HUVEC cells were incubated with 20 $\mu\text{g/ml}$ non-irradiated or irradiated SGC-7901 and BGC-823 exosomes for 36 h, then migrated cells were stained with DAPI and imaged under microscope. For each experiment 5 images were taken and representative images were shown. Bars = 50 μm . **B:** Migration Cell number after exosome treatment as shown in A. Data indicate means + SD, Student's t test; * $P < 0.05$, ** $P < 0.01$.

Effects of exosome released by irradiated gastric cancer cells on HUVEC proliferation, motility and migration are counteracted by Apatinib

Previously, it has been demonstrated that in GC cells, radiation induces HIF-1-mediated expression of VEGF, thereby promoting ECs survival [20]. Therefore, we decided to address whether the VEGFR-2 selective inhibitor Apatinib may reverse the effect of the irradiated gastric cell exosomes to promote HUVEC cells proliferation and migration capacity. First, we determined by IC₅₀, 60 $\mu\text{g/ml}$ as the optimal Apatinib concentration at which the division of HUVEC cells incubated with exosomes from irradiated GC is reduced by half (unpublished data).

Subsequently, in order to determine the SF of ECs in the presence of Apatinib we carried MTS cell proliferation assay. We monitored over 72 hours of time course the proliferation of HUVEC cells not incubated with exosomes, as control, or such incubated with exosomes from irradiated GC cells. The survival fraction of ECs in the absence of exosomes was enhanced in the no Apatinib negative control but was lower compared to the SF of the HUVEC cells incubated with exosomes from irradiated SGC-7901 and BGC-823 cell lines after 24 hours (Figure 5A). As expected, the proliferation of HUVEC cell was significantly boosted over the time period after their incubation with 20 $\mu\text{g/ml}$ of exosomes extracted from irradiated SGC-7901 and BGC-823 cancer cells irradiated at 6 Gy (Figure 5A).

Importantly, this effect was significantly reduced in the presence of Apatinib suggesting a negative effect of the VEGFR-2 inhibitor on ECs proliferation (Figure 5A).

Next, we sought whether Apatinib has the capacity to diminish the motility of ECs incubated with exosomes from irradiated GC cells. In accordance with the MTS cell proliferation assay, the motility of HUVEC cells in the no exosome control was enhanced but significantly lower than the motility of HUVEC cells incubated with 20 $\mu\text{g/ml}$ exosomes from irradiated SGC-7901 ($P = 0.0079$) and BGC-823 ($P = 0.0090$) cancer cells (Figure 5B, 5C). However, Apatinib significantly reduced the motility of the ECs 24 hours post incubation with irradiated SGC-7901 ($P = 0.0256$) and BGC-823 ($P = 0.0058$) cancer cells-derived exosomes respectively (Figure 5B, 5C).

Finally, we addressed whether Apatinib exhibits a negative effect on the invasion properties of ECs incubated with exosomes from irradiated GC cell lines. As expected, the migration capacity was enhanced in the absence of Apatinib regardless whether HUVEC cells were alone or incubated with exosomes from irradiated SGC-7901 and BGC-823 cell lines, but significantly increased in the latter condition ($P = 0.0160$ and $P = 0.0159$, respectively) (Figure 6A, 6B). Importantly, the presence of Apatinib hindered the ability of the ECs to proliferate after incubation with irradiated SGC-7901 ($P = 0.0079$) and BGC-823 ($P = 0.0067$) cancer cells-derived exosomes, respectively (Figure 6A, 6B), compared to HUVEC incubated only with exosomes extracted from irradiated GC cell lines. Taken together, the results

above suggest that GC cell radiation-induced exosome content-driven enhancement of the endothelial cells proliferation, motility and invasion

capacities are counteracted by the VEGFR-2 inhibitor Apatinib.

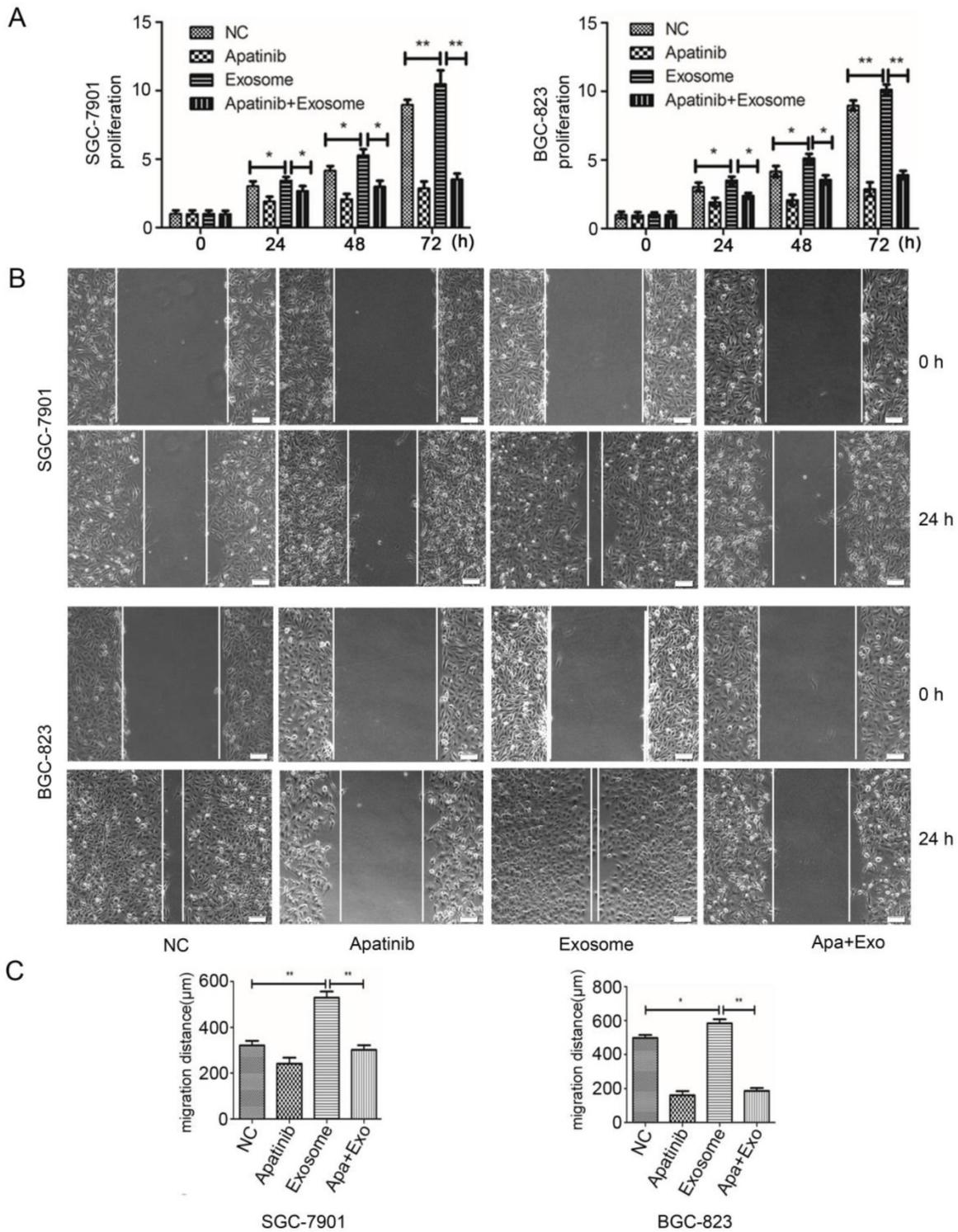


Figure 5. Combined effect of Apatinib and irradiated exosomes on proliferation of HUVEC cell. **A.** Cell proliferation assay of HUVEC cells after incubation with 60 µg/ml Apatinib, 20 µg/ml irradiated exosomes (SGC-7901 or BGC-823), or both for the indicated time. Each experiment was performed with four replicates. Data indicate means + SD, Student's t test; *P < 0.05, **P < 0.01. **B.** representative results of wound-healing assay. 60 µg/ml Apatinib or 20 µg/ml irradiated exosomes, or them both were incubated with 7×10⁵ HUVEC cells for indicated time before pictures were taken. Vertical white lines define the area lacking cells. Each experiment was performed in triplate. Bars = 100 µm. **C:** Migration distance calculated for treatments shown in B. Data indicate means + SD, Student's t test; *P < 0.05, **P < 0.01.

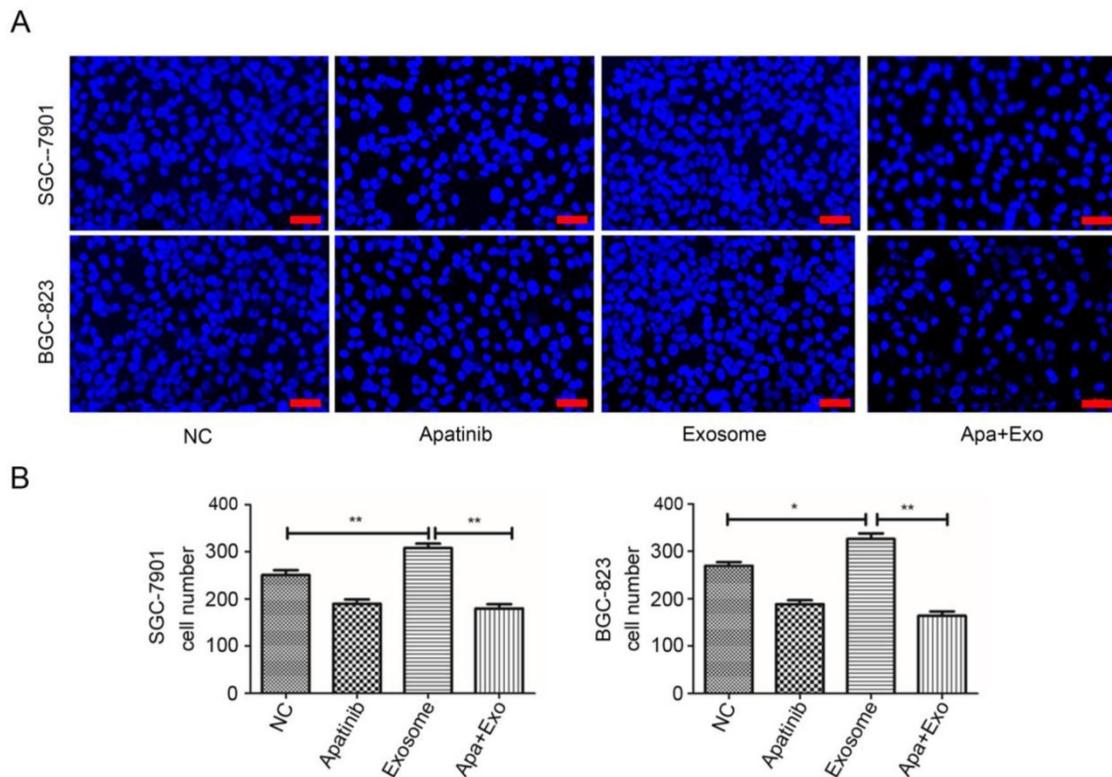


Figure 6. Combined effect of Apatinib and irradiated exosomes on motility of HUVEC cell. **A:** Transwell migration assay of HUVEC cells incubated with 60 $\mu\text{g/ml}$ Apatinib or 20 $\mu\text{g/ml}$ irradiated exosomes, or them both. 1×10^5 HUVEC cells were treated for 36 h, then migrated cells were stained with DAPI and imaged under microscope. For each experiment 5 images were taken and representative images were shown. Bars = 50 μm . **B:** Migration cell number after treatment shown in A. Data indicate means + SD, Student's t test; * $P < 0.05$, ** $P < 0.01$.

Discussion

It has been demonstrated that upon stress, such as irradiation, cells alter their exosomal cargo which they employ as means for intercellular communication [21, 22]. Furthermore, studies conducted in mice demonstrate that in cancer cells, radiation induces HIF-1-mediated expression of VEGF, thereby promoting endothelial cell survival [23]. Potential consequence of such phenomenon is generation of new vascularity, which has been shown not only to penetrate pre-existing cancerous growths, providing them with nutrients and oxygen, but also to condition the formation of metastasis.

Presently, as indicated by growing evidence, extracellular vesicles released from irradiated cancer cells boost the survival and promote migration capacities of glioblastoma, lung as well as head and neck cancer cells [4, 11, 19, 24]. Nevertheless, few have studied the impact of the aforementioned extracellular vesicles on the bystander normal tissues and the mechanisms by which such interaction affects the development of localized and distant environments hospitable for cancer growth and metastasis. Moreover, for decades *de novo* vascularization has been associated with successful

tumour growth, however anti-VEGF therapies remain controversial to this day [16].

Despite that exosomes released by gastric cells, with or without irradiation treatment, affect similarly EC proliferation and invasion, in this study we show that exosomes released by irradiated GC cell lines enhance the capacity of ECs to proliferate on a dose- and time-dependant manner. Moreover, 20 $\mu\text{g/ml}$ of exosomes derived from irradiated SGC-7901 and BGC-823 GC lines promote the motility and the invasiveness of HUVEC cells 24 hrs post incubation. These results are in agreement with the mentioned above previous studies which have demonstrated that ionizing radiation promotes the ability of cells to survive and proliferate within the tumour microenvironment and enhance their capacity to migrate [25, 26]. The latter have therapeutic consequences on tumour progression by endowing cancer cells resistance to radiotherapy.

Our data also demonstrates that exosomes derived from non-irradiated SGC-7901 and BGC-823 GC lines also promote ECs proliferation, migration and invasion capacities, however significantly strengthened upon incubation with irradiated GC cells-derived exosomes. Finally, we show that VEGFR-2 selective inhibitor Apatinib counteracts the

proliferation, migration and invasiveness of HUVEC cells treated with exosomes released by irradiated GC cells. Therefore, combining ionizing radiation and VEGFR inhibitors treatment can provide a potentially substantial impact in decreasing cancer death rates by averting the negative effect of radiotherapy regimens and by providing better disease management.

Interestingly, in a preliminary clinical investigation, we selected 20 patients with gastric cancer to undergo radiotherapy or non-radiotherapy (10 patients per group). Subsequently, the exosomes extracted from the serum of each patient were co-cultured with HUVEC cells. We observed similar results to those demonstrated by the *in vitro* experiments, indicating that our *in vitro* findings may provide guidance to clinical application. The latter, being a part of an ongoing research will be published upon the completion of the study. Additionally, further analysis of exosome content is required in order to decode how these extracellular vesicles mediate cell-to-cell transfer from cancer to normal cells, leading to the development of microenvironments amenable to tumour growth, invasion and metastasis. Deciphering and controlling the underlying mechanisms of intercellular communication bears the potential to further our insight on cancer's intricate effects on one's organism and to ameliorate treatment regimens for patients suffering cancer.

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Competing Interests

The authors have declared that no competing interest exists.

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